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PKCδ inhibition as a novel medical countermeasure for radiation-induced vascular damage

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ABSTRACT: In the event of a radiologic catastrophe, endothelial cell and neutrophil dysfunction play important roles in tissue injury. Clinically available therapeutics for radiation-induced vascular injury are largely supportive. PKCδ was identified as a critical regulator of the inflammatory response, and its inhibition was shown to protect critical organs during sepsis. We used a novel biomimetic microfluidic assay (bMFA) to interrogate the role of PKCδ in radiation-induced neutrophil–endothelial cell interaction and endothelial cell function. HUVECs formed a complete lumen in bMFA and were treated with 0.5, 2, or 5 Gy ionizing radiation (IR). At 24 h post-IR, the cells were treated with a PKCδ inhibitor for an additional 24 h. Under physiologic shear flow, the role of PKCδ on endothelium function and neutrophil adherence/migration was determined. PKCδ inhibition dramatically attenuated IR-induced endothelium permeability increase and significantly decreased neutrophil migration across IR-treated endothelial cells. Moreover, neutrophil adhesion to irradiated endothelial cells was significantly decreased after PKCδ inhibition in a flow-dependent manner. PKCδ inhibition downregulated IR-induced P-selectin, intercellular adhesion molecule 1, and VCAM-1 but not E-selectin overexpression. PKCδ is an important regulator of neutrophil–endothelial cell interaction post-IR, and its inhibition can serve as a potential radiation medical countermeasure.—Soroush, F., Tang, Y., Zaidi, H. M., Sheffield, J. B., Kilpatrick, L. E., Kiani, M. F. PKCδ inhibition as a novel medical countermeasure for radiation-induced vascular damage. FASEB J. 32, 000–000 (2018). www.fasebj.org

KEY WORDS: adhesion · microfluidics · leukocytes · transmigration · acute radiation syndrome

In the event of a radiologic catastrophe, affected individuals will be exposed to a wide range of radiation doses with the extent of injury depending on the victims' distance from the epicenter, the duration of exposure, and their inherent sensitivity/resistance to ionizing radiation (IR). Radiation-induced damage to the vascular endothelium plays a key role in the early and late onset of radiation-induced pathologies in several organs (1) and is an active participant in the recruitment and activation of neutrophils through the production of chemokines/cytokines and expression of adhesion molecules (2–5). The downregulation of endothelial inflammatory activation protects normal tissue from radiation-induced damage (6, 7).

The key to radiation-induced tissue damage is the excessive migration of activated neutrophils across the vascular endothelium (8, 9), and reduction in neutrophil infiltration is associated with better outcomes following skin irradiation (6). Postradiation injury, systemic inflammation leads to increased endothelial cell-adhesion molecule expression resulting in increased neutrophil–endothelial cell interaction, vascular endothelial cell damage, and organ dysfunction (2–5, 10–12). Neutrophil–endothelial cell interaction starts with neutrophil rolling on the endothelium, which is mediated by selectins, whereas firm adhesion and migration into the tissue are mediated by a combination of integrins/Ig and chemoattractants in the tissue. As a result of the significance of the neutrophil–endothelial cell interactions and given the complexity of existing *in vivo* models of the inflammatory process, several *in vitro* models have been developed to study different aspects of the neutrophil adhesion cascade. Unfortunately, for the most part, these models cannot characterize adhesion and migration in a single assay. To overcome these limitations, a novel

ABBREVIATIONS: 3D, 3-dimensional; bMFA, biomimetic microfluidic assay; EGM, endothelial cell growth medium; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; ICAM-1, intercellular adhesion molecule 1; IR, ionizing radiation; PKCδ-i, PKCδ-transactivator of transcription peptide inhibitor; TAT, transactivator of transcription

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biomimetic microfluidic assay (bMFA) has been developed that resolves and facilitates real-time assessment of individual steps, including rolling, firm arrest, spreading, and migration of neutrophils into the extravascular tissue space in a single system, which allows direct observation and quantification of neutrophil–endothelial cell interaction over time in a realistic microvasculature geometry with physiologic shear conditions (13–15). This is the first *in vitro* system that realistically models *in vivo* geometrical features (*e.g.*, bifurcations, vascular morphology) and flow conditions (*e.g.*, converging or diverging flows at bifurcations) of the microvasculature and allows for interrogation of the role of various factors in IR-induced leukocyte–endothelial cell interaction and endothelial cell damage (16). Thus, we used an integrated microfluidic assay to study specific steps in the leukocyte adhesion cascade induced by exposure to radiation.

Whereas the use of Neupogen and Neulasta for treating hematopoietic acute radiation syndrome was recently approved by the U.S. Food and Drug Administration, treatment strategies for radiation-induced vascular injury are largely supportive, and there are no specific pharmacologic therapies available that protect from radiation-mediated tissue damage (17, 18). Potential therapeutic target sites include local control of the vascular endothelial response to systemic inflammation, as well as direct modulation of leukocyte migration. Previously, the serine/threonine kinase PKCδ has been identified as a critical regulator of the inflammatory response (19–23). Moreover, PKCδ$^{-/-}$-deficient mice were protected from radiation-induced damage to the salivary gland and thymus (24, 25). Thus, we hypothesize that PKCδ is activated in response to radiation, and PKCδ inhibition modulates radiation-induced endothelial permeability and protects tissue against neutrophil-mediated damage. To test this hypothesis, we explored the role of PKCδ inhibition as a potential radiation countermeasure to reduce human neutrophil–endothelial cell interaction after endothelial cells were exposed to IR under physiologically relevant shear flow conditions. With the use of bMFA, we investigated the effect of PKCδ inhibition on irradiated endothelial cell permeability/integrity, temporal and spatial distribution of adhering/migrating neutrophils through irradiated endothelium, and adhesion molecule expression on endothelial cells.

