Published in final edited form as:

J Leukoc Biol. 2019 June ; 105(6): 1225–1234. doi:10.1002/JLB.3VMA0918-374R.

Adenosine from a biologic source regulates neutrophil extracellular traps (NETs)

Kai Xu^{1,2}, Kimberly A. Cooney¹, Eric Y. Shin¹, Lanfang Wang¹, Juline N. Deppen¹, Sydney C. Ginn¹, and Rebecca D. Levit¹

¹Division of Cardiology, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia, USA

²Department of Cardiovascular Medicine, Xiangya Hospital, Changsha, China

Abstract

Neutrophil extracellular traps (NETs) are implicated in autoimmune, thrombotic, malignant, and inflammatory diseases; however, little is known of their endogenous regulation under basal conditions. Inflammatory effects of neutrophils are modulated by extracellular purines such as adenosine (ADO) that is inhibitory or ATP that generally up-regulates effector functions. In order to evaluate the effects of ADO on NETs, human neutrophils were isolated from peripheral venous blood from healthy donors and stimulated to make NETs. Treatment with ADO inhibited NET production as quantified by 2 methods: SYTOX green fluorescence and human neutrophil elastase (HNE)-DNA ELISA assay. Specific ADO receptor agonist and antagonist were tested for their effects on NET production. The ADO _{2A} receptor (A_{2A}R) agonist CSG21680 inhibited NETs to a similar degree as ADO, whereas the A_{2A}R antagonist ZM241385 prevented ADO's NET-inhibitory effects. Additionally, CD73 is a membrane bound ectonucleotidase expressed on mesenchymal stromal cells (MSCs) that allows manipulation of extracellular purines in tissues such as bone marrow. The effects of MSCs on NET formation were evaluated in coculture. MSCs reduced NET formation in a CD73-dependent manner. These results imply that extracellular purine balance may locally regulate NETosis and may be actively modulated by stromal cells to maintain tissue homeostasis.

Keywords

inflammatory; receptor; signaling; agonist; antagonist

Correspondence: Rebecca D. Levit, Division of Cardiology, Department of Medicine, Emory University School of Medicine, 101 Woodruff Circle, Room 319 Atlanta, GA 30322. rlevit@emory.edu.

AUTHORSHIP

K.X. collected and analyzed data, and wrote and revised original drafts. K.A.C. collected and analyzed data, and significantly edited and revised manuscript. L.W., J.N.D., S.C.G., and E.Y.S. collected data and revised manuscript. R.D.L. conceived the project, collected and curated data, and edited the manuscript. K.X. and K.A.C. contributed equally to the work.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

1 | INTRODUCTION

In response to inflammatory triggers, neutrophils expel their chromatin coated with effector molecules into the extracellular space.^{1,2} This inflammatory process of neutrophil extracellular trap (NET) formation evolved to contain infectious pathogens, but has been linked to autoimmune, thrombotic, malignant, and inflammatory disorders.^{3–8} NETs contain chromatin, histones, and neutrophil-derived antibiotic, proteolytic, and proinflammatory molecules that include neutrophil elastase and myeloperoxidase (MPO). Release of these molecules can directly damage the extracellular matrix and amplify the immune response.^{9–11} NETs also function to link inflammation with coagulation by triggering platelet activation, preventing fibrinolysis, promoting thrombin release, and activating the coagulation cascade.^{5,7,12,13} Together these functions immobilize pathogens and attract other immune cells contributing to host immune defense.

There is increasing evidence that NETs play a pathologic role in human disease. NETs are implicated in autoimmune diseases including rheumatoid arthritis and lupus,^{4,14,15} hyperinflammatory states such as sepsis, gout, diabetes, and acute respiratory distress syndrome,^{16,17} and cardiovascular diseases including atherosclerosis, deep venous thrombosis, and coronary thrombi.^{6,12,18} In vivo, local and systemic factors that modulate NETosis are unknown. Endogenous DNAase can degrade NETs, but does not inhibit their formation. Increased understanding of endogenous regulation of NETosis is warranted.

Neutrophil activation is tightly regulated in the bone marrow where mesenchymal stromal cells (MSCs) set up an anti-inflammatory bone marrow niche and have a strong anti-inflammatory effect on cells of the innate and adaptive immune systems.²² MSCs release chemokines and exosomes to effect immune cell activation,^{26,27} but may also influence surrounding cells by modulating extracellular purine balance.^{28–30} ATP is a ubiquitous nucleotide necessary for almost all forms of life on earth and thus is sensed by many immune and vascular cells as indicators of tissue health, energetic state, and homeostasis.^{31–33} MSCs and other cell types express several nucleoside triphosphate diphosphohydrolases including CD39 and ectonucleotidase (CD73) that can convert extracellular ATP into adenosine (ADO), influencing the purinergic environment sensed by surrounding cells.^{28–30}

ATP is normally tightly sequestered inside of cells, but is released into the extracellular space when discharged from damaged cells. ATP receptors on macrophages, neutrophils, and dendritic cells trigger proinflammatory cytokine production, inflammasome assembly, migration, and adaptive immune activation.³² ADP activates platelets, launching a cascade of events resulting in thrombus formation.³³ In contrast, ADO exerts anti-inflammatory and antithrombotic effects. In neutrophils, ADO inhibits chemotaxis, inflammatory cytokine secretion, ROS production, and adhesion molecule expression.^{34–36} These effects are mediated by the localized expression and activation of 4 ADO G-protein coupled receptors A₁, A_{2A}, A_{2b}, and A₃. Agonism of the A_{2A} and A_{2b} increases intracellular cAMP, inhibiting cell adhesion, migration, and infiltration.³⁷ Pharmacologic targeting of the A_{2A} and A_{2b} receptor is tissue protective under diverse experimental and clinical conditions.^{38–40}

The endogenous factors that limit NETosis are largely unknown but may be of therapeutic benefit. Thus we tested the hypothesis that ADO inhibits NET formation via agonism of the A_{2A}R and that MSCs exert NET-inhibitory effects via CD73-dependent ADO production. Using venous blood-derived neutrophils from healthy donors, we show this pathway is indeed active and may be a therapeutic target for diseases of excessive NETosis.

