Inhibition of Neutrophil Extracellular Trap Formation after Stem Cell Transplant by Prostaglandin E₂

Racquel Domingo-Gonzalez^{1*}, Giovanni J. Martínez-Colón^{1*}, Alana J. Smith², Carolyn K. Smith^{1,3}, Megan N. Ballinger⁴, Meng Xia⁵, Susan Murray⁵, Mariana J. Kaplan³, Gregory A. Yanik⁶, and Bethany B. Moore^{7,8}

¹Immunology Graduate Program, ²Summer Undergraduate Research Program, ⁵Biostatistics Department, School of Public Health, ⁶Department of Pediatrics, Division of Hematology-Oncology, Medical School, ⁷Pulmonary and Critical Care Medicine Division, Department of Internal Medicine, and ⁸Department of Microbiology and Immunology, University of Michigan, Ann Arbor, Michigan; ³Systemic Autoimmunity Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland; and ⁴Pulmonary, Allergy, Critical Care and Sleep Division, Department of Internal Medicine, The Ohio State University, Columbus, Ohio

ORCID IDs: 0000-0003-4387-7673 (S.M.); 0000-0003-3051-745X (B.B.M.).

Abstract

Rationale: Autologous and allogeneic hematopoietic stem cell transplant (HSCT) patients are susceptible to pulmonary infections, including bacterial pathogens, even after hematopoietic reconstitution. We previously reported that murine bone marrow transplant (BMT) neutrophils overexpress cyclooxygenase-2, overproduce prostaglandin E₂ (PGE₂), and exhibit defective intracellular bacterial killing. Neutrophil extracellular traps (NETs) are DNA structures that capture and kill extracellular bacteria and other pathogens.

Objectives: To determine whether NETosis was defective after transplant and if so, whether this was regulated by PGE₂ signaling.

Methods: Neutrophils isolated from mice and humans (both control and HSCT subjects) were analyzed for NETosis in response to various stimuli in the presence or absence of PGE₂ signaling modifiers.

Measurements and Main Results: NETs were visualized by immunofluorescence or quantified by Sytox Green fluorescence. Treatment of BMT or HSCT neutrophils with phorbol 12-myristate 13-acetate or rapamycin resulted in reduced NET formation relative to control cells. NET formation after BMT was rescued both *in vitro* and *in vivo* with cyclooxygenase inhibitors. Additionally, the EP2 receptor antagonist (PF-04418948) or the EP4 antagonist (AE3-208) restored NET formation in neutrophils isolated from BMT mice or HSCT patients. Exogenous PGE₂ treatment limited NETosis of neutrophils collected from normal human volunteers and naive mice in an exchange protein activated by cAMP- and protein kinase A-dependent manner.

Conclusions: Our results suggest blockade of the PGE₂-EP2 or EP4 signaling pathway restores NETosis after transplantation. Furthermore, these data provide the first description of a physiologic inhibitor of NETosis.

Keywords: granulocyte; transplant; NETosis; prostaglandin; bacteria

Approximately 50,000 hematopoietic stem cell transplants (HSCTs) are performed annually (57% autologous; 43% allogeneic) (1). HSCT patients exhibit susceptibility to

pulmonary infections even late after engraftment (2–4). Neutrophil recruitment and function is important for innate immunity. In response to inhaled

pathogens, neutrophils are recruited by chemotactic signals released from activated alveolar macrophages and epithelial cells (5). Patients with chronic granulomatous

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*These authors contributed equally to this work.

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Correspondence and requests for reprints should be addressed to Bethany B. Moore, Ph.D., 4053 BSRB, 109 Zina Pitcher Place, Ann Arbor, MI 48103-2200. E-mail: bmoore@umich.edu

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At a Glance Commentary

Scientific Knowledge on the

Subject: Hematopoietic stem cell transplant patients are at increased risk for lung infections. Animal models have demonstrated that impaired innate immunity after transplant is related to prostaglandin E₂ (PGE₂) signaling. NETosis is important for killing extracellular bacteria and other pathogens, but NETosis has not been studied in the setting of transplantation.

What This Study Adds to the

Field: This study demonstrates the first physiologic inhibitor of NETosis by showing that PGE₂ blocks neutrophil extracellular trap formation through EP2 or EP4-mediated protein kinase A and exchange protein activated by cAMP-dependent pathways. Inhibition of PGE₂ signaling improves neutrophil extracellular trap release, supporting the therapeutic potential for improving neutrophil function after hematopoietic stem cell transplant. This also suggests that EP2/EP4 agonists could be used to block pathologic NETosis in autoimmune diseases.

disease with mutations in the NADPH oxidase complex exhibit functional defects in neutrophils (e.g., reduced formation of neutrophil extracellular traps [NETs]) that render them more susceptible to pathogens also afflicting HSCT patients, such as *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Aspergillus* species (6, 7). Interestingly, patients with chronic granulomatous disease receiving gene therapy complementing NADPH oxidase function restore anti-*Aspergillus* responses via restored NETs (7, 8).

