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## PROTEIN KINASE C-DELTA (PKC $\delta$ ) TYROSINE PHOSPHORYLATION IS A CRITICAL REGULATOR OF NEUTROPHIL-ENDOTHELIAL CELL INTERACTION IN INFLAMMATION

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### Abstract

**Background**—Neutrophil dysfunction plays an important role in inflammation-induced tissue injury. Previously, we identified Protein Kinase C-delta (PKC $\delta$ ) as a critical controller of neutrophil activation and trafficking but how PKC $\delta$  is regulated in inflammation hasn't been delineated. PKC $\delta$  activity is regulated by tyrosine phosphorylation on multiple sites. Tyrosine155 is a key regulator of apoptosis and gene expression, but its role in proinflammatory signaling is not known.

**Methods**—In vitro studies: superoxide anion (O<sub>2</sub><sup>-</sup>) and neutrophil extracellular traps (NETs) were measured in bone marrow neutrophils (BMN) isolated from wild type (WT) and PKC $\delta$ Y155F knock-in mice (PKC $\delta$  tyrosine 155  $\rightarrow$  phenylalanine). Our novel 3D biomimetic microfluidic assay (bMFA) was used to delineate PKC $\delta$ -mediated regulation of individual steps in neutrophil adhesion and migration using WT and PKC $\delta$ Y155F BMN and lung microvascular endothelial cells (MLMVEC). In vivo studies: WT and PKC $\delta$ Y155F knock-in mice underwent sham or cecal ligation and puncture (CLP) surgery and the lungs harvested 24 hrs post-surgery.

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#### Conflict of Interest Disclosure

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**Results**—*In vitro*: PKC $\delta$ Y155F BMN had significantly reduced O<sub>2</sub><sup>-</sup> and NETS release compared to WT. WT BMN, but not PKC $\delta$ Y155F BMN, demonstrated significant adhesion and migration across TNF-activated MLMVEC in bMFA. PKC $\delta$  inhibition significantly reduced WT BMN adhesion and migration under low shear and near bifurcations, but had no effect on PKC $\delta$ Y155F BMN. *In vivo*: mutation of PKC $\delta$  tyrosine 155 significantly decreased neutrophil migration into the lungs of septic mice.

**Conclusions**—PKC $\delta$  tyrosine 155 is a key phosphorylation site controlling proinflammatory signaling and neutrophil-endothelial cell interactions. These studies provide mechanistic insights into PKC $\delta$  regulation during inflammation.

### Keywords

PKC $\delta$ ; tyrosine phosphorylation; superoxide anion; NETS; neutrophil adhesion and migration

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### Introduction

Neutrophil dysfunction plays an important role in inflammatory diseases. Key to inflammation-induced tissue damage is the excessive migration of activated neutrophils across the vascular endothelium (1). While neutrophils are critical to host defense against pathogens, neutrophil dysregulation has a critical role in the early course of organ damage through release of proteases, oxygen radicals and neutrophil extracellular traps (NETs) that can damage the vascular endothelium (1, 2). During inflammation, the release of PAMPS (pathogen-associated molecular patterns) or DAMPS (damage-associated molecular patterns) activate immune cells to release cytokines/chemokines and other proinflammatory mediators that lead to increased adhesion molecule expression on neutrophils and endothelial cells resulting in enhanced neutrophil-endothelial cell interaction, vascular endothelial damage, and organ dysfunction. Neutrophil recruitment is a multi-step cascade that requires crosstalk between neutrophils and endothelial cells and is composed of a series of interactions between receptors and ligands which orchestrate rolling, adhesion, and transmigration (3, 4). Ultimately, arrested neutrophils extravasate to inflamed tissues across endothelial cells via a multi-step process controlled by concurrent chemoattractant-dependent signals, adhesive events, and hemodynamic shear forces (3, 4).

The inflammatory response is composed of multiple overlapping and redundant mechanisms, and recent research has shifted the focus to common control signaling points which are activated by diverse signals. We identified Protein Kinase C-delta (PKC $\delta$ ) as a critical regulator of the inflammatory response (5–8). PKC $\delta$  is a member of the PKC family, a phospholipid-dependent family of serine/threonine kinases, and is expressed in multiple cell types and is an important regulator of neutrophil and endothelial proinflammatory signaling (5, 7, 9). In neutrophils, PKC $\delta$  regulates inflammatory signaling, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) activation, secretion of cytokines/chemokine, and reactive oxygen species (ROS) production (5, 9). In endothelial cells, PKC $\delta$  is involved in NF- $\kappa$ B activation, adhesion molecule expression, and the release of inflammatory mediators important in neutrophil transmigration (7, 10). PKC $\delta$  is activated by inflammatory mediators involved in the inflammatory response including lipopolysaccharide (LPS), tumor necrosis factor (TNF) and interleukin-1 (IL-1) (11, 12). Studies with PKC $\delta$ <sup>-/-</sup>

mice and PKC $\delta$  inhibitors indicate a role for PKC $\delta$  in regulating neutrophil trafficking to the lung in response to inflammation triggered by stroke/reperfusion injury, LPS or pancreatitis (13–15). In recent studies, we demonstrated that PKC $\delta$  is activated in the lungs of septic animals, and PKC $\delta$  inhibition reduced neutrophil influx and was organ protective (6, 7, 10, 16). *In vitro*, PKC $\delta$  inhibition reduced human neutrophil migration across endothelial cells (7, 10). While these studies indicate a role for PKC $\delta$  in regulating neutrophil flux into the lung, they do not address specific mechanisms.

PKC $\delta$  activation requires multi-phosphorylation steps which triggers translocation from the cell cytosol to different subcellular compartments (17). PKC $\delta$ , in contrast to other PKC isotypes, is regulated by tyrosine phosphorylation patterns on multiple sites that determine activation, localization and substrate specificity (17–19). Thus, discrete cellular functions can be regulated by a single kinase through specific phosphorylation patterns. Phosphorylation of PKC $\delta$  tyrosine 155 in the regulatory domain regulates apoptosis and gene expression (19). However, the role of tyrosine 155 on pro-inflammatory signaling has not been studied. Our recent studies in a rodent model of sepsis (cecal ligation and puncture) demonstrated that sepsis triggered PKC $\delta$  activation and tyrosine 155 phosphorylation in lung endothelium suggesting a role for PKC $\delta$  tyrosine 155 phosphorylation in neutrophil-endothelial interaction (6, 16).

In this study, we used our novel 3D biomimetic microfluidic assay (bmFA) to investigate the role of PKC $\delta$  and PKC $\delta$  tyrosine 155 phosphorylation in neutrophil activation and neutrophil-endothelial cell interaction using pharmacologic (PKC $\delta$ -TAT peptide inhibitor) and genetic (PKC $\delta$  knock-in mice where PKC $\delta$  tyrosine 155 was mutated to phenylalanine: PKC $\delta$ Y155F KI mice) approaches. We then investigated the impact of mutation of PKC $\delta$  tyrosine 155 on neutrophil migration into the lungs of septic mice. We tested the hypothesis that PKC $\delta$  is an important regulator of neutrophil activation and migration and that PKC $\delta$  tyrosine 155 is a critical phosphorylation site.

## Materials and Methods

### Materials and Reagents

Mouse fibronectin (FN) was obtained from BD Biosciences (San Jose, CA). Mouse lung microvascular endothelial cells (MLMVEC) and mouse microvascular endothelial Growth Medium (EGM) were purchased from Cell Biologics (Chicago, IL). Carboxyfluorescein diacetate succinimidyl ester (CFDA/SE) and SYTOX green probes from Molecular Probes (Carlsbad, CA), Hanks' Balanced Salt Solution (HBSS), Trypsin/EDTA, Formalin, Triton X-100, Draq5, 40kDa Texas Red conjugated dextran, and Hoechst 33342 from Thermofisher Scientific (Rockford, IL), and Alexa Fluor® 488 Phalloidin from Life Technologies Corporation (Carlsbad, CA). Recombinant mouse TNF- $\alpha$  was purchased from EMD Millipore (Burlington, MA). Phorbol myristate acetate (PMA), N-Formylmethionyl-leucyl-phenylalanine (fMLP), cytochalasin B, and cytochrome c were purchased from Sigma-Aldrich (St. Louis, MO).