MATERIALS AND METHODS

Materials, equipment, and reagents

A mouse monoclonal anti-human intercellular adhesion molecule 1 (ICAM-1), a mouse monoclonal anti-human VCAM-1, a mouse monoclonal anti-human E-selectin, a mouse monoclonal anti-human VE-cadherin, and an Alexa Fluor 594 goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). A mouse monoclonal anti-human P-selectin was purchased from Abcam (Cambridge, MA, USA). Human fibronectin was obtained from BD Biosciences (San Jose, CA, USA). Protein A was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fluorescent 9.9 μm microparticles (green: excitation 468 nm, emission 508 nm) were purchased from Duke Scientific (Palo Alto, CA, USA). HUVECs were purchased from Lonza Walkersville (Walkersville, MD, USA). A carboxyfluorescein diacetate succinimidyl ester probe, HBSS, Trypsin/EDTA, formalin, Triton X-100, Draq5, 40 kDa Texas Red-Conjugated Dextran, Hoechst 33342, and Alexa Fluor 488 phalloidin were from Thermo Fisher Scientific. Bovine serum albumin was purchased from MilliporeSigma (Burlington, MA, USA).

A Nikon TE200 Fluorescence Microscope (Nikon Instruments, Melville, NY, USA) equipped with an automated stage was used for performing experiments. An Olympus FluoView FV1000 Confocal Microscope (Center Valley, PA, USA) equipped with a fully automated stage was used for capturing confocal image stacks. Images were acquired using an Orca Flash 4 Camera (Hamamatsu, Bridgewater, NJ, USA). A PHD Ultra Syringe Pump (Harvard Apparatus, Holliston, MA, USA) was used for injecting media, permeability dye, or neutrophil suspension to the bMFA with high precision. A stage warmer was used to keep the bMFA at 37°C. NIS Elements Software (Nikon Instruments) was used to control the microscope stage and the camera.

PKCδ inhibitor peptide synthesis

PKCδ activity was selectively inhibited by a peptide antagonist that consisted of a peptide derived from the first unique region (V_1) of PKCδ (SFNSYELGSL: aa 8–17) coupled *via* an N-terminal Cys–Cys bond to a membrane-permeant peptide sequence in the HIV transactivator of transcription (TAT) gene product (YGRKKRRQRRR: aa 47–57 of TAT) (26). The PKCδ TAT peptide produces a unique dominant-negative phenotype that effectively inhibits activation of PKCδ, but not other PKC isotypes. The peptide was synthesized by Mimotopes (Melbourne, VIC, Australia) and purified to >95% by HPLC.

Design and fabrication of the microfluidic assay

We have published the methods for design and fabrication of the novel microfluidic assay and *in vivo* validation previously (14, 27, 28). In brief, a modified Geographic Information System approach was used to digitize the *in vivo* microvascular networks (**Fig. 1*A***) that were lithographically patterned on polydimethylsiloxane (Fig. 1*B*). Microfabricated pillars (10 μm diameter) were used to fabricate the 3 × 100-μm pores, resulting in a network of vascular channels connected to a tissue compartment *via* a 3-μm porous barrier (Fig. 1*C*), which is the optimum size for neutrophil migration.

Seeding of endothelial cells in bMFA

Endothelial cells (HUVECs) were cultured in growth media [endothelial cell growth medium (EGM), PrimaPure; Genlantis, San Diego, CA, USA] and used between passages 3 and 6. The bMFA was coated with fibronectin, and endothelial cells were cultured under shear flow (inlet flow rate of 0.5 μl/min) for 24 h (28). Consistent with our published data (27, 29, 30), endothelial cells in bMFA form a confluent lumen and aligned in the direction of flow (Fig. 1*D*). Formation of the 3-dimensional (3D) lumen in vascular channels under physiologic conditions was confirmed using confocal microscopy (Fig. 1*E*) (27, 29). In agreement with the observed complexity of *in vivo* flow conditions (16), shear stress is different in different vessels of the bMFA as a result of flow distribution in the complex *in vivo*-like geometry. However, by keeping inlet flow conditions constant, shear stress in a given vessel in bMFA is the same across different measurements. Assays in which neutrophils freely entered the tissue compartment without attachment were discarded.