Long-term defects in neutrophil functions have previously been noted in HSCT patients (6). Neutrophils from autologous HSCT patients exhibit a diminished capacity to produce respiratory burst (6, 9, 10), whereas allogeneic HSCT patients exhibit defects in neutrophil chemotaxis in addition to impaired respiratory burst (6, 11, 12). However, the cause for neutrophil dysfunction has remained unclear.

Furthermore, the ability of neutrophils from HSCT patients to undergo NETosis is unknown.

NETosis is a cell death pathway characterized by release of extracellular weblike structures composed of chromatin, histones, and granular proteins (13–15). NETs serve as antimicrobial defenses against extracellular pathogens including bacteria (16). Takei and coworkers (17) described this as a novel form of cell death, distinct from apoptosis and necrosis, because of its dependence on chromatin decondensation, increase in membrane permeability, and its independence from necrosis-inducing or apoptosis-inducing stimuli (18). Studies have shown NETosis may be dependent on NADPH oxidase or myeloperoxidase-generated reactive oxygen species (ROS), autophagy, neutrophil elastase, and histone citrullination by peptidylarginine deiminase 4 (19–21). Live cells can also participate in a process called “vital NETosis” where neutrophils maintain their membrane integrity while rapidly releasing NETs and continuing to chemotax and phagocytize bacteria (22, 23).

We previously demonstrated that host defense against *P. aeruginosa* and *S. aureus* is impaired after bone marrow transplant (BMT) in mice (24–26). Because NETs can effectively kill both *S. aureus* and *P. aeruginosa* (14, 18, 27), it is unclear whether the bactericidal defects relate to impaired NETosis after transplant. We showed defective neutrophil function is attributable to overproduction of prostaglandin E₂ (PGE₂) (25). PGE₂ is generated using cyclooxygenase (COX) enzymes (basal COX-1 or inducible COX-2) (28). Inhibition of COX with indomethacin rescued the functional bactericidal defects *in vivo* (25). Similar pathways can be involved in intracellular killing and NETosis. NADPH oxidase activity and autophagy (29) can promote NETosis and killing, but regulation is poorly understood. Although much is known about inducers of NETosis (e.g., phorbol 12-myristate 13-acetate [PMA], bacterial components, and IL-8), nothing is known of physiologic inhibitors or negative regulators. Here, we propose a novel role for PGE₂ as an inhibitor of NETosis.

Methods

Detailed methods can be found in the online supplement.

Human Subjects

Neutrophils collected from the bronchoalveolar lavage (BAL) were obtained from HSCT patient 1. Studies using neutrophils collected from the peripheral blood originated from HSCT patients 2–12 and from six healthy volunteers. Table 1 provides human subject characteristics. Written informed consent was received and all experiments were approved by the University of Michigan institutional review board.

Animals

C57BL/6 and Balb/c mice from Jackson Laboratory (Bar Harbor, ME) were housed under specific pathogen-free conditions and killed by CO₂ asphyxiation. University of Michigan Committee on Use and Care of Animals approved all procedures.

Transplantation

Murine syngeneic (syn) and allogeneic (allo) BMT was performed after 13-Gy total body irradiation as described (30).

Murine Neutrophils

Neutrophils were recruited via an intratracheal injection of 25 µg of *P. aeruginosa* LPS in 50 µl. After 18–20 hours, neutrophils were collected by BAL (25). Alternatively, bone marrow-derived neutrophils were isolated as previously described (31).

Human Neutrophils

Neutrophils were isolated from the peripheral blood of healthy volunteers and allogeneic HSCT patients using Ficoll-Paque PLUS, or by BAL.

H₂O₂ Detection Assay

Cellular H₂O₂ secretion was determined from LPS-recruited neutrophils via Amplex Red reagent.

Western Blotting for NETs

Supernatant from neutrophils exposed to multiple conditions was collected; DNA was removed with DNase I; and protein was acetone precipitated before being run on a gel, transferred, and blotted for expression of myeloperoxidase.

Sytox Green Fluorescence Assays

NETs were quantified using a cell-impermeable nucleic acid dye, Sytox Green.