### Generation of PKC $\delta$ Y155F knock-in mice

In PKC $\delta$ , tyrosine 155 is located in the C1 domain of the regulatory motif and is a conserved site expressed in humans and mice. PKC $\delta$  knock-in mice were generated at the University of Connecticut as outlined in Figure 1 where PKC $\delta$  tyrosine 155 was mutated to phenylalanine. Murine PKC $\delta$  is a 674-amino acid protein consisting of 18 exons and tyrosine 155 is located on exon-5 of PKC $\delta$ . The Y155F mutation was introduced into exon-5 of PRKCD locus in mice embryonic stem cells by homologous recombination (Figure 1). The clones positive for Y155F mutation were confirmed by dual selection using G418 and Gancyclovir along with PCR and sequencing. The resulting chimeras were bred with transgenic mice expressing Cre recombinase to remove the PGKneo cassette. The PKC $\delta$ Y155F mice were identified by PCR containing a copy of LoxP in intron-6 which is 271 bp product compared to 181 bp wildtype littermate control (Figure 1B). The mutation in the PCR product was confirmed by DNA sequence analysis (not shown). The Y155F mutation was further confirmed by another PCR using oligonucleotides that recognize the mutant allele (Figure 1C). PKC $\delta$ Y155F mice are viable and follow predicted mendelian ratios. Age matched male and female C57BL6 mice (Jackson Laboratories) and C57BL6/jX129sv mice (in house breeding) were used as wild type (WT) controls. There were no significant differences in neutrophil activity ( $O_2^-$  production and NETs release) or endothelial cell activity between the two strains.

### Inhibitor Peptide Synthesis

As described previously (5–7, 9, 10, 16), PKC $\delta$  activity was selectively inhibited by a peptide antagonist that consisted of a peptide derived from the first unique region (V1) of PKC $\delta$  (SFNSYELGSL: amino acids 8–17) coupled via an N-terminal Cys-Cys bond to a membrane permeant peptide sequence in the HIV TAT gene product (YGRKKRRQRRR: amino acids 47–57 of TAT) (20). Extensive *in vitro* and *in vivo* studies demonstrate that, when taken up by cells, the PKC $\delta$  TAT peptide produces a unique dominant-negative phenotype that effectively inhibits activation of PKC $\delta$  but not other PKC isotypes (5, 20). Further studies demonstrate that the TAT peptide alone is nontoxic and does not alter PKC $\delta$  activity (5, 9). The peptide was synthesized by Mimotopes (Melbourne, Australia) by 9-fluorenylmethoxycarbonyl solid-phase chemistry. Peptides were purified to >95% by preparative reverse-phase HPLC.

### Animal Protocols

Animal procedures and handling were conducted in accordance to National Institutes of Health standards and were approved by the Institutional Animal Care and Use Committee at the Lewis Katz School of Medicine at Temple University (Philadelphia, PA, USA). Male and female mice (25–30g) were housed in a climate controlled facility and given free access to food and water.

### CLP Model

Sepsis was induced by cecal ligation and puncture (CLP) as we described previously (6, 7, 16, 21). Sham surgery animals are subjected to sham laparotomy without cecal ligation or puncture. Briefly for studies using septic mice, under isoflurane anesthesia, a midline laparotomy was performed, the cecum identified, the mesentery trimmed, and the stalk

joining the cecum to the large intestine was ligated. The cecum was punctured with a 21 gauge needle, stool expressed, the cecum returned to the abdomen, and the incision was closed in two layers. Mice were fluid resuscitated with sterile saline administered subcutaneously. At 24hrs post-surgery, the mice were anesthetized and the lungs perfused with sterile PBS, harvested and stored at  $-70^{\circ}\text{C}$ .

### Mouse Bone Marrow Neutrophil Isolation

To obtain mouse bone marrow neutrophils, PKC $\delta$ Y155 and WT mice were euthanized and the femur and tibiae from both hind legs were harvested. The distal tip of each bone was cut off, bones were rinsed using HBSS, and cell clumps were dispersed. Neutrophils were isolated using a Percoll gradient sedimentation, followed by hypotonic lysis to remove erythrocytes.

### Mouse lung endothelial cell isolation

To obtain mouse pulmonary endothelial cells, PKC $\delta$ Y155 and WT mice were anesthetized with isoflurane, the lungs removed aseptically and the animals euthanized. The lungs were minced, digested with collagenase/dispase, and mechanically dispersed to produce a single cell solution (22). The pulmonary endothelial cells are isolated by positive selection using a PECAM-1 antibody conjugated to magnetic beads. The isolated cells were cultured on gelatin coated flasks until confluent and then further purified using ICAM-2 antibody conjugated to magnetic beads. The purified endothelial cells were maintained in culture until use.

### Superoxide Anion Generation

Superoxide anion ( $\text{O}_2^-$ ) generation was measured spectrophotometrically as superoxide-dismutase (SOD)-inhibitable cytochrome c reduction. Bone marrow neutrophils ( $2 \times 10^6$ ) were activated with PMA ( $1\mu\text{g/ml}$ ) or fMLP ( $10^{-8}\text{M}$ ) in the presence of  $5\mu\text{g/ml}$  cytochalasin B and the generation of  $\text{O}_2^-$  monitored over a 10 min time period (9). For experiments employing TNF as a stimulus, 96 well plates were coated with fibronectin, and cells were allowed to adhere for 30 minutes prior to addition of TNF ( $50\text{ng/ml}$ ) and  $\text{O}_2^-$  production measured over a 120 min time period. To examine the effects of complete PKC $\delta$  inhibition, WT neutrophils were pretreated with buffer or the PKC $\delta$ -TAT peptide inhibitor (PKC $\delta$ -*i*) ( $5\mu\text{M}$ ) as described previously (9).

### NETs formation

NETs production was measured fluorometrically using an excitation wavelength of 492 nm and an emission wavelength of 530nm by monitoring DNA release from WT and 155KI bone marrow neutrophils.  $2 \times 10^5$  neutrophils/well were seeded in 96 well black plates with 0.5% FBS and the membrane-impermeable DNA binding dye SYTOX green (Molecular Probes, Invitrogen) ( $5\mu\text{M}$ ). Neutrophils were incubated with buffer, TNF ( $50\text{ ng/ml}$ ), PMA ( $30\text{ nM}$ ) or IL-1 ( $10\text{ U/ml}$ ). WT neutrophil NETs production was measured  $\pm$   $\delta$ -PKC TAT inhibitor ( $5\mu\text{M}$ ). The plates were incubated at  $37^{\circ}\text{C}$ , and DNA release from bone marrow neutrophils was monitored by Sytox Green fluorescence at 0, 1, 2, 3, and 4 hours. Specificity of the reaction was determined by treatment with DNase ( $200\text{U/ml}$ ).

## Lung Myeloperoxidase Activity

Myeloperoxidase enzymatic activity in lung tissue was measured as we described previously (21). Lung tissue was homogenized and sonicated. Homogenates were cleared by centrifugation, and MPO levels were determined using a MPO assay kit according to the manufacturer's instructions (Cayman, USA).