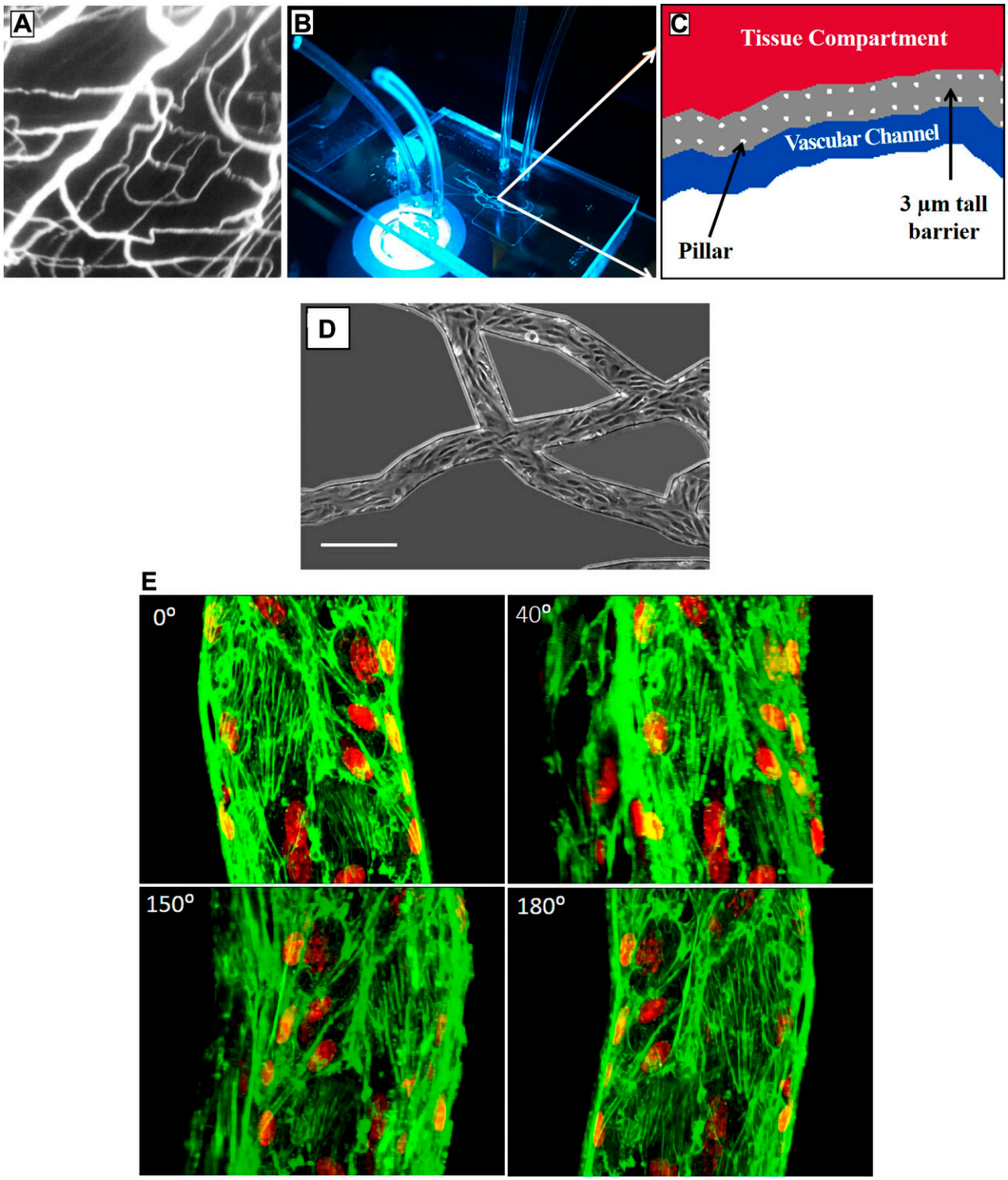

Figure 1. *A*) Intravital microscopy was used to map microvascular networks in animals. *B*) These maps are then used to fabricate the vascular network on polydimethylsiloxane and assemble the bMFA. *C*) The bMFA includes vascular channels that are connected to the tissue compartment through a 3 μm barrier. *D*) Endothelial cells are aligned in the direction of flow in the bMFA (original scale bar, 250 μm). Confocal microscopy demonstrates that endothelial cells form a complete 3D lumen in the vascular channel. *E*) F-actin is labeled in green, and nuclei are labeled in red.

IR treatment of endothelial cells

Endothelial cells in the bMFA were exposed to 0 (no treatment), 0.5, 2, or 5 Gy (1.13 Gy/min) of radiation treatment from an X-Rad 320 Irradiator (PXI, North Branford, CT, USA). At 24 h after ionizing irradiation (post-IR) treatment, EGM or EGM containing the PKCδ-TAT peptide inhibitor (PKCδ-i; 5 μM) was injected into the bMFA. This treatment timepoint (*e.g.*, 24 h post-IR) was selected to approximate clinically relevant treatment scenarios following radiologic disasters (31). At 48 h post-IR, the tissue compartment was filled with EGM containing a chemoattractant [*N*-formylmethionyl-leucyl-phenylalanine (fMLP), 1 μM] or

EGM (control) before introducing neutrophils or antibody-coated microparticles into the vascular compartment (**Fig. 2**).