Table 1. Human Subject Characteristics

Subject ID	Age	Sex	HSCT Type	Days after HSCT	Indication for HSCT	Conditioning Regimen	Chronic GVHD	Current Immunosuppressive Regimen
HSCT 1 (BALF)	65	M	Allo	806	Acute myelomonocytic leukemia	Fludarabine and busulfan	Present	Dexamethasone prednisone
HSCT 2	47	F	Allo	158	Acute myelomonocytic leukemia	Fludarabine and busulfan	Absent	Tacrolimus
HSCT 3	64	F	Allo	616	Acute myeloid leukemia	Fludarabine and busulfan	Present	Prednisone
HSCT 4	70	F	Allo	58	Myelodysplastic syndrome	Fludarabine and busulfan	Absent	Tacrolimus methotrexate (low dose)
HSCT 5	63	F	Allo	91	Myelofibrosis	Fludarabine and busulfan	Absent	Tacrolimus prednisone
HSCT 6	67	M	Allo	20	Acute myeloid leukemia	Clofarabine busulfan	Absent	Tacrolimus cellcept
HSCT 7	30	M	Allo	176	Acute myeloid leukemia	Fludarabine and busulfan	Absent	Tacrolimus restasis
HSCT 8	27	M	Allo	26	T-cell acute lymphoblastic leukemia	Fludarabine and busulfan	Absent	Tacrolimus
HSCT 9	55	M	Allo	55	Myelodysplastic syndrome	Fludarabine and busulfan	Absent	Tacrolimus carfilzomib
HSCT 10	71	M	Allo	35	Myelodysplastic syndrome-refractory anemia with excess blasts-2	Fludarabine and busulfan	Absent	Tacrolimus restasis
HSCT 11	46	M	Allo	83	Philadelphia chromosome plus acute lymphoblastic leukemia	Fludarabine and total body irradiation	Present	Prednisone sirolimus
HSCT 12	55	F	Allo	51	Angioimmunoblastic T-cell lymphoma after autotransplant	Fludarabine and busulfan	Present	Prednisone tacrolimus
Control 1	50	F	None					
Control 2	23	M	None					
Control 3	23	F	None					
Control 4	26	F	None					
Control 5	51	F	None					
Control 6	53	M	None					

Definition of abbreviations: Allo = allogeneic; BALF = bronchoalveolar lavage fluid; GVHD = graft-versus-host disease; HSCT = hematopoietic stem cell transplant.

Immunofluorescence Studies

Using poly-lysine-coated cover slips, 200,000 neutrophils were seeded and treatments were added directly for 5–7 hours (murine) or 3 hours (human), before cells were fixed and stained with antineutrophil elastase and Hoescht. Slides were analyzed by confocal microscopy.

Pharmacologic Agents

Information is provided in the online supplement.

Statistical Analysis

Prism 6.0 statistical program (Graphpad Software, San Diego, CA) and SAS version 9.4 (SAS Institute Inc., Cary, NC) were used for analyses. Comparisons of continuous measures between two experimental murine groups were determined using the Student's *t* test, and error bars denote the SEM. Comparison

between three or more murine groups used analysis of variance analyses followed by Tukey post-test, and error bars also denote SEM. In human studies, the following steps were used to normalize patient data according to patient-specific outcomes in the media treatment group. First the mean across an individual's media replicates was calculated. This individual-specific mean was then used to rescale outcomes from all experimental conditions for that individual, via division, giving a mean media outcome in each individual of 100. Other experimental conditions are then summarized as percentage of control units (above or below 100% with media group as reference). Mixed models with a random intercept for individual were used to estimate experimental condition means and corresponding 95% confidence intervals used in the figures; correlation between outcomes within individual are accounted for via the

random intercept term (32). A *P* value less than 0.05 was considered statistically significant.

Results

NETosis Is Impaired after Syn and Allo BMT

To determine whether the capacity to undergo NETosis was deficient or intact after BMT, neutrophils were recruited into the lungs of untransplanted control or syn or allo BMT mice. We next examined NET formation after treatment with PMA or infection with *S. pneumoniae*. PMA, a known inducer of NETosis, stimulated NETs in untransplanted control neutrophils; however, PMA-treated syn and allo BMT neutrophils exhibited impaired extracellular DNA release as measured by extracellular Sytox fluorescence versus control neutrophils (Figures 1A and 1B). These observations were supported by