## Design and Fabrication of the Microfluidic Assay

The methods for design and fabrication of the novel microfluidic assay and its *in vivo* validation have been previously published (10, 23). This microfluidics system used in our studies (bMFA) is one of the few devices that realistically reproduces the entire leukocyte adhesion cascade in a single assay encompassing circulation, rolling, adhesion and migration of leukocytes in a physiologically realistic three-dimensional environment under physiologically-relevant flow conditions. Briefly, a modified Geographic Information System (GIS) approach was used to digitize the *in vivo* microvascular networks which were lithographically patterned on polydimethylsiloxane (PDMS). To mimic the *in vivo* conditions, this novel microfluidics system consists of vascular channels in which cultured endothelial cells form a continuous lumen that is in communication with a tissue compartment filled with chemoattractants (e.g. fMLP). Microfabricated pillars (10  $\mu\text{m}$  diameter) were used to fabricate the  $3 \times 100 \mu\text{m}$  pores resulting in a network of vascular channels connected to a tissue compartment via a 3  $\mu\text{m}$  porous barrier, which is the optimum size for neutrophil migration. Leukocytes circulate in the vascular channels and interact with the endothelial cells under physiologic shear flow conditions. In agreement with the flow parameters measured in the vessels of original microvascular network *in vivo* (23), shear rates used in the vascular channels of bMFA ranged from 0–150  $\text{sec}^{-1}$ . The vascular channels form a realistic microvascular network with realistic geometry (including bifurcating capillaries) reproduced from microvascular networks observed *in vivo* (23). We have extensively validated this system and demonstrated that the adhesion pattern of neutrophils in bMFA is very similar to those observed *in vivo* by intravital microscopy (23).

## Seeding of Endothelial Cells in the biomimetic MicroFluidic Assay (bMFA)

Mouse lung microvascular endothelial cells isolated from WT and PKC $\delta$ Y155 mice were cultured in EGM and used between passages 3–6. The bMFA were coated with fibronectin and endothelial cells were cultured at 37 °C and 5% CO<sub>2</sub> under shear flow (inlet flow rate of 0.1  $\mu\text{l}/\text{min}$ ) for 48h (23). Consistent with our published data (10, 24, 25), endothelial cells in bMFA form a confluent lumen and aligned in the direction of flow (Figure 2). Formation of the 3D lumen in vascular channels under physiological conditions was confirmed using confocal microscopy (Figure 2) (10, 24). In agreement with the observed complexity of *in vivo* flow conditions, shear stress is different in different vessels of bMFA due to flow distribution in the complex *in vivo*-like geometry. However, by keeping inlet flow conditions constant, shear stress in a given vessel is the same across different measurements. Assays in which neutrophils freely entered the tissue compartment without attachment were discarded.

A Nikon TE200 fluorescence microscope equipped with an automated stage was used for performing experiments. Images were acquired using an ORCA Flash 4 camera (Hamamatsu Corp., USA). PhD Ultra Syringe pump (Harvard Apparatus) 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 Inc., Melville, NY) was used to control the microscope stage and the camera.

### Adhesion and Migration Experiments

Isolated neutrophils were labeled in suspension using CFDA/SE probe for 10 min at room temperature. Neutrophils were treated with either TNF- $\alpha$  (10U/mL) or TNF- $\alpha$  (10U/mL) + PKC $\delta$ -TAT peptide inhibitor (5 $\mu$ M, PKC $\delta$ -*i*) for 10 min before injection into bMFA. Neutrophils were introduced in vascular channels at an inlet flow rate of 1  $\mu$ l/min. WT or PKC $\delta$ Y155F KI lung endothelial cells were treated with TNF (10U/ml) for 4 hrs. For PKC $\delta$ -*i* treatment, a solution of TNF- $\alpha$  and PKC $\delta$ -*i* (5 $\mu$ M) was injected into the network. At 4 hours post TNF- $\alpha$  with or without PKC $\delta$ -*i* treatment, the tissue compartment was filled with buffer (control) or fMLP (1  $\mu$ M) before injecting neutrophils in the vascular compartment. Fluorescently labeled BMN isolated from WT or PKC $\delta$ Y155F KI mice were introduced in the vascular compartment (5 X 10<sup>6</sup> cells/ml). Cells that did not move for 30 s were considered adherent. Adhesion level of neutrophils to endothelial cells 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 every 3 min for 60 min. Nikon Elements and Fiji software were used to collect and analyze the data (10).

### Permeability Measurements

The vascular compartment of the bMFA was connected to a Hamilton gas tight syringe filled with Texas Red 40 kDa dextran (25  $\mu$ M in EGM) mounted on a syringe pump. Permeability was measured by imaging the bMFA every minute for 2h while the dextran solution flowed through the vascular channels (flow rate 1  $\mu$ l/min). Using our previously published method (24), the following equation was used to calculate permeability (P) of dextran across the endothelium in bMFA:

$$P = \frac{1}{I_{v0}} \frac{V}{S} \frac{dI_t}{dt} \quad (1)$$

where  $I_t$  is the average intensity in the tissue compartment,  $I_{v0}$  is the maximum fluorescence intensity of the vascular channel and V/S is the ratio of vascular channel volume to its surface area.

### Statistical Analysis

Data are presented as Mean  $\pm$  SEM. All numerical data passed the Shapiro-Wilk normality test. Statistical significance was determined by one-way or two-way analysis of variance (ANOVA) with Holm-Šidák method post-hoc using SigmaPlot software for multiple group comparisons. For comparison of 2 groups, a Student t-test was employed. Differences were considered statistically significant if  $p < 0.05$ .

## Results

### Impact of PKC $\delta$ Tyr155 phosphorylation on neutrophil function

The role of PKC $\delta$  Tyr155 phosphorylation in neutrophil activation has not been delineated. In these studies, we examined the role of PKC $\delta$  and PKC $\delta$  Tyr155 phosphorylation in superoxide anion ( $O_2^-$ ) generation in response to PMA (1 $\mu$ g/ml), fMLP ( $10^{-8}$ M) and TNF (50ng/ml).  $O_2^-$  production was measured as SOD-inhibitable cytochrome c reduction in bone marrow neutrophils isolated from WT and PKC $\delta$ Y155F KI mice. As shown in Figures 3 and 4, mouse bone marrow neutrophils generated significant quantities of  $O_2^-$  in response to PMA, fMLP and TNF. In the absence of PKC $\delta$  Tyr155 phosphorylation, fMLP-stimulated  $O_2^-$  production was inhibited by 42% as compared to WT  $O_2^-$  production but had no significant effect on PMA, an activator of multiple PKC isotypes, mediated  $O_2^-$  generation (Figure 3).

Full activation of neutrophils by proinflammatory mediators, such as TNF, requires adherence and ligation of integrins (9). Adherence of human neutrophils to extracellular matrix (ECM) proteins such as FN produces significant alterations in the kinetics of oxygen radical production in response to soluble mediators. There is a significant delay of approximately 60 min, followed by  $O_2^-$  generation, which is enhanced significantly as compared with non-adherent neutrophil  $O_2^-$  generation. In WT BMN, PKC $\delta$  inhibition significantly reduced  $O_2^-$  production in response to TNF (Figure 4A,  $p < 0.01$ ). In PKC $\delta$ Y155F KI BMN,  $O_2^-$  production was also significantly decreased in response to TNF (Figure 4A,  $p < 0.05$ ). The  $V_{max}$  of the reaction was also significantly reduced in PKC $\delta$ Y155F KI BMN and WT BMN treated with the PKC $\delta$  inhibitor as compared to WT BMN (Figure 4B,  $p < 0.01$ ). Further analysis of the kinetics of  $O_2^-$  production demonstrated a significant increase in the lag time to  $O_2^-$  production in both the PKC $\delta$ Y155F KI BMN and WT BMN treated with the PKC $\delta$  inhibitor as compared to WT BMN suggesting a role for PKC $\delta$  in the assembly of the activated NADPH oxidase (Figure 4C,  $p < 0.05$ ).