2 | MATERIALS AND METHODS

2.1 | Source and culture of human MSCs

Human MSCs were obtained from healthy donors via iliac crest bone marrow biopsy, then cultured in Minimum Essential Medium Eagle, Alpha Modification (#SH30265.01; GE Health Life Sciences, Chicago, IL) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (#S11150H and #B21110; Atlanta Biologicals, Atlanta, GA). All studies involving bone marrow-derived human MSCs were approved by the Emory University Institutional Review Board and were in accordance with institutional guidelines. MSCs were plated on 75 cm tissue culture flasks (#H353133; Corning, Corning, NY) and maintained in culture at 37°C in the presence of 5% carbon dioxide. Only cell passages 2–5 were used to conduct experiments. The phenotypic markers CD73, CD90, and CD105 were confirmed by flow cytometry. MSCs for co-culture experiments were grown to 80% confluence then seeded onto 96-well plates (Corning) at a density of 5×10^3 cells/well. Some MSCs were treated for 12 hours with and without 100 μ M of the irreversible CD73 inhibitor, adenosine 5' (α , β -methylene) diphosphate sodium salt (APCP; #363310; Tocris, Minneapolis, MN).

2.2 | Isolation and in vitro stimulation of human neutrophils

Human neutrophils were obtained from peripheral blood of healthy volunteers as previously described.⁴¹ All studies that involve drawing human blood were approved by the Emory University Institutional Review Board and were in accordance with institutional guidelines. All donors gave consent before blood draw. Ten milliliter of venous blood was collected through a 21 gauge needle in EDTA containing Vacutainer (#366643; Becton, Dickinson and Company, Franklin Lakes, NJ) and overlaid on 5 ml lympholyte cell separation media (#CL5071; Cedarlane Labs, Burlington, NC). The tubes were then centrifuged at 1600 rpm for 35 min at 20°C. Neutrophils were collected from the interphase and diluted in 10 ml of Hanks' balanced salt solution without calcium/magnesium (#SH30588.02; GE Health Life Sciences). The cell suspension was then spun at 1600 rpm for 10 min at 20°C and the supernatant was removed. The pellet was washed and lysed with red blood cell lysis buffer (#11814389001; Sigma–Aldrich, St. Louis, MO), centrifuged at 1200 rpm for 5 min at 20°C, and the supernatant was removed. The cells were resuspended in Hanks' balanced salt solution without calcium/magnesium and washed with a total volume of 10 ml. Finally, the tubes were centrifuged at 1600 rpm at 20°C for 10 min and the pellet was resuspended in RPMI 1640 medium without the addition of L-glutamine and phenol red (#R7509–1L; Sigma–Aldrich). Once neutrophils were isolated, they were cocultured with or without MSCs for 30 min prior to treatment with 324 nM PMA (# P8139; Sigma–Aldrich) or with LPS (#L4391; Sigma–Aldrich) to promote the formation of NETs. Neutrophil sample purity was confirmed by Wright–Giemsa Staining of human blood cells and flow cytometry.

2.2.1 | Stimulation of PMNs with PMA or LPS—Neutrophils were seeded onto 96-well plates (#165305, Thermo Fisher Scientific, Waltham, MA, and #655061, Greiner Bio-One, Monroe, NC) at a density of 5×10^4 cells/well in 100 μ l RPMI 1640 media then stimulated with 324 nM PMA or 50 μ g/ml LPS to induce NETs formation.

2.2.2 | Treatment of neutrophils with ADO and agonists—Freshly isolated human neutrophils were seeded onto 96-wells plates and pretreated for 30 min with either 10 μ M ADO (#A9251; Sigma–Aldrich), 5 μ M CGS21680 (#ab120453; Abcam, Cambridge, UK), 10 μ M 5'-N-ethylcarboxamideadenosine (NECA), 2'-MeCCPA, CGS21680, BAY60–6583, or HEMADO30 receptor agonists (#1691, #2281, #4472, and #1579; R&D Systems, Minneapolis, MN) before stimulation with PMA or LPS. Optimal treatment doses were determined by literature search and confirmed by dose–response curves ranging from 1 nM to 10 μ M.^{42–47}

2.2.3 | Stimulation of neutrophils with ADO receptor antagonists—Freshly isolated human neutrophils were seeded onto 96-wells plates and pretreated with ADO receptor-specific antagonists including 1 μ M ZM241385, 100 nM MRS1754, and 100 nM MRS3777 (#1036, #2752, and #2403; R&D Systems) for 15 min followed by 30 min of ADO or NECA treatment. The cells were then stimulated with PMA, and NET formation was quantified as previously described. Optimal antagonist dose was determined by dose–response curves ranging from 1 nM to 10 μ M.

2.3 | Quantification of extracellular DNA using SYTOX green assay

Abundance of extracellular DNA, a surrogate of NETs, was quantified using the SYTOX green assay. Once isolated neutrophils were plated onto a 96-well plate and treated as described, 5 μ M SYTOX green dye (#S7020; Invitrogen, Carlsbad, CA) was added to each well and the fluorescence was read with filter setting at 485-nm excitation/525-nm emission using a Synergy H1 Microplate Reader and Gene5 software (Biotek, Winooski, VT). The fluorescence was read every 5 min for a total of 2 h at 37°C. The fluorescence of each treatment was measured in triplicate between 90 and 120 min of each experiment at the early plateau of fluorescence.