Figure 1. NETosis is impaired after syngeneic (syn) and allogeneic (allo) bone marrow transplant (BMT). LPS-recruited neutrophils from the lung of (A) syn BMT or (B) allo BMT and untransplanted control mice were stimulated for 5 hours with phorbol 12-myristate 13-acetate (PMA; 100 nM) or *Streptococcus pneumoniae* (multiplicity of infection, 5) or left untreated, and NETosis was measured by Sytox Green fluorescence. (C) NETosis after 5 hours of PMA treatment in LPS-recruited neutrophils from untransplanted control, syn BMT, and allo BMT mice was visualized by immunofluorescence via staining of DNA (Hoechst, blue) and neutrophil elastase (green, control and syn BMT $\times 20$; allo BMT $\times 40$ magnification; arrowheads denote neutrophil extracellular traps in BMT groups). (D) Lung neutrophils were harvested 18–20 hours after LPS intratracheal injection, and H_2O_2 production was measured colorimetrically via the Amplex Red reagent; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ relative to control. In A, $n = 6/\text{group}$ for media and PMA stimulations and $n = 3/\text{group}$ for *S. pneumoniae* stimulation; in B, $n = 3/\text{group}$; in D, the $n = 15/\text{group}$.

immunofluorescence that showed few visible NETs in untreated groups (Figure 1C, top row), but extensive, intact NETs on PMA stimulation in control cells. In contrast, NETs from both syn and allo BMT cells were significantly decreased and appeared structurally less intact (Figure 1C, bottom row). Defective NETosis was also noted in neutrophils from BMT mice exposed to *S. pneumoniae*, a relevant HSCT pathogen (Figure 1A). We confirmed that neutrophils from syn BMT mice exhibited basal defects in production of hydrogen peroxide (Figure 1D).

COX Inhibitors Restore NET Formation after BMT

To determine whether NETosis was negatively regulated by increased COX-2 activity, syn BMT mice were intraperitoneally injected with indomethacin, a nonselective COX inhibitor before the recruitment of neutrophils. After *in vivo* inhibition of COX, NETosis was restored in neutrophils from syn BMT mice (Figure 2A). *In vivo* treatment with indomethacin did not affect neutrophil recruitment because LPS exposure recruited $89 \pm 1.77\%$ neutrophils in syn BMT receiving indomethacin, which was comparable with the $88.7 \pm 1.34\%$ neutrophils recruited in untreated control animals and $87.1 \pm 1.32\%$ in vehicle-treated syn BMT mice. Immunofluorescence studies again confirmed the stimulatory effects of indomethacin on NETosis, because *in vivo* inhibition of COX visibly enhanced NET production from syn BMT neutrophils compared with untransplanted control neutrophils (Figure 2C). NETosis was increased in allo BMT mice on *in vitro* COX inhibition with indomethacin (Figure 2B). The ability of PMA to induce NETs in syn BMT mice was also restored by diclofenac, an inhibitor with higher affinity for COX-2 than COX-1, or by addition of anti-PGE₂ (see Figures E1A and E1B in the online supplement). Similarly, neutrophils from mice deficient in PGE synthase showed enhanced PMA-induced NETosis compared with cells from wild-type mice (see Figure E1C).

PGE₂ Inhibits PMA-induced NETs from Murine and Human Neutrophils Neutrophils from BMT mice have elevated levels of cytosolic phospholipase A₂ and COX-2 (see Figure E2A). PGE₂ causes

Figure 2. Indomethacin (Indo) rescues impaired NETosis after bone marrow transplant (BMT). LPS-recruited pulmonary neutrophils from untransplanted control, syngeneic (syn) BMT, or syn BMT mice after intraperitoneal injection with indomethacin (1.2 mg/kg) were treated with phorbol 12-myristate 13-acetate (PMA; 100 nM) or left untreated for 5 hours, and NETosis was measured via (A) Sytox fluorescence or (C) immunofluorescence studies (gray scale of Hoechst DNA staining at $\times 20$ magnification; arrowheads denote neutrophil extracellular traps). (B) Sytox fluorescence was performed in LPS-recruited allogeneic BMT neutrophils to measure NETosis after 5 hours *in vitro* treatment with indomethacin (10 μ M), PMA (100 nM), PMA and indomethacin, or media alone. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. In A and B, $n = 4$ /group.

functional defects in alveolar macrophages and neutrophils that occur after BMT (25, 26, 33). Thus, we focused on the effects of PGE_2 on NETosis. Interestingly, *in vitro* treatment with PGE_2 was able to decrease NET release from control murine neutrophils despite stimulation with PMA (Figure 3A). This effect was reversed in the presence of anti- PGE_2 antibodies (Figure 3B). PGE_2 was also able to inhibit NETosis in cells from normal volunteers (Figure 3C). Activation of the COX pathway in syn BMT mice results in approximately 4 ng/ml (11.34 nM) PGE_2 in the BAL fluid (25) and elevated levels are also seen in HSCT BAL fluid (see Figure E2B). Figure 3D demonstrates that physiologic levels of PGE_2 (10 nM) are sufficient to limit PMA-induced NETosis in a control subject. Immunofluorescence studies showed a significant absence of intact

NET formation on the simultaneous treatment of human or murine neutrophils with PMA and PGE_2 (Figure 3E).