PKCδ phosphorylation and translocation

HUVECs grown in 6-well plates to confluency were exposed to 0 (no treatment), 0.5, 2, or 5 Gy (1.13 Gy/min) of radiation treatment and then incubated for 1 h at 37°C. The cells were placed on ice and harvested, and the membrane and cytoplasm fractions were isolated according to the manufacturer's instructions using a Subcellular Protein Fractionation Kit for Cells (Thermo Fisher Scientific). Samples for Western blot analysis were prepared by mixing an aliquot of the samples with 2 times sample buffer and heating for 5 min at 95°C. Purity of membrane fractions was routinely determined by probing fractions for cytoplasmic (glyceraldehyde 3-phosphate dehydrogenase) and membrane (VE-cadherin) markers. Proteins (30 μg/lane) were separated on 4–12% SDS-PAGE gels and transferred to nitrocellulose membranes, as previously described. Translocation of phosphorylated PKCδ was determined by immunoblotting using a phospho-specific PKCδ (Ser643/676) antibody (Cell Signaling Technology, Beverly, MA, USA), as previously described (19, 32, 33). Translocation of PKCδ to the membrane (particulate fraction) was quantitated by densitometry analysis of Western blots with ImageJ software v.1.46r [National Institutes of Health (NIH), Bethesda, MD, USA], and the values were expressed in arbitrary densitometry units.

Neutrophil isolation and labeling

Heparinized human blood was obtained from healthy adult donors following informed consent, as approved by the Institutional Review Board of our institute. Human neutrophils were isolated using Ficoll-Hypaque separation, Dextran sedimentation, and hypotonic lysis to remove erythrocytes (19, 22). After isolation, neutrophils were counted, suspended in HBSS (5×10^6 cells/ml), and labeled using the carboxyfluorescein diacetate succinimidyl ester probe for 10 min at room temperature. Neutrophils were introduced into the vascular channels of the bMFA at a flow rate of 1 μl/min.

Preparation of antibody-coated microparticles

With the use of our established methodology (4), the level of adhesion of antibody (*e.g.*, anti-ICAM-1)-coated microparticles to endothelial cells was used as an index of the level of upregulation of adhesion molecules post-IR (2 Gy). In brief, 9.9-μm fluorescent polystyrene microparticles were washed with a sodium bicarbonate buffer and coated with protein A (300 μg/ml) *via* passive adsorption and incubated overnight at room temperature. Microparticles were then washed and incubated in a blocking buffer (1% bovine serum albumin in HBSS) at room temperature. Microparticles (5×10^6 particles/mm) were counted, diluted in blocking buffer, and incubated with antibodies to ICAM-1, VCAM-1, P-selectin, or E-selectin for 30 min. Antibody-coated microparticles were then suspended in EGM and introduced into the bMFA as described before (27). The level of adhesion of given antibody (*e.g.*, anti-ICAM-1)-coated microparticles to endothelial cells was used as an index of the level of upregulation of that adhesion molecule (4). Similar to the protocol previously described for neutrophils, the number of adhered antibody-coated microparticles in a given vessel was quantified under the same shear stress across different measurements.

Permeability measurements

The vascular compartment was connected to a Hamilton gas tight syringe filled with Texas Red 40 kDa Dextran (25 μM in EGM) mounted on a syringe pump. Permeability was measured by imaging the bMFA every minute for 2 h while the Dextran solution flowed through the vascular channels (flow rate 1 μl/min). With the use of our previously published method (29), the following equation was used to calculate permeability (P) of Dextran across the endothelium in bMFA:

$$P = \frac{1}{I_{v_0}} \times \frac{V}{S} \times \frac{dI_t}{dt}$$

where I_t is the average intensity in the tissue compartment, I_{v_0} is the maximum fluorescence intensity of the vascular channel, and V/S is the ratio of vascular channel volume to its surface area.

Immunofluorescence staining

The formation of endothelial cell–cell adherens junctions in microvessels of the bMFA was characterized using immunostaining against VE-cadherin (30). To study morphologic changes in cells post-IR, actin filaments were stained with phalloidin, and cell nuclei were stained with Hoechst 33342. Images were taken using the microscope and camera system as previously described.

Data analysis

Cells that did not move for 30 s were considered adherent. Adhesion level of neutrophils or antibody-coated microparticles to the endothelium reached steady state after 10 min of flow and was quantified by scanning the entire network. The number of migrated neutrophils was quantified using time-lapse imaging

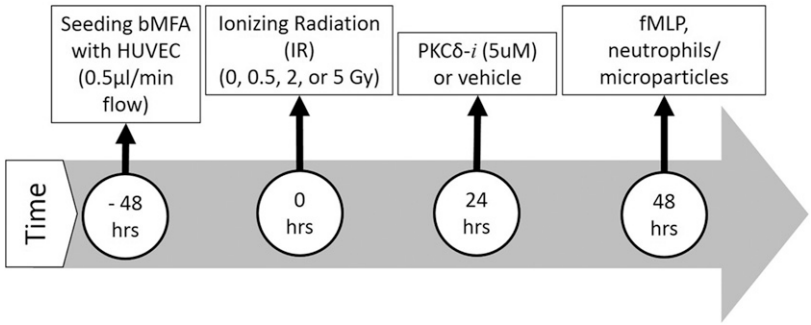

Figure 2. Timeline of experiments. bMFA is prepared and seeded with endothelial cells. After endothelial cells reach confluency under shear flow and form a complete lumen in bMFA (usually in 48 h after seeding), they are treated with IR. At 24 h post-IR, the cells are treated with either PKCδ-i or vehicle. At 48 h post-IR, fMLP is added to the tissue compartment, and interaction of either fluorescent-labeled neutrophils or microparticles with endothelial cells is studied.

every 3 min for 60 min. Nikon Elements and Fiji Software (NIH) were used to collect and analyze the data (34). Data are presented as means ± SEM. Statistical significance were determined by Student's *t* test, or 1- or 2-way ANOVA using SigmaPlot software. Differences were considered statistically significant if $P < 0.05$.