2.4 | Quantification of NETs by ELISA

HNE-DNA complexes are highly specific markers of NET formation and can be detected by ELISA.⁴⁸ For this assay, neutrophils were treated as described above for 4 h. Prior to collecting the supernatant containing the generated NETs, a DNase digestion was performed by adding 2 U/ml DNase (#10104159001; Sigma–Aldrich) to each well for a total of 15 min. The digestion was stopped with the addition of 2.5 mM EGTA (#E0396; Sigma–Aldrich, St. Louis, MO) for 5 min. The supernatant was collected in clean micro centrifuge tubes and spun at $300 \times g$ for 5 min at room temperature to discard cell debris from the samples. A “NET Standard” was prepared from a mixture of NETs from 5 separate donors. A 1:2 serial dilution of the “NET Standard” was prepared in $1 \times$ PBS and 2.5 mM EGTA and used for the assay. Both the standards and samples were added onto an ELISA plated precoated with anti-HNE-DNA capture Abs and incubated over night at 4°C to allow binding of NET DNA to the plate. The next morning, the plate was washed 3 times with $1 \times$ PBS. Each well was

further incubated with 100 μ l of horse-radish peroxidase conjugated anti-DNA Ab (#11544675001; Roche, Penzberg, Germany) and incubated in the dark for 1 h. The plate was washed 4 times with 1 $\times$ PBS and 100 μ l of TMB peroxidase substrate (#34028; Thermo Scientific) was added to each well for 30 min causing a blue coloration indicating peroxidase activity and the presence of NETs. The peroxidase reaction was stopped by adding 100 μ l of 1 M HCL resulting in a color shift from blue to yellow. The absorbance of the plate was read at 450 nm using a Synergy H1 Micro plate Reader (Biotek).

2.5 | Detection of NET formation and ADO receptor expression by immunofluorescence

Neutrophils were seeded in 8 well chambers (#354632; Corning) at a density of $3-5 \times 10^6$ cells/well in 500 μ l of RPMI 1640 medium and stimulated with 324 nM PMA for 4 h. Cells were then fixed with 4% paraformaldehyde, washed with 1 $\times$ PBS, and permeabilized using 3% Triton X-100 reagent (#9002-93-1; Sigma-Aldrich). To detect the presence of NETs, anti-histone-3 Ab (1:200, #ab5103; Abcam), anti-MPO Ab (1:100, #ab11729; Abcam), and secondary Ab, Alexa Fluor 568 goat anti-rabbit IgG (1:2000, #a11011; Invitrogen) was used.

For visualization of A_{2A}R expression, cells were fixed and permeabilized as described above and an anti-A_{2A} receptor Ab (1:30, A_{2A}R Ab, #ab3461; Abcam) was used to detect the expression levels of the ADO receptor. Images were acquired using the ZEISS LSM 800 AIRYSCAN fluorescent microscope.

2.6 | Expression analysis of human ADO receptors

Quantitative real-time PCR (QRT-PCR) was performed to measure the expression levels of the ADO receptor family genes. RNA was isolated using a TRIzol extraction method following the manufacturer's protocol (Invitrogen). Synthesis of cDNA was performed using High-Capacity cDNA Reverse Transcription Kit's (#4374966; Thermo Fisher Scientific) protocol. The human neutrophil housekeeping gene (PPIA, Taqman:Hs04194521) was used as an endogenous control and all of the genes expressed were normalized to the PPIA housekeeping gene using ddCT method. The human ADO receptor primers used are as follows: A₁, TaqMan:Hs00181231_m1; A_{2A}, TaqMan: Hs00169123_m1; A_{2B}, TaqMan:Hs00386497_m1; and A₃, TaqMan: Hs00181232_m1. QRT-PCR was done on samples from the same population of neutrophils ran in separate experiments.

2.7 | Statistical analysis

The relative inhibition percent of NET accumulation was calculated as the decline in NETs produced under specified treatment conditions as compared to PMA only stimulated neutrophils isolated from the same donor. The PMA only treated group is used as the positive control having no relative inhibition percentage. All other treatment groups were calculated in respect to the PMA only treated group. Values are presented as mean $\pm$ SD. Data between 2 independent groups were compared using unpaired two-tailed *t*-test. Data among multiple groups were compared using one-way ANOVA followed by Tukey's post hoc analysis. A *P* < 0.05 was considered significant. All statistical analysis was performed on GraphPad Prism version 5.0 (GraphPad Prism Software Inc., San Diego, CA).

3 | RESULTS

3.1 | ADO inhibits NETosis

ADO exerts anti-inflammatory effects on neutrophils and other immune cells, inhibiting migration, priming, and inflammatory mediator production.^{28,30,32,34–37} We investigated whether exogenous ADO, as well as the stable ADO analogue NECA, can reduce NETs in freshly isolated human neutrophils. Staining of DNA with the cell impermeant dye SYTOX green showed robust NET production from neutrophils treated with PMA (Fig. 1A). Pretreatment of neutrophils with ADO or NECA reduced SYTOX staining (Fig. 1A). ADO inhibited NET production in a dose-responsive manner across a physiologic dose range (Fig. 1B). Using SYTOX green fluorescence quantification and HNE-DNA ELISA, both ADO and the ADO analogue NECA inhibit NET formation (Figs. 1C and D). Together these results demonstrate the NET-inhibitory effects of ADO.

3.2 | Quantification of mRNA transcripts by PCR confirmed immunohistochemical staining with robust A_{2A}R expression 2-fold higher than the next highest subtype A₃R

In mammals, there are 4 ADO receptor subtypes with 7 conserved transmembrane domains.^{32,49} ADO concentration, downstream effects on cAMP, and location of receptor expression dictate the cellular response to ADO. We utilized immunohistochemistry and PCR to determine the relative receptor expression in neutrophils freshly isolated from 7 healthy volunteers. Robust A_{2A}R expression was seen in all donors with lower and variable expression of other receptor subtypes (Fig. 2A). Quantification of mRNA transcripts by PCR confirmed immunohistochemical staining with robust A_{2A}R expression 2-fold higher than the next highest subtype, A₃R. The higher numbers of A_{2A}R ADO receptors suggest that this subtype is important in mediating the ADO-NET effects.