PGE_2 Signaling Inhibits NETosis via Both Protein Kinase A- and Epac-mediated Pathways

PGE_2 signals through EP receptors 1–4 and we previously showed that both EP2 and EP4 were enhanced on BMT neutrophils when compared with their respective levels on control cells (25) (Figure 4A, inset); however, it should be noted that EP2 levels are still higher than EP4 levels on BMT neutrophils when compared with each other (Figure 4A, large graph). To determine whether EP2 signaling regulated NETosis, BMT neutrophils were treated with an EP2 receptor antagonist (PF-04418948). Treatment with the EP2 antagonist enhanced NETosis in both syn

and allo murine BMT neutrophils (Figures 4B and 4C). Because EP2 and EP4 signal through protein kinase A (PKA) and/or Epac (34–36), the effects of PKA- or Epac-agonists on NETosis were analyzed. Activation of either the PKA or Epac pathway was able to effectively block NET production (Figure 4D).

Blocking PGE_2 Signaling Rescues NETosis in Allogeneic HSCT Patients

We next investigated the effects of EP receptor antagonism on NETosis in neutrophils from the peripheral blood (Figures 5A and 5C) or BAL (Figure 5B) of allogeneic HSCT patients. Blocking EP2 or EP4 signaling enhanced NET formation in HSCT samples. Healthy human peripheral blood neutrophils were treated with PMA, PKA agonist, Epac agonist, or a combination of these treatments. Similar to our murine data, PMA was able to induce

Figure 3. Prostaglandin E₂ (PGE₂) inhibits phorbol 12-myristate 13-acetate (PMA)-induced murine and human neutrophil extracellular traps. LPS-recruited neutrophils from untransplanted mice were stimulated with PGE₂ (10 μ M), PMA (100 nM), or PMA plus PGE₂ or left untreated for 5 hours and measured for NETosis via (A) Sytox fluorescence ($n = 4$ –6/group) or (E, top row) immunofluorescence. (B) LPS-recruited neutrophils from untransplanted mice were left untreated or treated with PMA, PMA plus 1 μ M PGE₂, or this combination along with an antibody against PGE₂ (1:1000 dilution); $n = 5$ –12/group combined from two experiments. (C) Neutrophils isolated from peripheral blood of normal volunteers ($n = 4$) were stimulated with PMA (100 nM), PGE₂ (10 μ M), or PMA plus PGE₂ or were left untreated for 3 hours before NETosis was measured by Sytox fluorescence. Graph shows the mean $\pm$ 95% confidence interval as estimated via mixed effect linear models (see Table E1A). (D) Neutrophils isolated from peripheral blood of a normal volunteer (control 6) were left untreated or were cultured with PMA or PMA plus low-dose PGE₂ (10 nM); $n = 5$ replicates from the same subject. (E, bottom row) Neutrophils isolated from peripheral blood of a normal volunteer (control 2) were stimulated with PMA (100 nM), PGE₂ (10 μ M), or PMA plus PGE₂ or were left untreated for 7 hours (Hoechst, DNA, blue; neutrophil elastase [green]; $\times 60$ magnification; arrowheads denote neutrophil extracellular traps). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 4. Inhibition of prostaglandin E_2 signaling rescues murine neutrophil extracellular trap (NET) production after bone marrow transplant (BMT). (A) Total RNA was isolated from neutrophils collected from control and syngeneic (syn) BMT mice ($n = 6$ per group), and real-time reverse transcriptase polymerase chain reaction was performed to analyze expression of EP2 and EP4 receptors. *Inset* shows levels of EP2 and EP4 in BMT samples compared with their respective levels in cells from control untransplanted mice (normalized to 1). The *large graph* shows the levels of EP4 when compared with EP2 (normalized to 1) in the syn BMT cells alone. LPS-recruited neutrophils collected from (B) syn ($n = 5$ – 6 /group) and (C) allogeneic ($n = 4$ – 5 /group) BMT mice were treated with phorbol 12-myristate 13-acetate (PMA; 100 nM), an EP2 antagonist (EP2 anta; 10 nM), or PMA and EP2 anta for 5 hours, and NETs were measured by Sytox fluorescence. (D) Effects of downstream prostaglandin E_2 signaling on NETosis were determined with 5-hour protein kinase A (PKA) agonist (PKA ag = 6-Bnz-cAMP; 500 μ M) and Epac agonist (EPAC ag = 8-pCPT-2'-O-Me-cAMP; 500 μ M) treatment plus or minus PMA on untransplanted control neutrophils. NET production was determined by Sytox fluorescence ($n = 4$ – 6 pooled from two separate experiments; shown as "percent of control"). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