RESULTS

Radiation activates PKCδ in endothelial cells

Activation of PKCδ is a multistep process that requires both phosphorylation and translocation of PKCδ from the cytosol to the membranous sites. Phosphorylation of PKCδ on Thr505 leads to an autophosphorylation step and phosphorylation on Ser643 in the PKCδ activation loop (19, 33, 35), a site that regulates enzymatic activity and protein: protein interactions (36). As shown in **Fig. 3**, there is little phosphorylated PKCδ present in membrane fractions of control HUVECs (0 Gy). In contrast, exposure to IR results in a significant increase in translocation of phosphorylated PKCδ exposed to 0.5 and 2 Gy. PKCδ translocation in response to 5 Gy IR was variable and did not reach statistical significance. Thus, irradiation induces phosphorylation and translocation of PKCδ in endothelial cells.

PKCδ inhibition preserves integrity of irradiated endothelial cells

Endothelial cells formed a complete lumen and aligned in the direction of flow (**Fig. 4*A***). Irradiated endothelial cells in the bMFA showed significant changes in morphology, decreased expression of F-actin filaments, lack of F-actin alignment with flow direction, and decreased adherens junction expression 48 h post-IR, indicating damage to the endothelial barrier integrity (Fig. 4*B*). At 24 h post-IR, treatment with the inhibitor for 24 h attenuated this damage, as indicated by alignment of F-actin filaments with flow and stronger VE-cadherin expression (Fig. 4*C*).

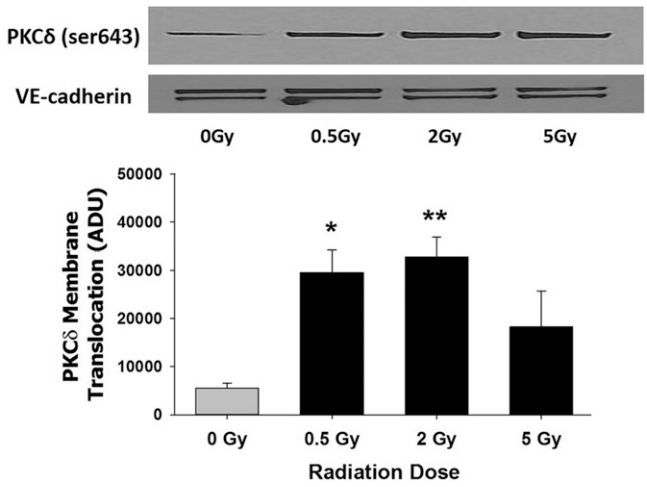

Figure 3. Radiation exposure induces PKCδ activation through phosphorylation and translocation. HUVECs were exposed to varying levels of radiation, and PKCδ Ser643 phosphorylation and translocation to membrane fractions were determined, as described in Materials and Methods. *A*) Representative Western blot of PKCδ membrane translocation response to 0, 0.5, 2, and 5 Gy and VE-cadherin as a marker for membrane fractions ($n = 4$ separate experiments). Membrane extracts were prepared, as described in Materials and Methods. *B*) Densitometry analysis of PKCδ (Ser643) in the membrane fraction. Values are expressed in arbitrary densitometry units (ADU). Means ± SEM ($n = 4$); 1-way ANOVA. $*P < 0.05$, $**P < 0.01$.

PKCδ inhibition modulates the increase in permeability of irradiated endothelial cells

Integrity of endothelial cell-barrier post-IR was directly assessed in the bMFA by measuring the Dextran permeation from the vascular channels to the tissue compartment. Permeability rates across the endothelial barrier were measured 48 h post-IR ± the PKCδ inhibitor administered at 24 h post-IR. Exposure to 0.5, 2, or 5 Gy IR significantly increased Dextran permeability from control levels to 49–83% at 48 h post-IR exposure (**Fig. 5**). Treatment of cells with the PKCδ inhibitor for 24 h significantly reduced permeability, back to control levels for 0.5 Gy IR treatment, while reducing it by 67 and 70% for 2 and 5 Gy IR exposure, respectively.

PKCδ inhibition attenuates neutrophil migration following endothelial cell irradiation

Neutrophil migration across the endothelium into the tissue compartment was used to assess further endothelial barrier function after irradiation. In bMFA, neutrophil migration across irradiated endothelial cells, in response to fMLP, significantly increased over 60 min at each dose of radiation (**Fig. 6**). Of interest, the increase in neutrophil migration after 5 Gy IR treatment was significantly less pronounced compared with other irradiated groups. Neutrophil migration across IR-treated endothelial cells was significantly reduced after inhibition of PKCδ by 84, 78, and 86% for IR doses of 0.5, 2, and 5 Gy, respectively (Fig. 6). Thus, PKCδ inhibition attenuated neutrophil migration. Furthermore, the PKCδ inhibitor was able to decrease neutrophil migration significantly, even at 5 Gy when neutrophil migration was reduced compared with lower doses of radiation.