3.3 | The A_{2A} receptor mediates anti-NET effects of ADO

Chemical agonist and antagonist were used to evaluate the role of A_{2A}R on NETs suppression. The A_{2A}R agonist CGS21680 has been used in in vitro and in vivo studies.^{38,50,51} Treatment of freshly isolated human neutrophils with 5 μ M CGS21680 suppressed NET formation to a similar degree as ADO when detected by SYTOX green fluorescence (Figs. 3A and D). Neutrophils were also treated with the A_{2A}R antagonist ZM241385 (1 μ M) prior to ADO or NECA.^{44,52} ZM241385 impaired the NET-suppressive effects of ADO and NECA by SYTOX green fluorescence (Figs. 3B and C) and HNE-DNE ELISA (Figs. 3E and F). ADO and CGS21680 also suppressed LPS-induced NET formation as measured by SYTOX green fluorescence (Supplementary Fig. 1). Together, these findings suggest the importance of A_{2A}R signaling in ADO-mediated NET inhibition.

3.4 | A₁, A_{2B}, and A₃ ADO receptor subtypes demonstrate variable and less prominent NET-suppressive effects

Although the A_{2A}R is the most logical receptor to mediate NET-inhibitory effects of ADO due to its high expression, we investigated A₁, A_{2B}, and A₃ anti-NET effects. A₁ and A₃ are G_i protein-coupled receptors that inhibit adenylyl cyclase, reducing intracellular cAMP and activating cells. Although both the A_{2A}R and A_{2B}R receptors are G_s, increasing cAMP

levels, A_{2A}R has higher affinity for ADO, functioning at physiologic ADO concentrations. As predicted, the A₁ R-specific agonist 2'-MeCCPA did not inhibit NET formation and increased NETs in some donors across a broad range of doses (1 nM–10 μ M, Fig. 4A). The A₃R-specific agonist HEMADO also had variable effects on NETosis with variability in suppression and induction of NETs (Fig. 4A). The A_{2B}R agonist BAY60–6583 did have NET inhibitory effects by SYTOX green fluorescence (Fig. 4A).^{40,53} However, the more specific HNE-DNA ELISA assay failed to confirm the BAY60–6583 NET-inhibitory effects (Fig. 4B). To further evaluate the ADO receptor subtypes, antagonist studies were performed with ADO and NECA pretreatment before stimulant (PMA) administration. MRS1754 is a potent and selective A_{2B}R antagonist, while MRS3777 is an A₃R antagonist. Neither MRS1754 nor MRS3777 impaired NET regulation by ADO or NECA (Fig. 4C). These data support the primary role of A_{2A}R in mediating ADO's anti-NET effects, however involvement of other subtypes may occur in some people under physiologic or pathologic conditions.

3.5 | Human MSCs suppress NET formation by producing extracellular ADO

Recently, we showed that CD73 activity is critical for MSC's anti-inflammatory effects in myocardial ischemia-reperfusion, but the importance of MSCs, and their ADO production on NETosis, is unknown.²⁸ Freshly isolated human neutrophils were cocultured with bone marrow–derived MSCs. After PMA stimulation, neutrophils with MSC coculture showed reduced NETosis (Figs. 5A and B). These findings were confirmed by HNE-DNA ELISA, which showed a large NET inhibitory effect of MSCs (Fig. 5C). Ecto-5'-nucleotidase (CD73) converts extracellular adenosine monophosphate (AMP) into ADO, and can be blocked with the irreversible inhibitor APCP.^{28,29} The conditioned media of cultured MSCs supplemented with CD73 substrate AMP had increased ADO (Fig. 6A). However, MSCs pretreated with APCP have almost undetectable ADO in the conditioned media despite having normal morphology and viability (Fig. 6A). MSCs were washed and unbound APCP was removed prior to combination with neutrophils. Then, we evaluated the NET inhibitory effects of CD73-inhibited MSCs. Using both SYTOX green fluorescence and HNE-DNA ELISA assay, APCP-treated MSCs had a significantly reduced ability to inhibit PMA-induced NET formation (SYTOX green: $n = 10$; HNE-DNA ELISA: $n = 7$; Figs. 6B and C). The purinergic modulatory ability of MSCs may be a key component of their NET modulatory abilities.

4 | DISCUSSION

Like most aspects of inflammation, NETs perform important anti-pathogen function, but also have pathologic potential.^{1,2} Anti-citrullinated histone auto-antibodies have long been marker of disease in rheumatoid arthritis.⁴ Chronic inflammatory conditions such as gout, asthma, diabetes, and cardiovascular disease are initiated or perpetuated by NETs.^{3,6,7,9,11,12,17–19} Excessive inflammation in response to pathogens in acute respiratory distress syndrome, sepsis, and diffuse intravascular coagulation is tied to NETs.^{5,54} Better understanding of the endogenous regulation of NETs may direct therapeutic strategies for these pathophysiologic conditions.

The process of NETosis is inherently explosive, designed to rapidly activate the body's immune resources. This does not mean that this process is without modulatory mechanisms. Here, we demonstrate for the first time that ADO signaling through the A_{2A}R reduces NET formation (Figs. 1 and 3). The influence of A_{2A}R agonism in this effect is not surprising given the importance of this receptor in modulating neutrophil effector functions such as migration and reactive oxygen species formation.^{34,35,55} MSCs also have the ability to significantly reduce NET formation in response to PMA, relying at least partially on purinergic modulation to do so (Fig. 5). Inhibition of the MSC's ecto-5'-nucleotidase CD73 both impairs production of extracellular ADO and significantly reduces their NET modulatory abilities (Fig. 6). The ubiquitous nature of purines means that many cell types have ADO receptors that convey information about the extracellular milieu, such as energy state and tissue homeostasis.³¹⁻³³ Thus it is not surprising that these receptors then trigger dramatic responses in cell phenotype, especially from neutrophils that are particularly attuned to tissue damage or disease.