NETs and this function was decreased by concurrent treatment with PKA agonist or the Epac agonist (Figure 6A). In HSCT patients, as expected PMA did not significantly induce NETs alone, but was able to in the presence of a PKA antagonist (Figure 6B). The Epac antagonist was toxic in the HSCT neutrophils (data not shown).

PGE₂ Inhibits Autophagy-induced NET Release

The mechanisms by which NETs are released into the extracellular space may involve autophagy and ROS, myeloperoxidase, and neutrophil elastase expression (29). In support of this, PMA treatment can stimulate NADPH oxidase activity and autophagy (37). Autophagy has

been shown to play an important role in promoting clearance of intracellular pathogens (38). In the presence of nutrients, mammalian target of rapamycin functions to inhibit autophagy (39). Rapamycin, a mammalian target of rapamycin inhibitor, promotes NET release in neutrophils from control mice but not in cells from syn BMT mice (Figure 7A),

Figure 5. Blocking EP receptor signaling rescues neutrophil extracellular traps in allogeneic hematopoietic stem cell transplant (HSCT) patients. Neutrophils collected from peripheral blood (A and C) or bronchoalveolar lavage (B) from allogeneic HSCT patients were incubated with media, phorbol 12-myristate 13-acetate (PMA; 100 nM), EP2 antagonist (EP2 anta, 10 nM), or EP4 antagonist (EP4 anta, AE3-208, 1 μ M) as indicated, and NETosis was measured by Sytox fluorescence. A reports the mean $\pm$ 95% confidence interval as estimated via mixed effect linear models for $n = 5$ HSCT patients (see Table E1B), and C reports data from $n = 6$ HSCT patients (see Table E1C). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

suggesting impaired autophagy-mediated NETosis after transplant. Treatment with PGE₂ conferred defective NET release in control murine neutrophils despite concomitant stimulation with rapamycin (Figure 7B). This inhibition was corroborated by Western blot analysis because less myeloperoxidase was detected in the supernatant of rapamycin plus PGE₂-treated neutrophils than was released from cells treated with rapamycin alone (Figure 7C). Furthermore, EP2 receptor antagonism restored rapamycin-induced NET formation in syn BMT lung neutrophils (Figure 7D).

NETosis is dependent on NADPH oxidase and autophagy (29), so we next modulated these pathways in neutrophils collected from the bone marrow of control mice. PMA effectively induced NET release from bone marrow neutrophils, a process significantly inhibited after inhibition of autophagy (wortmannin treatment) or inhibition of ROS (diphenyleneiodonium treatment) (Figure 7E). Interestingly, when examining rapamycin-induced NETosis, PGE₂ was more effective in limiting this pathway than either wortmannin or diphenyleneiodonium treatment (Figure 7F).

When neutrophils from an age-matched control subject and HSCT patient were collected and analyzed on the same day, the cells from the HSCT patient were defective in NETosis induced both by PMA and rapamycin relative to the control subject (Figure 8A). In human control neutrophils, rapamycin effectively induced NETosis (Figure 8B). This rapamycin-stimulated NETosis was reduced by PGE₂, PKA agonists, or Epac agonists (Figure 8B).

Overall, these data suggest that the inhibition of NETs by PGE₂ signaling is a universal feature of neutrophils obtained from both lung and bone marrow compartments in mice and from lung and peripheral blood of humans. In addition, PGE₂ can inhibit NETosis induced by a variety of stimuli.

Discussion

Neutrophil responses have been shown to be particularly important for host response to pulmonary infection. We have previously

shown that neutrophils are unable to clear engulfed pathogens after BMT (25). Here we show that neutrophils recruited to the lung also exhibit impaired NETosis after either syngeneic or allogeneic BMT implying that extracellular killing mechanisms may also be defective after BMT. This impairment is caused by overproduction of PGE₂ and signaling via the EP2 or EP4 receptor. We verified the ability of physiologic doses of PGE₂ to limit NETosis and used anti-PGE₂ antibodies to confirm specificity. We previously measured EP4 receptor expression to be 4.8-fold higher in BMT PMNs, whereas EP2 was 2.4-fold higher compared with