NET formation was measured fluorometrically in BMN by monitoring DNA release. WT BMN produced NETs in response to PMA, IL-1 and TNF. PKC $\delta$  inhibition in WT BMN significantly decreased NETs in response to TNF ( $p < 0.05$ ) and IL-1 ( $p < 0.05$ ), but not PMA (Figure 5A and 5B). NET formation was also significantly attenuated in response to IL-1 ( $P < 0.05$ ) and TNF ( $P < 0.05$ ) in PKC $\delta$ Y155F KI BMN as compared to WT BMN (Figure 5C). Thus, PKC $\delta$  is an important regulator of  $O_2^-$  and NETs release, key components of neutrophil-mediated damage of vascular endothelium during inflammation. Furthermore, PKC $\delta$  tyrosine155 is a key phosphorylation site controlling neutrophil proinflammatory signaling.

### Impact of PKC $\delta$ Tyr155 phosphorylation on endothelial cell function

PKC $\delta$  is also expressed in endothelial cells and plays an important role in cell function, but the role of PKC $\delta$  tyrosine155 phosphorylation in endothelial cell activation is not known. We next examined the role of PKC $\delta$  tyrosine155 phosphorylation on endothelial cell structure and function. We utilized our novel bMFA to determine the role of PKC $\delta$  and Tyr155 phosphorylation on endothelial cell permeability in response to inflammation. The



expression of f-actin filaments in lung microvascular endothelial cells from WT and PKC $\delta$ Y155F KI mice under physiologically relevant flow condition was investigated using immunofluorescence staining and confocal microscopy. Under flow conditions, WT and PKC $\delta$ Y155F KI endothelial cells completely covered the vascular channels, formed a 3D lumen and aligned in the direction of flow (Figure 2).

The integrity of the endothelial cell barrier was directly assessed in the bMFA by measuring 40 kDa dextran permeation from the vascular channels to the tissue compartment. Permeability rates across the endothelial barrier were measured in WT and PKC $\delta$ Y155F KI endothelial cells under the following conditions: no treatment, TNF- $\alpha$  treatment (4h activation) and TNF- $\alpha$  +PKC $\delta$ -TAT peptide inhibitor (PKC $\delta$ -*i*) treatment (4 h activation). Permeability was similar between PKC $\delta$ Y155F KI endothelial cells and WT endothelial cells under control conditions (No Treatment) (Figure 6). TNF- $\alpha$  treatment significantly increased dextran permeability by 84% in WT but not to PKC $\delta$ Y155F KI endothelial cells (Figure 6). Treatment of cells with the PKC $\delta$ -*i* significantly reduced the permeability rates in WT endothelial cells to control levels (No Treatment) from  $7.9 \times 10^{-7}$  to  $4.7 \times 10^{-7}$  cm/s in WT cells but had no significant effect on permeability of PKC $\delta$ Y155F KI cells (Figure 6).

### **PKC $\delta$ Tyr155 Phosphorylation differentially impacts adhesion and migration of neutrophils to endothelial cells in bMFA**

To delineate the mechanism by which PKC $\delta$  regulates individual steps in neutrophil-endothelial cell interaction during inflammation, we utilized the bMFA to ascertain the role of PKC $\delta$  and PKC $\delta$  Tyr155 phosphorylation on the spatial distribution of adhering/migrating neutrophils. As shown in Figure 7A, there was significant migration of WT BMN across TNF-activated WT endothelial cells in response to the chemoattractant fMLP as compared to No Treatment (Figure 7A). Incubation with the PKC $\delta$  TAT peptide inhibitor resulted in a significant ( $p < 0.001$  at 60 minutes) decrease in WT BMN migration. In contrast, while baseline (No Treatment) migration of PKC $\delta$ Y155F BMN across WT lung endothelial cells (Figure 7B) was similar to baseline (No Treatment) migration of WT BMN across WT endothelial cells (Figure 7A), migration of PKC $\delta$ Y155F BMN across WT lung endothelial cells was not increased in response to TNF/fMLP activation (Figure 7B). Treatment with the PKC $\delta$  inhibitor did not result in further inhibition of migration of PKC $\delta$ Y155F BMN across WT lung endothelial cells. Furthermore, migration of PKC $\delta$ Y155F BMN across PKC $\delta$ Y155F KI lung microvascular endothelial cells was also significantly reduced as compared to WT BMN and was not increased in response to TNF/fMLP stimulation (Figure 7C). Again, treatment with the PKC $\delta$  inhibitor did not result in further inhibition of migration. The migration patterns of PKC $\delta$ Y155F BMN across WT endothelial cells (Figure 7B) were not significantly different from their migration pattern across PKC $\delta$ Y155F KI endothelial cells (Figure 7C) supporting the hypothesis that in neutrophils, and not endothelial cells, PKC $\delta$  Tyr155 phosphorylation is the dominant regulatory mechanism regulating migration across endothelial cells.

Neutrophil adhesion to endothelium under shear flow precedes its migration and is a determinant of its efficiency. Activation with TNF- $\alpha$  resulted in a significant increase in WT BMN adhesion to WT lung endothelial cells. As shown in Figure 8, BMN preferentially

adhered to activated endothelial cells near bifurcations and in regions of low shear with minimal adhesion in high shear regions (shear rate  $> 30 \text{ s}^{-1}$ ), indicating that flow conditions strongly influence cell adhesion in these microvascular networks (10). PKC $\delta$  inhibition resulted in a significant reduction in BMN adherence (Figure 8A). In contrast, TNF-activation or treatment with the PKC $\delta$ -*i* did not impact adhesion of PKC $\delta$ Y155F KI BMN to WT endothelial cells (Figure 8B). Similarly, TNF-activation did not increase adhesion of PKC $\delta$ Y155F KI BMN to PKC $\delta$ Y155F KI lung endothelial cells (Figure 8C). Furthermore, treatment with the PKC $\delta$  inhibitor did not significantly alter PKC $\delta$ Y155F KI BMN adhesion to PKC $\delta$ Y155F KI lung microvascular endothelial cells (Figure 8C). Moreover, the adhesion level of PKC $\delta$ Y155F BMN to PKC $\delta$ Y155F endothelial cells (Figure 8C) was significantly different from its adhesion level of WT BMN to WT endothelial cells (Figure 8A), but not significantly different from adhesion level of PKC $\delta$ Y155F BMN to WT endothelial cells (Figure 8B), supporting the hypothesis that both neutrophil and endothelial PKC $\delta$  Tyr155 phosphorylation play a role in regulating adhesion of neutrophils to endothelial cells.

### Impact of PKC $\delta$ Tyr155 phosphorylation on neutrophil recruitment to the lung in sepsis

Using our bMFA *in vitro* model system, we demonstrated that PKC $\delta$  is a critical regulator of murine neutrophil adhesion and migration through PMVEC in response to inflammation, and the Tyr155 phosphorylation site is critical to neutrophil adhesion and migration through pulmonary endothelium. We next sought to confirm that PKC $\delta$  Tyr155 phosphorylation had an important role regulating neutrophil migration into the lung *in vivo* in response to sepsis induced by CLP. As shown in Figure 9, lung MPO activity, a marker of neutrophil infiltration, increased significantly in WT mice 24 hrs post CLP surgery as compared to WT sham mice. When sepsis was induced in PKC $\delta$ Y155F KI mice, MPO activity was reduced 66% as compared to WT mice. Thus, we have demonstrated both *in vitro* and *in vivo* that this tyrosine phosphorylation site has an important role in regulating neutrophil activation and migration through pulmonary endothelium.