PKCδ inhibition attenuates neutrophil adhesion to irradiated endothelium

To explore further the effect of PKCδ inhibition on neutrophil–endothelial cell interaction post-IR, we investigated neutrophil adhesion to endothelial cells under shear flow conditions. IR treatment significantly increased neutrophil adhesion to endothelial cells compared with controls with no significant differences among 0.5, 2, and 5 Gy IR treatment groups (**Fig. 7**). PKCδ inhibition significantly reduced the total number of adhered neutrophils by 51, 64, and 36% for IR doses of 0.5, 2, and 5 Gy, respectively. The reduction in neutrophil adhesion after PKCδ inhibition was most pronounced in vessels with low shear flows and near bifurcations. These findings indicate that PKCδ inhibition significantly reduces neutrophil adhesion to irradiated endothelial cells.

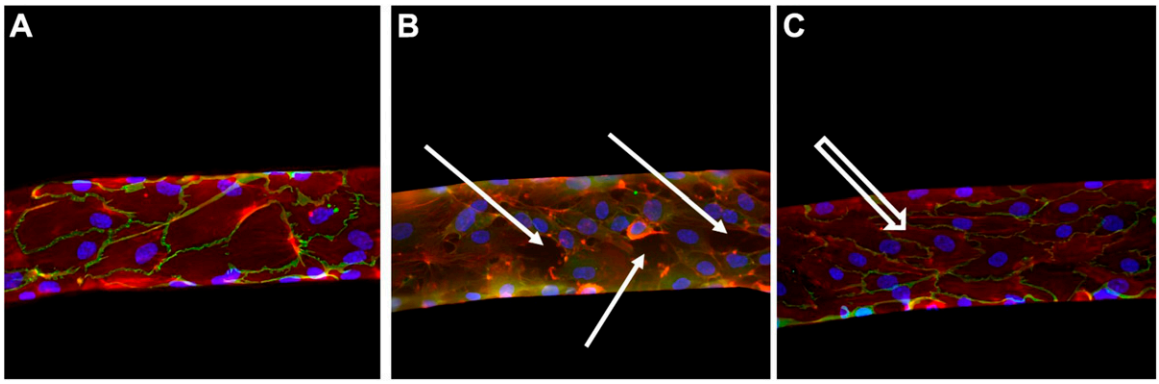

Figure 4. *A*) Under control conditions, endothelial cells are aligned in the direction of flow, whereas in response to 5 Gy IR, endothelial cells are not as well aligned and denuded (solid arrows; *B*). Inhibition of PKCδ, 24 h post-IR, prevents denuding of endothelial cells that align in the direction of flow (open arrow; *C*). Green, VE-cadherin (adherens junction); red, phalloidin (actin filament); blue, Hoechst 33342 (cell nucleus).

PKCδ inhibition downregulates expression of P-selectin, VCAM-1, and ICAM-1 on endothelial cells

Antibody-coated microparticles were used to characterize the role that PKCδ plays in upregulation of adhesion molecules on endothelial cells post-IR (37, 38). Adhesion of microparticles coated with antibodies to E-selectin, P-selectin, ICAM-1, or VCAM-1 to 2 Gy-irradiated endothelial cells was measured 48 h post-IR under the experimental conditions described earlier. Irradiation at 2 Gy significantly increased the adhesion of anti-ICAM-1- and anti-VCAM-1- but not anti-E-selectin- or anti-P-selectin-coated microparticles to endothelial cells (**Fig. 8**). VCAM-1 demonstrated the largest increase in expression. PKCδ inhibition significantly reduced adhesion of anti-P-selectin-, anti-VCAM-1-, and anti-ICAM-1- but not anti-E-selectin-coated microparticles to irradiated endothelial cells (Fig. 8). Hence, PKCδ regulates IR-induced ICAM-1, VCAM-1, and P-selectin expression in endothelial cells.

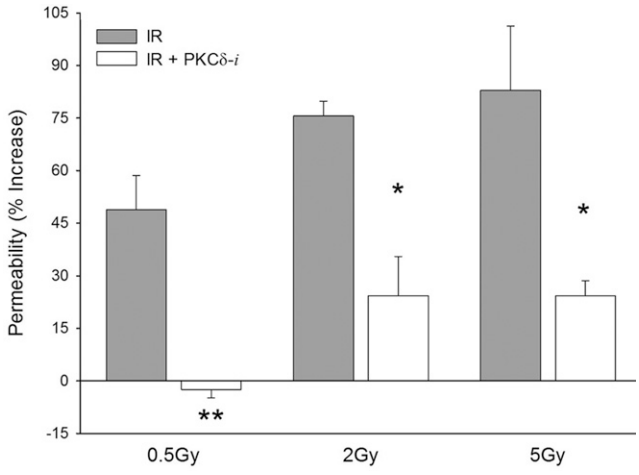

Figure 5. Dextran permeability of irradiated endothelial cells is significantly increased. Treatment of cells with PKCδ-i restores their permeability to control levels (0 Gy). Data are normalized with respect to the permeability of endothelial cells with no treatment. Means ± SEM (n = 3), 2-way ANOVA. *$P < 0.05$, **$P < 0.01$.