Figure 6. Alterations in prostaglandin E₂ signaling can influence NETosis in control subjects and hematopoietic stem cell transplant patients. Neutrophils were collected from peripheral blood of four normal subjects (A) or from eight hematopoietic stem cell transplant patients (B). Cells were cultured in the presence of media, phorbol 12-myristate 13-acetate (PMA; 100 nM), Epac agonist (EPAC ag, 8-pCPT-2'-O-Me-cAMP; 500 μ M), protein kinase A (PKA) agonist (PKA ag, 6-Bnz-cAMP; 500 μ M), or PKA antagonist (PKA anta, H89, 20 μ M; Sigma) as indicated for 3 hours, and NETosis was measured by Sytox fluorescence. *** $P < 0.001$, **** $P < 0.0001$. Graphs represent the mean $\pm$ 95% confidence interval as estimated via mixed effect linear models (see Tables E1D and E1E).

Figure 7. Prostaglandin E₂ (PGE₂) inhibits autophagy-induced murine NETosis. (A) LPS-recruited lung neutrophils of untransplanted control and syngeneic (syn) bone marrow transplant (BMT) mice were treated with 200 nM rapamycin (Rapa) or were left untreated for 5 hours before Sytox fluorescence detection; n = 6/group. (B) LPS-recruited lung neutrophils from untransplanted control mice were treated with 10 μ M PGE₂, rapamycin, or rapamycin plus PGE₂ for 5 hours before Sytox fluorescence detection; n = 6/group. (C) LPS-recruited lung neutrophils of untransplanted control mice were stimulated with rapamycin or rapamycin plus PGE₂ or were unstimulated for 5 hours before removal of supernatants and Western blot analysis of this supernatant for myeloperoxidase. The Western blot depicts dividing lines to denote altered lane order from the original blot. (D) LPS-recruited lung neutrophils from syn BMT mice were treated with EP2 antagonist (EP2 anta; 10 nM), rapamycin, or rapamycin plus EP2 antagonist or were untreated for

Figure 8. Prostaglandin E_2 (PGE $_2$) regulates NETosis induced by autophagy in human neutrophils. (A) Human peripheral blood neutrophils from control subject 5 (age 50) and hematopoietic stem cell transplant subject 2 (HSCT 2) (age 47) were collected on the same day and stimulated for 3 hours in the presence of media, phorbol 12-myristate 13-acetate (PMA; 100 nM), or rapamycin (Rapa; 200 nM) before being analyzed by Sytox fluorescence; $n = 10$ replicates per patient per group. (B) Peripheral blood neutrophils collected from four normal volunteers were cultured for 3 hours in the presence of media, PGE $_2$ (10 μ M), Epac agonist (EPAC ag, 8-pCPT-2'-O-Me-cAMP; 500 μ M), protein kinase A (PKA) agonist (PKA ag, 6-Bnz-cAMP; 500 μ M), or combinations of these agents with rapamycin (200 nM) for 3 hours before Sytox detection; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. B graphs the mean $\pm$ 95% confidence intervals as estimated via mixed effect linear models (see Table E1F).

untransplanted control neutrophils (Figure 4A, *inset*) (25). EP4 has higher affinity for PGE $_2$ than does EP2 (0.79 vs. 4.9 nM) (40), but given the fact that EP2 levels are over fourfold higher than EP4 levels (Figure 4A, *large graph*) when compared with each other on BMT neutrophils and both of these receptors signal via a cAMP intermediate, it is not surprising that both EP2 and EP4 antagonists are effective in restoring NETosis after transplant. We were only able to obtain one BAL fluid sample with sufficient neutrophils for analysis, but BAL fluid-derived cells behaved similarly to peripheral-blood-derived cells from other HSCT patients in responses to EP2 antagonists (Figures 5A and 5B). Although it is possible that other changes in the lung after BMT (e.g., elevated IL-6 and granulocyte-macrophage colony-stimulating factor; decreased tumor necrosis factor- α , IFN- γ , and leukotrienes) (24, 33, 41, 42) or other COX-2 effectors (e.g., thromboxane and prostacyclin) may also serve roles in regulating NETosis, our studies prove that PGE $_2$ signaling limits NETosis.

Because PGE $_2$ -EP2 or PGE $_2$ -EP4 intracellular signaling relies on PKA and/or

Epac (43), we explored the contribution of both pathways in NET regulation. Interestingly, the use of either PKA or Epac agonists was sufficient for the inhibition of NETosis in murine and human cells and the use of both agonists did not result in an additive effect. Similar inhibitory effects of PGE $_2$ on NETosis were observed regardless of source of neutrophils (BAL or peripheral blood), or species (humans or mice), or stimulus (PMA, rapamycin, bacteria), suggesting that this is a well-conserved mechanism. It is interesting that the neutrophils collected from allogeneic HSCT patients showed spontaneous NETosis after treatment with EP2 or EP4 antagonists alone (Figure 5), which may indicate that cells from these patients were stimulated *in vivo*, yet unable to respond.