Previously our lab has utilized the immune- and purinergic-modulatory abilities of MSCs to alter the innate immune response in response to myocardial ischemia.^{28,56} MSC delivered to the cardiac epicardium increased myocardial ADO levels, reducing inflammation and ultimately improving cardiac function.²⁸ In tissues, purines are rapidly metabolized and local concentrations can vary considerably. This has made pharmacologic attempts to augment ADO levels largely unsuccessful and may be an argument in favor of cell-based therapy. By delivering MSCs to the site of injury, they may be more effective at modulating and maintaining the purinergic environment than a systemically administered drug.^{28,56}

We extensively characterized the effects of ADO signaling through the 4 receptor subtypes, concluding that the A_{2A}R is the strongest mediator of the anti-NET effects of ADO in this context. The A_{2A}R is well known to down-regulate inflammatory activation of neutrophils, including reductions in reactive oxygen species production.³⁵ The higher abundance of this receptor on neutrophils from healthy donors likely contributed to the dominance of this receptor in reducing NETosis (Fig. 2). Neutrophils from diseased patients may have different relative receptor subtype expression that could change neutrophils response to ADO in respect to NETosis. Another unknown is if ADO directly influences PAD4 (protein arginine deiminase) activation.²¹ Future work will evaluate if increases in intracellular cAMP caused by A_{2A}R G_s activation directly change PAD4 activity in neutrophils. Another possibility is that the reduction in reactive oxygen species production due to ADO exposure reduced NETosis. Some studies, but not all, suggest that ROS are required for NETosis.⁵⁸⁻⁶⁰ A better understanding of the downstream signaling pathways influencing PAD4 will allow new therapeutic strategies for diseases of disordered NETosis.

A strength of these studies is our exclusive use of human neutrophils, a cell type that is short lived and has considerable biological variability. The universal response of these cells from a variety of donors to MSCs and ADO is reassuring in the accuracy of the conclusions. However, using human cells limits the use of genetic manipulation or viral-mediated receptor knockdown. Future work will confirm these findings using mouse neutrophils from various genetic backgrounds lacking ADO receptor subtypes. We will also utilize

neutrophils from donors with known chronic inflammatory diseases to identify if disordered purinergic detection or signaling contributes to excessive NETosis or inflammation.

Another strength of these experiments is our use of 2 distinct methods to quantify NETosis. Fluorescence techniques have been criticized for their lack of specificity and inability to distinguish apoptotic and NETing neutrophils. In most cases, our SYTOX green fluorescence quantification concurred with the more specific HNE-DNA ELISA data. Interestingly, these data did not agree in our assays of the A_{2B}R agonists with SYTOX showing a NET inhibitory effect and HNE-DNA ELISA showing no NET-inhibitory capacity of BAY60–6583 (Fig. 4). These experiments were conducted with neutrophils derived from different donors on different days, and differences in receptor subtype expression, downstream signaling, or inflammatory activation could explain these results. However, the lack of response of the A_{2B}R antagonist reversing the inhibitory effects of ADO and NECA gave more weight to our conclusion that the A_{2B}R does not play as prominent a role as A_{2A}R in NET regulation. However, more precise evaluation using neutrophils from A_{2B}R deficient mice will add further clarity to this issue.

In our experiments, we used PMA and LPS to stimulate NET formation and confirmed similar NET suppression with A_{2A}R agonism (Supplementary Fig. 1). PMA is a potent NET inducer, but is not present in physiologic inflammation. PMA NETs have been shown to follow similar intracellular signaling pathways as infectious stimuli.⁵⁸ However, different antigens have been shown to trigger NETs differently, most notably in their reliance on ROS. How these pathways interact to influence PAD4 activity is not yet known. The role of ADO in regulating PAD4 activity and therefore NETs from different stimuli will require additional in vitro and in vivo studies.

In conclusion, we find a strong effect of ADO, signaling through the A_{2A} receptor subtype, to powerfully inhibit NETs in neutrophils from a large number of healthy human donors. MSCs, which set up the anti-inflammatory environment in bone marrow, also suppress NETosis in a purinergic-dependent manner. Many diseases of pathologic inflammation have been linked to excessive or inappropriate NETosis, making these findings important for strategically identifying new therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

FUNDING

L30 HL129299 (RDL), 1RO1 HL140223 (RDL), and T32 5T32HL007745 (KAC).

Abbreviations:

ADO	adenosine
AMP	adenosine monophosphate
HNE	human neutrophil elastase

MPO	myeloperoxidase
MSCs	mesenchymal stromal cells
NET	neutrophil extracellular trap
PAD	protein arginine deiminase
QRT-PCR	quantitative real-time PCR