DISCUSSION

Interaction of the immune system and endothelial barrier plays an important role in radiation-induced damage through the release of reactive species and cytokines at different time points post-IR (7, 39, 40). Whereas recombinant growth factors, such as recombinant granulocyte colony-stimulating factor (G-CSF; or Neupogen) and recombinant granulocyte-M-CSF (GM-CSF; or Neulasta) have been recently approved by the U.S. Food and Drug Administration for treatment of hematopoietic acute radiation syndrome, their effectiveness depends on "highly trigger-based supportive care" (18, 41) and does not address the IR damage to the endothelium (42, 43). Hence, there is a need for discovery and development of new radiomitigators and radiation medical countermeasures.

Recent studies indicate a role for PKCδ in radiation-induced apoptosis (44–46), radiation-induced cell proliferation anomaly (47), and radiation-induced tissue damage in salivary gland, thymus (24, 25), and thyroid cells (25, 45, 46). However, less is known about how PKCδ is activated in endothelium following radiation exposure or its role in neutrophil–endothelial cell interaction and endothelial cell activation, in part as a result of the lack of realistic fluidic models for *in vitro* reconstitution of disease-related cell types and tissues (48). In this study, we have used a novel bMFA, a physiologically relevant *in vitro* environment (13, 14, 16, 28), to discover mechanisms by which PKCδ impacts neutrophil–endothelial cell interaction during radiation-induced inflammation and to show that a PKCδ-i can significantly downregulate the increased neutrophil–endothelial cell interaction post-IR. We showed that endothelial cells form a 3D lumen in the bMFA to provide a physiologically realistic environment to study cell–cell interactions (Fig. 1). Our results further demonstrate that PKCδ inhibition in irradiated endothelial cells is a potent downregulator of leukocyte–endothelial interaction,

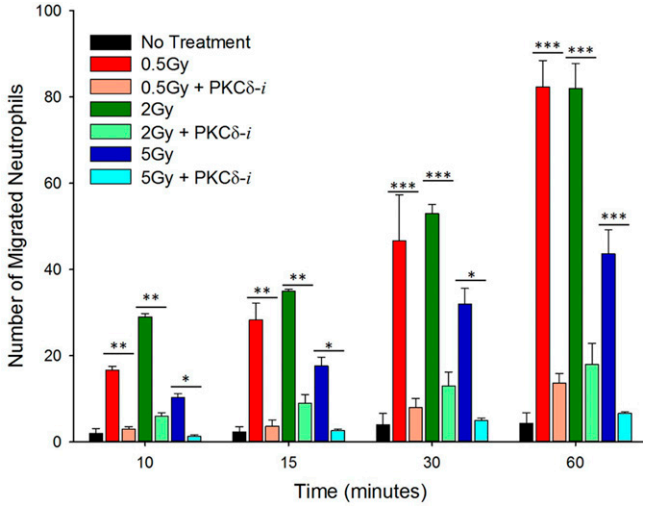

Figure 6. Neutrophil migration across irradiated endothelial cells increases over time by up to 20-fold at 60 min. PKCδ inhibition with PKCδ-i at 24 h post-IR significantly reduces neutrophil migration by up to 82% after 60 min. Means ± SEM ($n = 3$), 2-way ANOVA. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

protects endothelial barrier integrity, and reduces endothelial permeability to a control level even when the inhibitor is administered 24 h postradiation exposure.

A role for PKCδ in inflammation-induced neutrophil–endothelial cell interaction, under both static and flow conditions, has been demonstrated (22, 27). Neutrophil rolling and adhesion on endothelial cells are mediated by selectins (*e.g.*, E- and P-selectin) and integrins (*e.g.*, ICAM-1), whereas the transition to migration is mediated by ICAM-1 and VCAM-1 (49–51). A number of molecules involved in the leukocyte adhesion cascade are also involved in radiation-induced tissue damage. For example, intravital microscopy demonstrated that adhesion molecules (*e.g.*, ICAM-1) are upregulated in irradiated tissue *in vivo*, and the resulting increase in leukocyte adhesion could be modulated with administration of an anti-inflammatory agent (dexamethasone) or an anti-ICAM-1 antibody (5, 10, 52). In this study, we used the physiologically realistic 3D cell-culture environment of bMFA to show that radiation exposure increases neutrophil adhesion and migration across endothelial cells at all radiation doses studied. Interestingly, the increase in neutrophils migration levels was less pronounced at the 5 Gy dose compared with lower doses and may be a result of decreased PKCδ activation at this radiation dose. However, this dose-dependent difference was not observed in neutrophil adhesion levels, suggesting differential regulation by PKCδ. This differential impact of radiation on neutrophil migration *vs.* adhesion indicates that not all adhesion molecules are uniformly impacted by IR. Further studies of neutrophil–endothelial cell interaction and adhesion molecule upregulation under shear flow may be required to understand better the role of IR in the radiation-induced inflammatory process (11, 53, 54).

Consistent with previous reports (15, 27, 37), neutrophil adhesion, as well as its downregulation by PKCδ inhibition, was most pronounced in vessels with low shear flows and near bifurcations. Treatment with the PKCδ inhibitor, even 24 h post-IR, decreases expression of VCAM-1, ICAM-1, P-selectin, but not E-selectin. This decreased expression of adhesion molecules was associated with decreased permeability and decreased neutrophil adherence and migration through irradiated endothelial cells in response to fMLP.