## Discussion

Previous studies from our group (5–7, 9, 10, 16) have demonstrated a key role for PKC $\delta$  in the regulation of proinflammatory signaling. How PKC $\delta$  is activated under proinflammatory conditions has not been fully delineated but site-specific tyrosine phosphorylation may be a critical step. The results of the present study demonstrate for the first time that PKC $\delta$  tyrosine 155 phosphorylation plays an important regulatory role in neutrophil activation and migration. We demonstrate that this phosphorylation site on PKC $\delta$  is critical for O $_2$ -generation in response to TNF and the bacterial peptide fMLP but is not required for PMA-stimulated O $_2$ -production. We further demonstrate that PKC $\delta$  tyrosine 155 phosphorylation is also required for NETs formation and the extracellular release of DNA. The regulatory role of 155-tyrosine phosphorylation is not limited to neutrophils but also has a key role in the regulation of endothelial cell permeability, neutrophil adherence and transmigration. Lastly, we show that this phosphorylation site has a key role regulating neutrophil recruitment to the lung in sepsis induced by CLP. Thus, we have demonstrated both *in vitro* and *in vivo* that this tyrosine phosphorylation site has an important role in regulating

neutrophil activation and migration through pulmonary endothelium. These studies provide mechanistic insights into the regulation of PKC $\delta$  during inflammation.

PKC $\delta$ , in contrast to other members of the PKC family, is regulated by tyrosine phosphorylation on multiple sites including phosphorylation sites in PKC $\delta$  regulatory domain (Tyr<sup>52</sup>, Tyr<sup>155</sup>, and Tyr<sup>187</sup>), catalytic domain (Tyr<sup>512</sup> and Tyr<sup>523</sup>), and hinge region (Tyr<sup>311</sup> and Tyr<sup>332</sup>) (26). These tyrosine phosphorylation patterns determine activation, localization and substrate specificity (17–19). Tyrosine phosphorylation in the catalytic domain of PKC $\delta$  increases kinase activity, whereas tyrosine phosphorylation in the regulatory domain mediates cellular actions without influencing kinase activity (17). Furthermore, tyrosine phosphorylation may also regulate protein-protein interactions and serve as docking sites for other proteins (26). Thus, discrete cellular functions are regulated by a single kinase through specific phosphorylation patterns and can be positive or negative regulators of cell function. In support of this concept, we previously demonstrated that PKC $\delta$  differentially regulates dense granule secretion in human platelets (27), and specific PKC $\delta$  tyrosine phosphorylations were stimulus-dependent. These results indicate that PKC $\delta$  tyrosine residues are differentially phosphorylated, and phosphorylation can regulate discrete cellular events that are cell type and stimulus dependent (17).

Our previous studies demonstrated an important role for PKC $\delta$  in TNF-mediated O<sub>2</sub><sup>-</sup> production in systemic human neutrophils through PKC $\delta$  association and phosphorylation of the p47phox component of the NADPH oxidase enzyme complex (9). In this study, we establish that PKC $\delta$  is also a key regulator of murine O<sub>2</sub><sup>-</sup> production in bone marrow neutrophils and that tyrosine 155 phosphorylation is a key phosphorylation site. Treatment of WT bone marrow neutrophils with the PKC $\delta$ -TAT peptide inhibitor resulted in reduced O<sub>2</sub><sup>-</sup> production in response to TNF and fMLP, but not to PMA. A similar decrease in O<sub>2</sub><sup>-</sup> generation in response to TNF was reported in bone marrow neutrophils isolated from PKC $\delta$ <sup>-/-</sup> mice (13). In the current study, we demonstrate for the first time that PKC $\delta$  tyrosine 155 is a critical phosphorylation site in regulating O<sub>2</sub><sup>-</sup> generation stimulated by TNF and fMLP, but not PMA. The finding that the response to PMA was unchanged in response to the PKC $\delta$  inhibitor or in the PKC $\delta$ Y155F cells indicate that the requirement for PKC $\delta$  activity and tyrosine 155 phosphorylation is stimulus dependent.

During inflammation, activated neutrophils can release NETS composed of chromatin filaments studded with histones and granular proteins such as MPO, elastase and MMP9. While NETs are important in pathogen sequestration and killing, NETS can also damage the vascular endothelium and have an important role in the pathophysiology of sepsis-induced tissue damage (2). NETS formation can be triggered by inflammatory stimuli such as IL-1 and TNF (2), but the signaling pathways that regulate NET formation have yet to be fully elucidated. NET formation requires NADPH oxidase activity and activation of the MEK-ERK pathway (2, 28). PMA, an activator of multiple PKC isoforms including PKC $\delta$ , is a potent stimulus of NET formation suggesting that PKC $\delta$  may have a role in regulating NET formation. PKC $\delta$  regulates TNF-mediated ERK activation and the NADPH oxidase in neutrophils (this study and (5, 9). In this study, we found that PKC $\delta$  inhibition significantly decreased NET formation in response to TNF and IL-1, but not PMA. Furthermore, neutrophil PKC $\delta$  tyrosine 155 phosphorylation is required for TNF and IL-1 triggered NET

formation but not PMA-induced NET formation indicating that PMA-induced NETs is PKC $\delta$  independent. In support of this concept, a role for PKC $\beta$ , but not PKC $\delta$ , in PMA-induced NETs was previously reported (29).

During inflammation, mediators damage the vascular endothelium resulting in increased permeability and excessive neutrophil migration into critical organs, with the lung being an early target. The vascular endothelium is an active participant in the dynamic process of neutrophil recruitment and activation through production of chemokines/cytokines and adhesion molecule expression (1, 3). To further investigate the role of PKC $\delta$  Tyr155 phosphorylation, we have used a novel biomimetic microfluidic assay (bMFA) that reproduces the endothelial barrier function and entire neutrophil adhesion cascade in a physiologically realistic three-dimensional environment and has already been validated against *in vivo* data (23, 25). We have previously shown that PKC $\delta$  has a critical role in regulating human neutrophil-endothelial cells interaction (10) but its molecular mechanisms were not identified. In this study, our findings indicate that PKC $\delta$  is a critical regulator of pulmonary endothelial cells permeability and murine neutrophil-endothelial cells interaction cascade during the inflammation. Moreover, we show that the Tyr155 phosphorylation site is a critical regulator for microvascular endothelium barrier function, neutrophil adhesion to, and migration through pulmonary endothelium. Consistent with our previous findings (10, 30), PKC $\delta$  was found to play a more significant role in regulating migration of neutrophils across endothelial cells as opposed to their adhesion to endothelial cells.

Previously, we demonstrated that in CLP-induced sepsis, PKC $\delta$  is activated in the lungs, is phosphorylated on tyrosine 155, and the kinase translocated to the nucleus, a process regulated by tyrosine 155 phosphorylation (6, 16, 31, 32). In these studies, we demonstrate for the first time that PKC $\delta$  tyrosine 155 phosphorylation is an important regulator of PKC $\delta$  proinflammatory activity during sepsis regulating neutrophil influx into the lung. Our studies show that PKC $\delta$ Y155F mice had significantly decreased MPO activity in their lungs as compared to WT mice 24 hrs post CLP, similar to what we previously observed following treatment with the PKC $\delta$ -TAT inhibitor (6, 7, 10, 16). The finding that sepsis triggers PKC $\delta$  tyrosine155 phosphorylation and nuclear translocation is important as these cellular events are also associated with tissue injury following ischemia-reperfusion and radiation damage (33–35).