REFERENCES

1. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science*. 2004;303:1532–1535. [PubMed: 15001782]
2. McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P. Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell Host Microbe*. 2012;12:324–333. [PubMed: 22980329]
3. Toussaint M, Jackson DJ, Swieboda D, et al. Host DNA released by NETosis promotes rhinovirus-induced type-2 allergic asthma exacerbation. *Nat Med*. 2017;23:681–691. [PubMed: 28459437]
4. Pratesi F, Dioni I, Tommasi C, et al. Antibodies from patients with rheumatoid arthritis target citrullinated histone 4 contained in neutrophils extracellular traps. *Ann Rheum Dis*. 2014;73: 1414–1422. [PubMed: 23727635]
5. McDonald B, Davis RP, Kim SJ, et al. Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice. *Blood*. 2017;129:1357–1367. [PubMed: 28073784]
6. Mangold A, Alias S, Scherz T, et al. Coronary neutrophil extracellular trap burden and deoxyribonuclease activity in ST-elevation acute coronary syndrome are predictors of ST-segment resolution and infarct size. *Circ Res*. 2015;116:1182–1192. [PubMed: 25547404]
7. Fuchs TA, Brill A, Wagner DD. Neutrophil extracellular trap (NET) impact on deep vein thrombosis. *Arterioscler Thromb Vasc Biol*. 2012;32:1777–1783.
8. Wolach O, Sellar RS, Martinod K, et al. Increased neutrophil extracellular trap formation promotes thrombosis in myeloproliferative neoplasms. *Sci Transl Med*. 2018;10:eaan8292. [PubMed: 29643232]
9. Warnatsch A, Ioannou M, Wang Q, Papayannopoulos V. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. *Science*. 2015;349:316–320. [PubMed: 26185250]
10. Tillack K, Breiden P, Martin R, Sospedra M. T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses. *J Immunol*. 2012;188:3150–3159. [PubMed: 22351936]
11. Sangaletti S, Tripodo C, Chiodoni C, et al. Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity. *Blood*. 2012;120:3007–3018. [PubMed: 22932797]
12. de Boer OJ, Li X, Teeling P, et al. Neutrophils, neutrophil extracellular traps and interleukin-17 associate with the organisation of thrombi in acute myocardial infarction. *Thromb Haemost*. 2013;109: 290–297. [PubMed: 23238559]
13. Gould TJ, Vu TT, Swystun LL, et al. Neutrophil extracellular traps promote thrombin generation through platelet-dependent and platelet-independent mechanisms. *Arterioscler Thromb Vasc Biol*. 2014;34:1977–1984. [PubMed: 25012129]
14. Knight JS, Subramanian V, O'Dell AA, et al. Peptidylarginine deiminase inhibition disrupts NET formation and protects against kidney, skin and vascular disease in lupus-prone MRL/lpr mice. *Ann Rheum Dis*. 2015;74:2199–2206. [PubMed: 25104775]
15. Grayson PC, Kaplan MJ. At the bench: neutrophil extracellular traps (NETs) highlight novel aspects of innate immune system involvement in autoimmune diseases. *J Leukoc Biol*. 2016;99:253–264. [PubMed: 26432901]

16. Xu J, Zhang X, Pelayo R, et al. Extracellular histones are major mediators of death in sepsis. *Nat Med.* 2009;15:1318–1321. [PubMed: 19855397]
17. Wong SL, Demers M, Martinod K, et al. Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. *Nat Med.* 2015;21:815–819. [PubMed: 26076037]
18. Megens RT, Vijayan S, Lievens D, et al. Presence of luminal neutrophil extracellular traps in atherosclerosis. *Thromb Haemost.* 2012;107: 597–598. [PubMed: 22318427]
19. Ge L, Zhou X, Ji WJ, et al. Neutrophil extracellular traps in ischemia-reperfusion injury-induced myocardial no-reflow: therapeutic potential of DNase-based reperfusion strategy. *Am J Physiol Heart Circ Physiol.* 2015;308:H500–9. [PubMed: 25527775]
20. Shishikura K, Horiuchi T, Sakata N, et al. Prostaglandin E2 inhibits neutrophil extracellular trap formation through production of cyclic AMP. *Br J Pharmacol.* 2016;173:319–331. [PubMed: 26505736]
21. Lewis HD, Liddle J, Coote JE, et al. Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. *Nat Chem Biol.* 2015;11:189–191. [PubMed: 25622091]
22. Le Blanc K, Mougiakakos D. Multipotent mesenchymal stromal cells and the innate immune system. *Nat Rev Immunol.* 2012;12: 383–396. [PubMed: 22531326]
23. Forbes GM, Sturm MJ, Leong RW, et al. A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy. *Clin Gastroenterol Hepatol.* 2014;12:64–71. [PubMed: 23872668]
24. Le Blanc K, Frassoni F, Ball L, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet North Am Ed.* 2008;371:1579–1586.
25. Tan J, Wu W, Xu X, et al. Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. *JAMA.* 2012;307:1169–1177. [PubMed: 22436957]
26. Sze SK, de Kleijn DPV, Lai RC, et al. Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. *Mol Cell Proteomics.* 2007;6:1680–1689. [PubMed: 17565974]
27. Zhang B, Yin Y, Lai RC, Tan SS, Choo AB, Lim SK. Mesenchymal stem cells secrete immunologically active exosomes. *Stem Cells Dev.* 2014;23:1233–1244. [PubMed: 24367916]
28. Shin EY, Wang L, Zemskova M, et al. Adenosine production by biomaterial-supported mesenchymal stromal cells reduces the innate inflammatory response in myocardial ischemia/reperfusion injury. *J Am Heart Assoc.* 2018;7:e006949. [PubMed: 29331956]
29. Chen X, Shao H, Zhi Y, et al. CD73 pathway contributes to the immuno-suppressive ability of mesenchymal stem cells in intraocular autoimmune responses. *Stem Cells Dev.* 2016;25:337–346. [PubMed: 26650818]
30. Saldanha-Araujo F, Ferreira FI, Palma PV, et al. Mesenchymal stromal cells up-regulate CD39 and increase adenosine production to suppress activated T-lymphocytes. *Stem Cell Res.* 2011;7:66–74. [PubMed: 21546330]
31. Verkhratsky A, Burnstock G. Biology of purinergic signalling: its ancient evolutionary roots, its omnipresence and its multiple functional significance. *Bioessays.* 2014;36:697–705. [PubMed: 24782352]
32. Burnstock G, Boeynaems JM. Purinergic signalling and immune cells. *Purinergic Signal.* 2014;10:529–564. [PubMed: 25352330]
33. Burnstock G Purinergic signaling in the cardiovascular system. *Circ Res.* 2017;120:207–228. [PubMed: 28057794]
34. Sullivan GW, Lee DD, Ross WG, et al. Activation of A2A adenosine receptors inhibits expression of alpha 4/beta 1 integrin (very late antigen-4) on stimulated human neutrophils. *J Leukoc Biol.* 2004;75:127–134. [PubMed: 14525968]
35. Sullivan GW, Rieger JM, Scheld WM, Macdonald TL, Linden J. Cyclic AMP-dependent inhibition of human neutrophil oxidative activity by substituted 2-propynylcyclohexyl adenosine A(2A) receptor agonists. *Br J Pharmacol.* 2001;132:1017–1026. [PubMed: 11226132]