Whereas a role for PKCδ in radiation injury has been established in several cell types (25, 45, 46), the exact role of PKCδ in regulating radiation-induced adhesion molecule expression in endothelial cells is not well established. In endothelial cells, PKCδ is involved in NF-κB activation, adhesion molecule expression, and production of inflammatory mediators important in neutrophil transmigration (22, 55–58). *In vivo*, systemic inflammation (*e.g.*, induced by sepsis) produced increased expression of ICAM-1, which was attenuated by treatment with the PKCδ-i (22, 23). *In vitro* mechanistic studies demonstrated that PKCδ regulated adhesion molecule expression in HUVECs and microvascular endothelial cells (human pulmonary microvascular endothelial cells) (22, 27). Our findings (Fig. 8) support the hypothesis that PKCδ regulates these key components, which are critical to vascular endothelial cell activation after radiation exposure.

In summary, we have used a novel bMFA to study the role of PKCδ as a regulator of human neutrophil–endothelial cell interaction and endothelium integrity postradiation exposure. Our findings indicate a key role for PKCδ regulation of radiation-induced changes in endothelial cell barrier structure and function, expression of several key cell-adhesion molecules, leukocyte–endothelial cell interaction, and leukocyte migration through endothelium. Furthermore, our findings indicate that PKCδ-i can significantly downregulate the increased neutrophil–endothelial cell interaction and

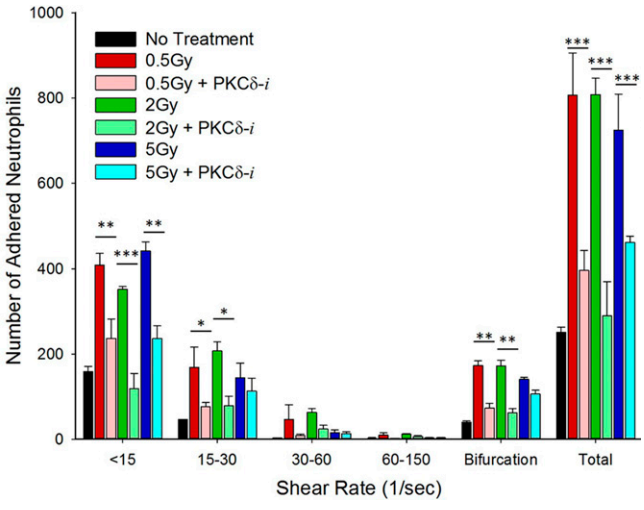

Figure 7. Neutrophil adhesion to endothelial cells significantly increases post-IR, especially in vessels with lower shear rates and near bifurcations. Inhibition of PKCδ with PKCδ-i significantly reduces neutrophil adhesion at shear rates lower than 60 s^{-1} and at bifurcations. Means ± SEM ($n = 3$), 2-way ANOVA. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

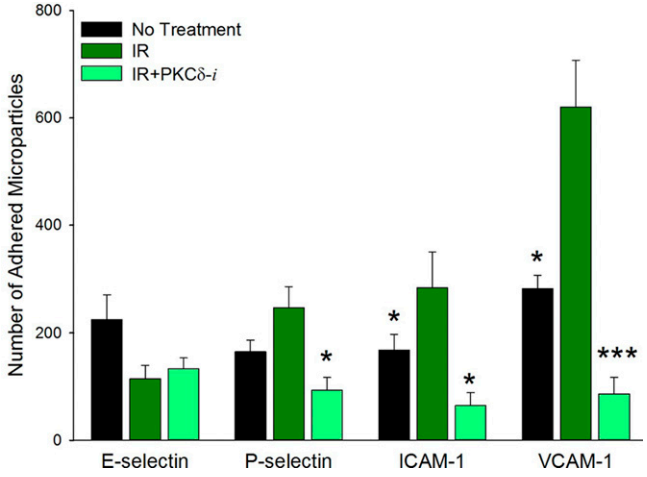

Figure 8. Adhesion of mAb-coated microparticles to endothelial cells. Adhesion of anti-ICAM-1-, anti-VCAM-1-, and anti-P-selectin- but not anti-E-selectin-coated microparticles to endothelial cells significantly increases post-IR compared with control. Inhibition of PKCδ with PKCδ-i, 24 h post-IR, significantly reduces adhesion of anti-ICAM-1-, anti-VCAM-1-, and anti-P-selectin-coated microparticles to endothelial cells. Means ± SEM (n = 3), 1-way ANOVA. $*P < 0.05$, $***P < 0.001$.

preserve endothelial cell integrity post-IR. Therefore, we propose that PKCδ inhibition may serve as a novel medical countermeasure for treating radiation-induced vascular damage. The novel bMFA provides a tool for rapid screening of novel therapeutics for treating radiation injury. FJ

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AUTHOR CONTRIBUTIONS

F. Soroush and Y. Tang performed research, analyzed data, and wrote the manuscript and were also responsible for statistical analyses; H. M. Zaidi and J. B. Sheffield performed, edited, and analyzed imaging and analysis tools; and F. Soroush, Y. Tang, L. E. Kilpatrick, and M. F. Kiani designed the study, organized experiments, analyzed the data, and wrote the manuscript.

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