In this study we used a novel microfluidic assay to mimic many aspects of the complex physiological conditions that regulate leukocyte adhesion/migration and leukocyte-endothelial interaction. Complete microvascular networks, including post-capillary venules, are reproduced in this *in vitro* model resulting in complex flows and a range of shear rates similar to those observed *in vivo* (10, 23–25, 30). Nevertheless, as with any other *in vivo* or *in vitro* model of inflammation, bMFA does not reproduce the entirety of the clinical condition. For example, in this study isolated neutrophils were in EGM as they interacted with endothelial cells that formed the 3D vessel lumen in the bMFA. Further studies may be required to determine whether the presence of other blood components may alter the observed neutrophil-endothelial interaction patterns. Similarly, the version of bMFA used in this study has a uniform diameter of 100  $\mu$ m in all vessels and additional studies may shed further light on the possible effects of diameter variability on neutrophil-endothelial

interaction patterns. Furthermore, other perivascular cell types, such as smooth muscle cells, can be co-cultured in the tissue compartment of bMFA with endothelial cells to provide a more realistic environment for studying the inflammatory response. Nevertheless, given the extensive validation of this bMFA against *in vivo* data (23–25) and the overall agreement between the findings from various methods presented in this study, we believe that our results provide a novel approach for better understanding the inflammatory response.

These findings provide a clear indication that PKC $\delta$  Tyr155 phosphorylation is not only an important molecular regulator of endothelial cell function but also plays a critical role in regulating the interaction of neutrophils with endothelial cells at a functional level. Overall, these findings indicate that regulating the PKC $\delta$  pathway may provide novel therapeutic strategies for treating inflammation.

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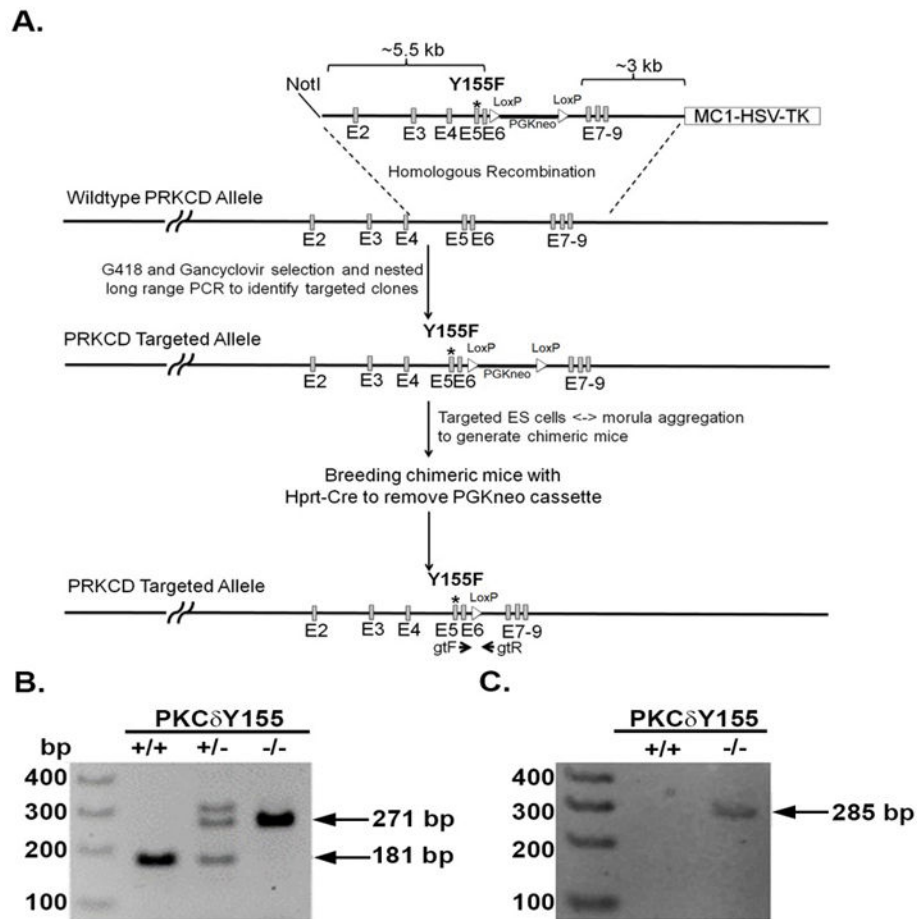
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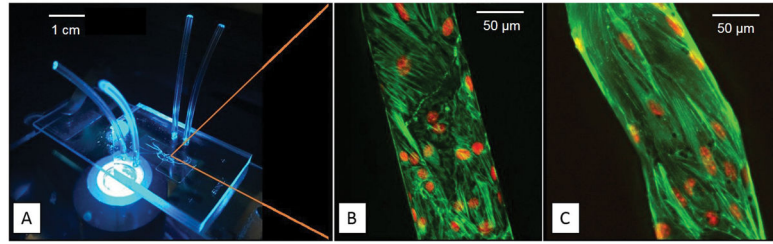
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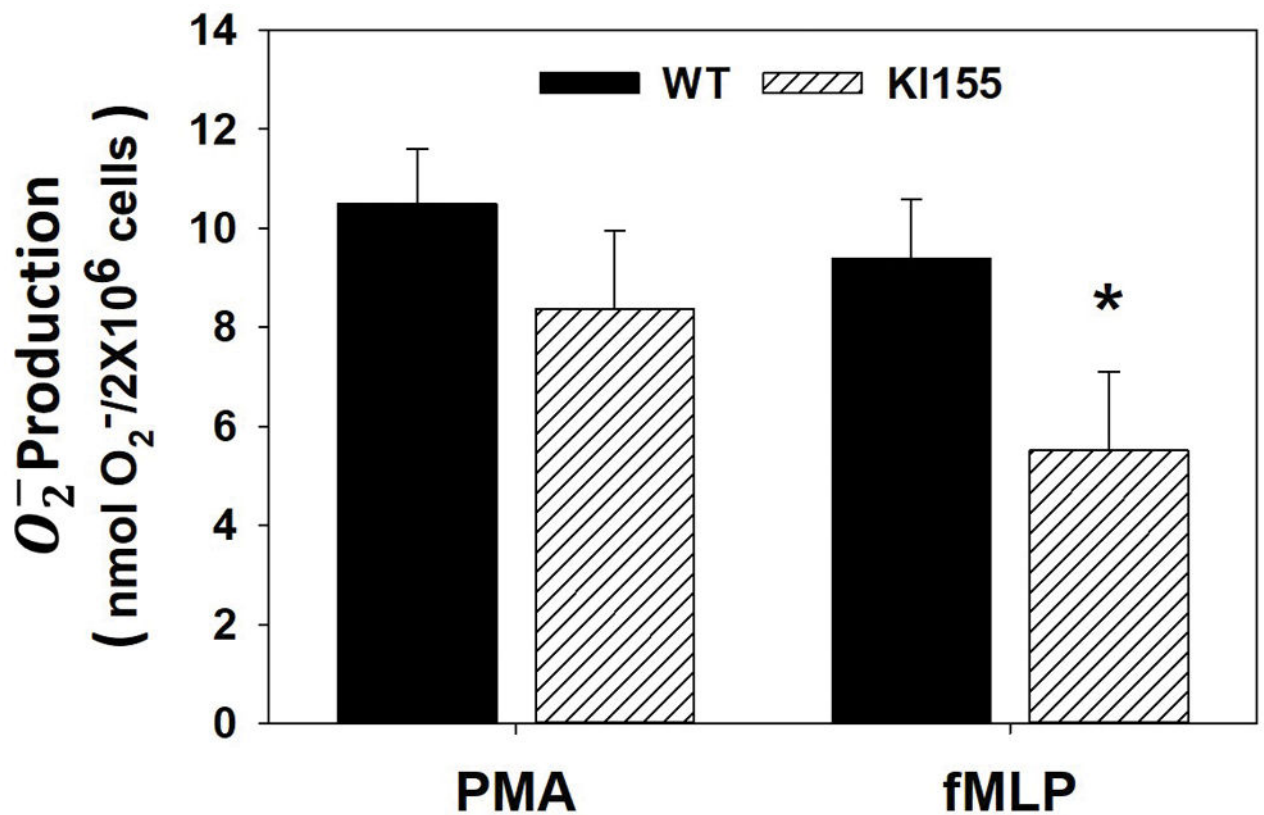


**Figure 1. Generation of PKC $\delta$ Y155F knock-in model**

(A.) Schematic representation of targeted generation of PKC $\delta$ Y155F knock-in mice. Exons 2–9 are represented as grey vertical lines. PKC $\delta$  tyrosine 155 is located on Exon 5 and is indicated with an asterisk. Identification of wildtype and PKC $\delta$ Y155F knock-in mice using (B.) gtf/gtR primer pair (indicated in panel A) and (C.) primers that specifically recognize PKC $\delta$ Y155F site by PCR.