36. van der Hoeven D, Wan TC, Auchampach JA. Activation of the A(3) adenosine receptor suppresses superoxide production and chemotaxis of mouse bone marrow neutrophils. *Mol Pharmacol*. 2008;74: 685–696. [PubMed: 18583455]
37. Barletta KE, Ley K, Mehrad B. Regulation of neutrophil function by adenosine. *Arterioscler Thromb Vasc Biol*. 2012;32:856–864. [PubMed: 22423037]
38. Arasa J, Martos P, Terencio MC, Valcuende-Cavero F, Montesinos MC. Topical application of the adenosine A2A receptor agonist CGS-21680 prevents phorbol-induced epidermal hyperplasia and inflammation in mice. *Exp Dermatol*. 2014;23:555–560. [PubMed: 24889129]
39. Konrad FM, Zwergel C, Ngamsri KC, Reutershan J. Anti-inflammatory effects of heme oxygenase-1 depend on adenosine A2A- and A2B- receptor signaling in acute pulmonary inflammation. *Front Immunol*. 2017;8:1874. [PubMed: 29326725]
40. Eckle T, Krahn T, Grenz A, et al. Cardioprotection by ecto-5'-nucleotidase (CD73) and A2B adenosine receptors. *Circulation*. 2007;115:1581–1590. [PubMed: 17353435]
41. Kjeldsen L, Sengeløv H, Borregaard N. Subcellular fractionation of human neutrophils on Percoll density gradients. *J Immunol Methods*. 1999;232:131–143. [PubMed: 10618515]
42. Liu YW, Yang T, Zhao L, et al. Activation of adenosine 2A receptor inhibits neutrophil apoptosis in an autophagy-dependent manner in mice with systemic inflammatory response syndrome. *Sci Rep*. 2016;6:33614. [PubMed: 27647162]
43. Jacobson KA, Park KS, Jiang JL, et al. Pharmacological characterization of novel A3 adenosine receptor-selective antagonists. *Neuropharmacology*. 1997;36:1157–1165. [PubMed: 9364471]
44. Poucher SM, Keddie JR, Singh P, et al. The in vitro pharmacology of ZM 241385, a potent, non-xanthine A2a selective adenosine receptor antagonist. *Br J Pharmacol*. 1995;115:1096–1102. [PubMed: 7582508]
45. Gao ZG, Kim SK, Biadatti T, et al. Structural determinants of A(3) adenosine receptor activation: nucleoside ligands at the agonist/antagonist boundary. *J Med Chem*. 2002;45:4471–4484. [PubMed: 12238926]
46. van der Hoeven D, Wan TC, Gizewski ET, et al. A role for the low-affinity A2B adenosine receptor in regulating superoxide generation by murine neutrophils. *J Pharmacol Exp Ther*. 2011;338: 1004–1012. [PubMed: 21693629]
47. Klotz K-N, Falgner N, Kachler S, et al. [3H]HEMADO— a novel tritiated agonist selective for the human adenosine A3 receptor. *Eur J Pharmacol*. 2007;556:14–18. [PubMed: 17126322]
48. Sil P, Yoo DG, Floyd M, Gingerich A, Rada B. High throughput measurement of extracellular DNA release and quantitative NET formation in human neutrophils in vitro. *J Vis Exp*. 2016 10.3791/52779
49. Hasko G, Cronstein BN. Adenosine: an endogenous regulator of innate immunity. *Trends Immunol*. 2004;25:33–39. [PubMed: 14698282]
50. Lupica CR, Cass WA, Zahniser NR, Dunwiddie TV. Effects of the selective adenosine A2 receptor agonist CGS 21680 on in vitro electrophysiology, cAMP formation and dopamine release in rat hippocampus and striatum. *J Pharmacol Exp Ther*. 1990;252: 1134–1141. [PubMed: 2156991]
51. Lebon G, Edwards PC, Leslie AG, Tate CG. Molecular determinants of CGS21680 binding to the human adenosine A2A receptor. *Mol Pharmacol*. 2015;87:907–915. [PubMed: 25762024]
52. Moura VJG, Alencar AKN, Calasans-Maia JA, et al. Novel agonist of adenosine receptor induces relaxation of corpus cavernosum in guinea pigs: an in vitro and in vivo study. *Urology*. 2015;85: 1214.e17–1214 e21.
53. Eckle T, Faigle M, Grenz A, Laucher S, Thompson LF, Eltzschig HK. A2B adenosine receptor dampens hypoxia-induced vascular leak. *Blood*. 2008;111:2024–2035. [PubMed: 18056839]
54. Gregoire M, Uhel F, Lesouhaitier M, et al. Impaired efferocytosis and neutrophil extracellular trap clearance by macrophages in ARDS. *Eur Respir J*. 2018;52:1702590. [PubMed: 29946009]
55. Yago T, Tsukamoto H, Liu Z, Wang Y, Thompson LF, McEver RP. Multi-inhibitory effects of A2A adenosine receptor signaling on neutrophil adhesion under flow. *J Immunol*. 2015;195:3880–3889. [PubMed: 26355151]
56. Levit RD, Landazuri N, Phelps EA, et al. Cellular encapsulation enhances cardiac repair. *J Am Heart Assoc*. 2013;2:e000367. [PubMed: 24113327]