Remijsen and colleagues (29) previously showed that autophagy was required for the productive release of NETs. Our data demonstrate that recruited lung neutrophils from untransplanted control mice and neutrophils isolated from murine bone marrow or normal volunteers induce NET formation after stimulation of autophagy via rapamycin (Figures 7B, 7F,

and 8B); however, NETosis was defective in syn BMT lung neutrophils despite exposure to rapamycin (Figure 7D). When cells from a control subject and HSCT patient were analyzed simultaneously, the response to rapamycin was diminished in the HSCT sample relative to the control subject (Figure 8A). There were differences in the collection of the cells because murine neutrophils recruited to the lung were exposed to LPS before treatment with rapamycin, whereas murine bone marrow and human peripheral blood neutrophils are unstimulated. LPS alone can induce NET formation (14, 18); however, it is likely that not all neutrophils produce NETs after the initial recruitment with LPS because our studies with murine lung neutrophils show clear changes in NET release after treatments. Furthermore, the fact that rapamycin was sufficient to induce NETs from human neutrophils in the absence of any priming factors, such as fMLP, suggests that autophagy induction is sufficient to induce NET release, challenging findings from a previous study (29).

Hydrogen peroxide, a ROS, was decreased in syn BMT neutrophils. Because

Figure 7. (Continued). 5 hours before detection of Sytox fluorescence; $n = 6$ /group. (E) Bone marrow neutrophils from control mice were treated with phorbol 12-myristate 13-acetate (PMA), PMA plus wortmannin (100 nM), or PMA plus diphenyleneiodonium (DPI; 10 μ M) for 5 hours before Sytox measurement; $n = 5$ /group. (F) Bone marrow neutrophils from control mice were treated as above with media, PGE $_2$, wortmannin, DPI, or combinations of these drugs with rapamycin for 5 hours before detection of Sytox fluorescence; $n = 5$ –8/condition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

ROS production is required for NETosis, it is possible that PGE₂-dependent inhibition of NETosis is mediated through the Epac-mediated inhibition of ROS. Recently, a renal ischemia-reperfusion study found that activation of Epac reduced mitochondrial ROS production via interaction with Rap1 in tubular epithelial cells (44). Thus, it is possible that Epac signaling upstream of NAPDPH oxidase or mitochondrial ROS production mediates inhibition of NETs. Alternatively, the effect of PGE₂ and Epac on autophagy is unknown. However, previous studies have shown a possible role for PKA in autophagy inhibition via phosphorylation of LC3 (45) or activation of TORC1(31). Thus, it is possible that BMT neutrophils exhibit defective NETosis as a consequence of reduced autophagy and/or ROS production secondary to PGE₂ stimulation. These pathways are likely linked and this may also explain why inhibition of either PKA or Epac restored NETosis after transplant.

Previous studies from our laboratory demonstrated that blocking EP2 signaling improves lung alveolar macrophage

function after HSCT by restoring bactericidal killing (25). Our current study extends this work to suggest that EP2 or EP4 antagonists may also restore intracellular and extracellular killing mechanisms in neutrophils after transplant. It is worth noting that the EP2 and/or EP4 antagonists or PKA antagonists restored function to neutrophils of HSCT patients despite the fact that these patients were often on immunosuppressive medications (Table 1). Thus, this strategy may be a way to improve innate immune function while maintaining immunosuppressive control of graft-versus-host disease. A limitation of our study is that the sample size for the human studies is small; however, findings were highly reproducible between mice and humans, and all findings are consistent with our conclusion that PGE₂ signaling inhibits NETosis.

Another implication of our work is that there may be a therapeutic role for EP2 or EP4 agonists for the inhibition of pathologic NETosis in diseases where uncontrolled NET formation induces exacerbation of

disease (46–48). Currently, therapies under development or in use for targeting NETs in autoimmune diseases like lupus include DNase (49, 50), antihistone antibodies (51, 52), and antiproteases (53). In the case of antihistone antibodies, the possibility of promoting autoimmunity may outweigh the benefits of this therapy. Before our studies, physiologic factors aside from DNase that could potentially suppress NETosis were unknown. Here, we reveal PGE₂ signaling via EP2 or EP4 as a negative regulator of NETosis. ■

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