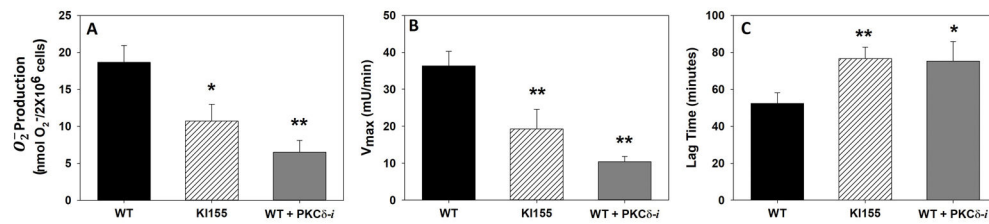


**Figure 2. The bmFA mimics a physiologically relevant microvascular environment**  
Microvascular network maps obtained *in vivo* are reproduced on PDMS to assemble the biomimetic microfluidic assay (scale bar 1 cm) (A). Microvascular endothelial cells, WT (scale bar 50 μm) (B) and KI155 (scale bar 50 μm) (C), formed a complete lumen in the vascular channel of bmFA (green indicates F-actin; red indicates cell nuclei).



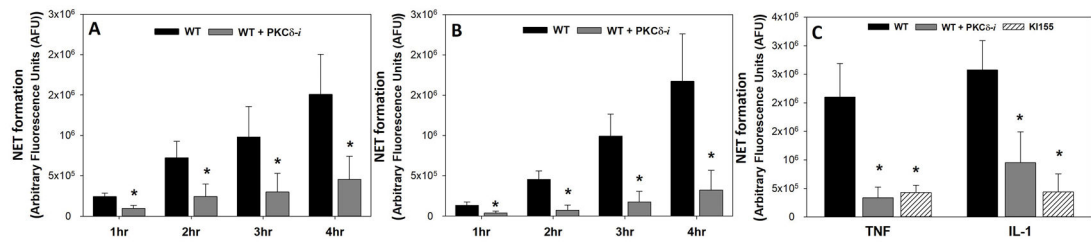
**Figure 3. PMA- and fMLP-stimulated Superoxide anion generation (O<sub>2</sub><sup>-</sup>)**

Superoxide anion generation by WT BMN and KI 155 BMN in response to PMA or fMLP was measured as SOD-inhibitable cytochrome c reduction. The measurements indicate significant quantities of superoxide anion generation in response to PMA and fMLP. While WT BMN O<sub>2</sub><sup>-</sup> generation in response to either PMA or fMLP was similar, lack of PKC $\delta$  Tyr 155 phosphorylation in KI155 BMN decreased O<sub>2</sub><sup>-</sup> generation significantly in response to fMLP, but not PMA. ( $N=11$ ;  $Mean\pm SEM$ ; \*  $p<0.05$  student  $t$ -test)



#### Figure 4. TNF-stimulated O<sub>2</sub><sup>-</sup> Generation

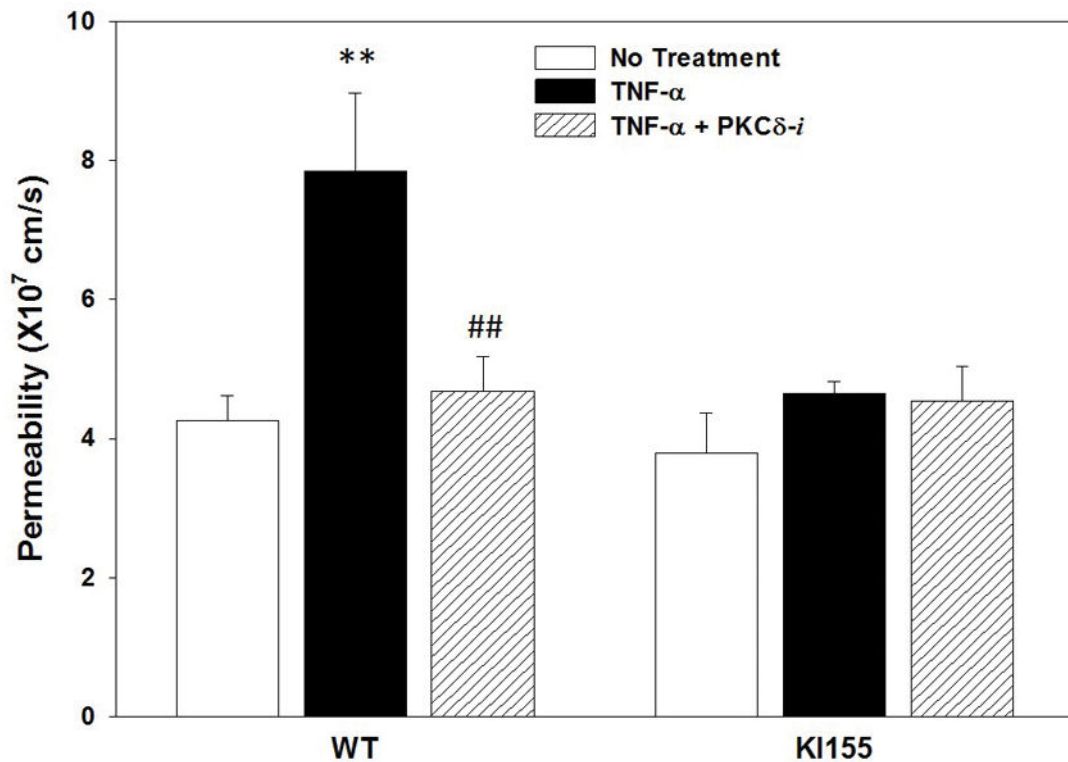
Superoxide anion generation in WT BMN with or without PKC $\delta$ -TAT peptide inhibitor (PKC $\delta$ -i) treatment and KI 155 BMN in response to TNF- $\alpha$  measured as SOD-inhibitable cytochrome c reduction. TNF- $\alpha$  stimulated O<sub>2</sub><sup>-</sup> generation was significantly decreased in KI155 BMN and in WT BMN treated with the PKC $\delta$ -i (A). V<sub>max</sub> of the reaction was also significantly reduced with KI155 BMN and WT BMN treated with the PKC $\delta$ -i as compared to WT BMN (B). There was a significant increase in the lag time to O<sub>2</sub><sup>-</sup> production in both the KI155 BMN and WT BMN treated with the PKC $\delta$  inhibitor as compared to WT BMN (C). (N=24 for WT group, N=9 for KI155 group, N=5 for WT+PKC $\delta$ -i group; Mean $\pm$ SEM; \*  $p$ <0.05, \*\*  $p$ <0.01 one-way ANOVA)