57. Masutomi H, Kawashima S, Kondo Y, et al. Induction of peptidylarginine deiminase 2 and 3 by dibutyryl cAMP via cAMP-PKA signaling in human astrocytoma U-251MG cells. *J Neurosci Res.* 2017;95: 1503–1512. [PubMed: 27704563]
58. Kenny EF, Herzig A, Kruger R, et al. Diverse stimuli engage different neutrophil extracellular trap pathways. *eLife.* 2017;6:e24437. [PubMed: 28574339]
59. Keshari RS, Verma A, Barthwal MK, Dikshit M. Reactive oxygen species-induced activation of ERK and p38 MAPK mediates PMA-induced NETs release from human neutrophils. *J Cell Biochem.* 2013;114:532–540. [PubMed: 22961925]
60. Rochael NC, Guimarães-Costa AB, Nascimento MTC, et al. Classical ROS-dependent and early/rapid ROS-independent release of neutrophil extracellular traps triggered by *Leishmania* parasites. *Sci Rep.* 2015;5:18302. [PubMed: 26673780]

FIGURE 1. Adenosine (ADO) suppresses human neutrophil extracellular trap (NET) formation.

After PMNs were seeded onto 96-well plates, ADO or NECA was applied to the culture medium for 30 min followed by stimulation with 324 nM PMA. Neutrophils treated with ADO or its analogue NECA made fewer NETs visualized using SYTOX green cell impermanent DNA dye (A). ADO inhibited NET formation in a dose-responsive manner (B). NET quantification by both SYTOX Green (C) staining and human neutrophil elastase-DNA complex ELISA (D) showed similar pattern of inhibition of NETosis with ADO and NECA treatment. ($n = 7-15$) Data are expressed as mean $\pm$ SD, * $P < 0.05$, *** $P < 0.001$. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc analysis

FIGURE 2. Isolated human neutrophils express high levels of adenosine $2A$ receptor subtype ($A_{2A}R$).

Human neutrophils were freshly isolated, fixed, and stained for the neutrophil specific marker myeloperoxidase (MPO) and $A_{2A}R$ (**A**). High cell purity and $A_{2A}R$ expression were seen. Quantitative PCR analysis for adenosine receptor subtype transcripts detected a predominance of $A_{2A}R$ transcripts (**B**; $n = 7$)

FIGURE 3. The adenosine receptor 2a ($A_{2A}R$) mediates the anti-NET effects of adenosine. Neutrophils were pretreated with specific $A_{2A}R$ agonist CSG21680 or antagonist ZM241385 prior to PMA stimulation. Treatment with 5 μ M of the $A_{2A}R$ -specific agonist CSG21680 suppress NET formation to a similar degree as ADO as detected by SYTOX green (**A**) and ELISA (**D**) ($n = 6-7$). Pretreatment with the A_{2A} antagonist (ZM 241385, 1 μ M) reversed adenosine and NECA's inhibition of NETosis by SYTOX green (**B** and **C**) and HNE-DNA ELISA (**E** and **F**; $n = 4-8$). Data are expressed as mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc analysis

FIGURE 4. Agonist and antagonism of adenosine (ADO) receptor subtypes A₁, A_{2B}, and A₃ have inconsistent effects on neutrophil extracellular trap (NET) production. Neutrophils were pretreated with specific ADO receptor agonists for 30 min and NET production was analyzed using the SYTOX green assay. Both the A₁ receptor agonist, 2'-MeCCPA (10 μ M), and HEMADO (1 μ M), an agonist for the A₃ receptor, failed to significantly reduce PMA-induced NET production (A; $n = 4-9$). However, BAY60-6583 (50 nM), an A_{2B} receptor agonist, significantly suppressed NET formation after PMA by SYTOX green fluorescence (A; $n = 5-9$). To confirm these findings, we tested the A_{2B} agonist effects on NET formation by the more specific HNE-DNA ELISA (B), which showed no NET inhibition with A_{2B} agonism. Furthermore, the A_{2B}-specific antagonist, MRS1754 (100 nM), and the A₃-specific antagonist, MRS3777 (100 nM), were ineffective in blocking the ADO- or NECA-induced NET inhibition (C; $n = 5-7$). Data are expressed as

mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc analysis

FIGURE 5. Mesenchymal stromal cells (MSCs) suppress neutrophil extracellular trap (NET) formation in vitro.

Human neutrophils were cocultured with MSCs for 30 min prior to stimulation with PMA. SYTOX green fluorescence staining (**A**) shows increase extracellular DNA, 2 h after PMA administration, and a reduction in cells cocultured with MSCs. MSCs alone without neutrophils showed no fluorescence (not shown). Quantification of green fluorescence (**B**; $n = 18$) as well as HNE-DNA ELISA assay (**C**) showed strong NET-inhibitory effect of MSCs ($n = 13$). Data are expressed as mean $\pm$ SD, *** $P < 0.001$ using the paired t -test analysis

FIGURE 6. Mesenchymal stromal cell (MSC)-derived adenosine (ADO) is a key mechanism of neutrophil extracellular trap (NET) suppression.

MSCs were pretreated overnight with 100 μ M APCP, a nonhydrolyzable inhibitor of CD73. HPLC analysis of conditioned media showed no ADO production without supplementation of the CD73 substrate AMP (**A**). MSC treatment with APCP had almost complete impairment of adenosine production from extracellular AMP (**A**). Quantification of SYTOX green fluorescence indicates that APCP impairs the ability of MSCs to inhibit NET production (**B**; $n = 10$). The relative inhibition percentage of APCP–MSC-treated PMN group was significantly reduced compared to the CD73 competent MSC-treated group by human neutrophil elastase-DNA ELISA (**C**; $n = 7$). Data are expressed as mean $\pm$ SD, * $P < 0.05$, *** $P < 0.001$. Statistical analysis was performed using one-way ANOVA followed by Tukey’s post hoc analysis