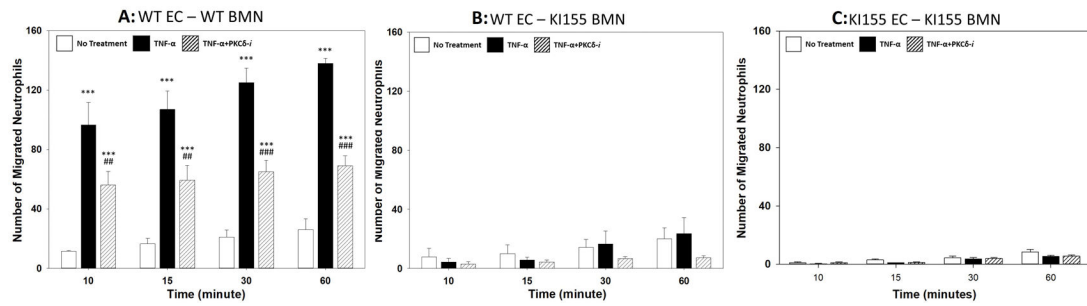
### Figure 5. Role of PKC $\delta$ in Neutrophil Extracellular Trap (NET) Formation

NET formation was measured fluorometrically in BMN by monitoring DNA release. NET formation in WT BMN in response to IL-1 (A) ( $N=5$ ) and fMLP (B) ( $N=5$ ) increases over 4 hrs. Inhibition of PKC $\delta$  with the inhibitor decreased NET formation in WT BMN significantly over the course of 4hrs ((A) and (B)). NET formation was also significantly attenuated in response to IL-1 and TNF- $\alpha$  in KI155 BMN as compared to WT BMN (C) ( $N=5$  for WT group,  $N=4$  for WT+PKC $\delta$ -i and KI155 groups). (Mean $\pm$ SEM; \* $p<0.05$  two-way ANOVA)



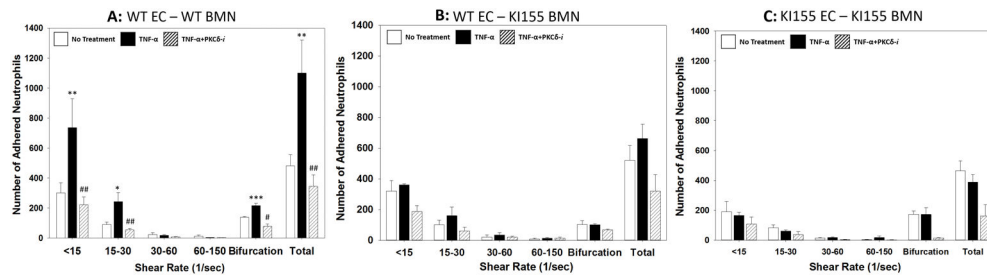


**Figure 6. Role of PKCδ Tyr155 phosphorylation on permeability of MLMVEC**  
WT MLMVEC permeability increased significantly after TNF-α activation. Pharmacological inhibition of PKCδ in WT MLMVEC using PKCδ-i reduced the permeability to No Treatment levels. In the absence of PKCδ Tyr 155 phosphorylation in KI155 MLMVEC, permeability was not altered in response to TNF-α activation or after treatment with PKCδ-i. ( $N=3$ ,  $Mean \pm SEM$ ,  $** p < 0.01$  compared to No Treatment;  $## p < 0.01$  compared to TNF-α, two-way ANOVA)



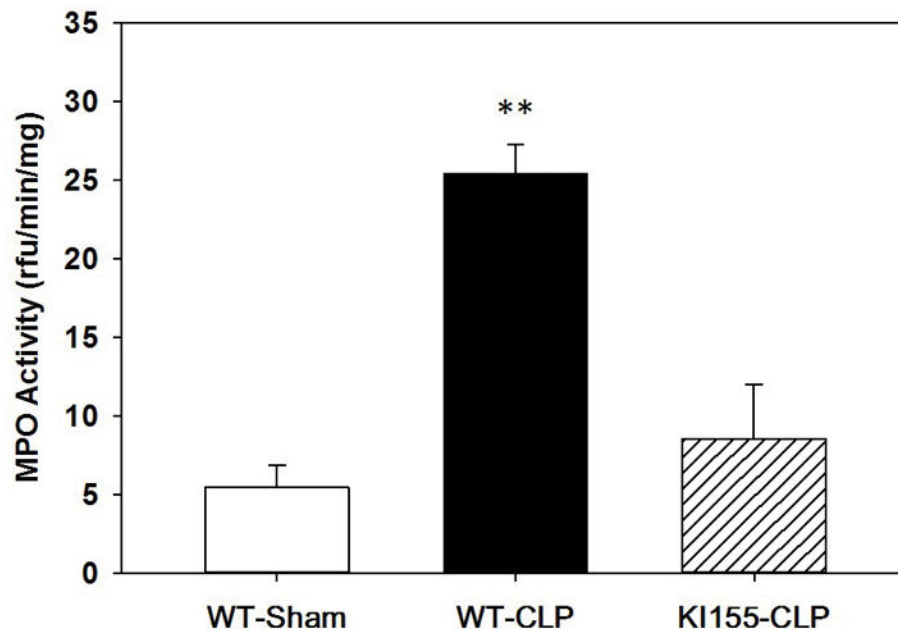
**Figure 7. Migration of WT and KI155 BMN across MLMVEC in bMFA**

There was a significant increase in migration of WT BMN across TNF-activated WT MLMVEC in response to fMLP. Pharmacological inhibition with the PKC $\delta$ -TAT inhibitor (PKC $\delta$ -i) significantly decreased the number of migrated WT neutrophils across MLMVEC (A). Migration of TNF-activated KI155 BMN across WT MLMVEC in response to fMLP was not significantly different as compared to No Treatment levels. PKC $\delta$  inhibition did not change migration of KI155 BMN significantly (B). Migration of KI155 BMN across TNF-activated KI155 MLMVEC in response to fMLP did not increase compared to No treatment levels. Treatment with PKC $\delta$ -i did not significantly impact KI155 BMN migration across KI155 MLMVEC (C). ( $N=3$ ;  $Mean \pm SEM$ ; \*\*\*  $p < 0.001$  compared to No Treatment; #  $p < 0.01$ , ###  $p < 0.001$  compared TNF- $\alpha$ , one-way ANOVA)



**Figure 8. Adhesion of WT and KI155 BMN to MLMVEC**

There was a significant increase in adhesion of WT BMN to TNF- $\alpha$  activated WT MLMVEC in the presence of fMLP, especially at low shear rates and near bifurcations. Pharmacological inhibition with the PKC $\delta$ -TAT inhibitor (PKC $\delta$ -i) significantly reduced the adhesion level of WT BMN to WT MLMVEC to No Treatment levels (A). TNF- $\alpha$  activation of KI155 BMN did not increase adhesion to WT MLMVEC above the No Treatment levels. PKC $\delta$ -i treatment did not significantly change the adhesion level of KI155 BMN to WT MLMVEC (B). Adhesion of KI155 BMN to TNF-activated KI155 MLMVEC was not significantly different from No Treatment. Treatment with PKC $\delta$ -i did not significantly alter the adhesion level of KI155 BMN to KI155 MLMVEC (C) ( $N=3$ ; Mean $\pm$ SEM; \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ , compared to No Treatment; #  $p<0.05$ , ##  $p<0.01$ , compared to TNF- $\alpha$ , one-way ANOVA)



**Figure 9. Role of PKC $\delta$  Tyr155 phosphorylation on Pulmonary MPO Activity in Sepsis**  
MPO analysis was performed in mouse lung samples harvested 24h post-surgery. There was a significant increase in MPO activity in the lungs from WT septic mice (WT-CLP, n=4) as compared to sham surgery WT mice (WT-sham, n=4). MPO activity was significantly lower in lungs from KI155 septic mice (KI155, n=3) as compared to WT septic mice (WT-CLP). Values are expressed as MEAN $\pm$ SEM in relative fluorescence units (RFU)/min/mg. (\*\* p<0.01, WT-CLP vs. WT-sham and WT-CLP vs. KI155-CLP, one-way ANOVA)