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Neutrophil extracellular trap formation is elicited in response to cold physical plasma

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ABSTRACT

Cold physical plasma is an ionized gas with a multitude of components, including hydrogen peroxide and other reactive oxygen and nitrogen species. Recent studies suggest that exposure of wounds to cold plasma may accelerate healing. Upon wounding, neutrophils are the first line of defense against invading microorganisms but have also been identified to play a role in delayed healing. In this study, we examined how plasma treatment affects the functions of peripheral blood neutrophils. Plasma treatment induced oxidative stress, as assessed by the oxidation of intracellular fluorescent redox probes; reduced metabolic activity; but did not induce early apoptosis. Neutrophil oxidative burst was only modestly affected after plasma treatment, and the killing of *Pseudomonas aeruginosa* and *Staphylococcus aureus* was not significantly affected. Intriguingly, we found that plasma induced profound extracellular trap formation. This was inhibited by the presence of catalase during plasma treatment but was not replicated by adding an equivalent concentration of hydrogen peroxide. Plasma-induced neutrophil extracellular trap formation was not dependent on the activity of myeloperoxidase or NADPH oxidase 2 but seemed to involve short-lived molecules. The amount of DNA release and the time course after plasma treatment were similar to that with the common neutrophil extracellular trap inducer PMA. After neutrophil extracellular traps had formed, concentrations of IL-8 were also significantly increased in supernatants of plasma-treated neutrophils. Both neutrophil extracellular traps and IL-8 release may aid antimicrobial activity and spur inflammation at the wound site. Whether this aids or exacerbates wound healing needs to be tested. *J. Leukoc. Biol.* 100: 791–799; 2016.

Introduction

Leukocytes play an important role in wound healing. During the inflammatory phase, neutrophils are the first cells to arrive at the wound followed by monocytes and lymphocytes [1]. The neutrophils' powerful arsenal of digestive and ROS-producing enzymes is toxic to bacteria and host tissue alike. Although neutrophil influx is indispensable for proper wound healing [2], their sustained presence is associated with nonhealing wounds [3] that often lack efficient therapy [4]. Recent evidence in rodents [5–7] and pilot studies in humans [8–10] support the idea that cold physical plasma may aid wound healing, but whether plasma impacts positively or negatively on neutrophil function is not known.

Plasma medicine is a young research field in which cold physical plasma is investigated for its potential application in areas, such as dentistry, implantology, oncology, dermatology, and wound healing [11]. In physics, plasma is referred to as the fourth state of matter. It is generated by applying large amounts of energy to a gas, up to a point where electrons dissociate from the molecules. Recent technological advances have allowed the generation of "cold" plasma that exhibits high chemical but low thermal energy. Cold plasma can be safely applied to cells and tissues and is well tolerated [12]. It is a multicomponent system, including photons, ions, electrons, UV, and infrared radiation, electrical fields, and ROS and nitrogen species [13]. A major stable reactive species in plasma is H_2O_2, which in our system, is generated at a rate of 60 μM/min [14]. All of these components are considered to contribute to the biologic effects of exposure to cold plasma.

We have previously found that plasma treatment of peripheral blood lymphocytes causes oxidation of intracellular redox probes and accelerates apoptosis [15]. Neutrophils will also be exposed to plasma during wound treatment, but it is not known how they are

Abbreviations: 4-ABAH = 4-aminobenzoic acid hydrazide, λ_{em} = emission, λ_{ex} = excitation, APF = aminophenyl fluorescein, CM-H_2DCF-DA = chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate, DHR123 = dihydrorhodamine 123, DPI = diphenyleneiodonium, GSH = glutathione (reduced form), H_2O_2 = hydrogen peroxide, MPO = myeloperoxidase, NAC = *N*-acetyL-L-cysteine, NET = neutrophil extracellular trap, NOX = NADPH oxidase, ROS = reactive oxygen species, SOD = superoxide dismutase

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affected by this physicochemical gas cocktail. Crucial effector functions of neutrophils are activation of their NOX to generate superoxide and the phagocytosis and intracellular killing of microorganisms [16]. Release of NETs—sticky DNA decorated with histones and antimicrobial cytoplasmic and granular proteins [17], such as MPO [18] and neutrophil elastase [19]—are also an increasingly characterized part of the neutrophil's response to danger-associated stimuli. NETs are believed to trap bacteria and help contain infection [20]. However, as a result of their cytotoxic and immunogenic components, dysregulated NET formation or clearance is associated with a variety of pathologies, such as sepsis, cancer, or autoimmunity [21–23]. We have examined how the above functions are affected when isolated human neutrophils are exposed to cold plasma. We report only minor effects on the ability of neutrophils to produce superoxide in response to further stimulation and on phagosomal killing of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. However, plasma treatment was a potent inducer of NET formation. We have used a range of inhibitors to probe the mechanism of the reaction. Furthermore, experimentally added oxidant and antioxidants were added to determine their role in plasma-mediated NETosis. Plasma treatment also led to the release of the proinflammatory cytokine IL-8 after 6 h, potentially as the cells died, as a result of NETosis. Our results are a first step toward a better understanding of neutrophil behavior in response to cold physical plasma.

MATERIALS AND METHODS

Materials

Dextran and Ficoll were purchased from VWR International (Radnor, PA, USA). Catalase, cytochrome C, H_2O_2, PMA, SOD, Triton X-100, 4-ABAH, DPI, L-GSH, NAC, uric acid, and DAPI were obtained from Sigma (St. Louis, MO, USA). Amplex UltraRed, APF, CellEvent Caspase-3/7 Detection Reagent Kit, CM-H_2DCF-DA, DHR123, PrestoBlue resazurin, goat anti-rabbit Alexa Fluor 488, and Sytox Green and Orange were obtained from Thermo Fisher Scientific Life Sciences (Waltham, MA, USA). The polyclonal goat anti-human neutrophil elastase and rabbit anti-citrullinated histone H3 antibodies were purchased from Abcam (Cambridge, United Kingdom). Protein block was obtained from Dako (Carpinteria, CA, USA), and fixation buffer and anti-human IL-8 ELISA were purchased from BioLegend (San Diego, CA, USA). Tryptone soya broth was purchased from Oxoid (Thermo Scientific, Hampshire, United Kingdom), and Vectashield containing DAPI was obtained from Vector Laboratories (Burlingame, CA, USA). Tissue-culture plastic was obtained from Becton Dickinson (Franklin Lakes, NJ, USA), Nunc (Roskilde, Denmark), and Sarstedt (Nümbrecht, Germany).

Neutrophil isolation

Neutrophils were isolated from the venous blood of healthy controls, obtained with informed consent and the approval of the New Zealand Health and Disability Ethics Committee. Neutrophil isolation was performed at room temperature using a modified method of Böyum [24]. In brief, dextran (final concentration 1%) sedimentation of erythrocytes was followed by Ficoll density gradient separation of white blood cells. Contaminating red cells were removed by hypotonic lysis. Neutrophils were resuspended in HBSS (10 mM PBS, pH 7.4, 500 μM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml glucose). Percentages of dead cells and contaminating PBMCs were <5%, as routinely tested by flow cytometry.

Treatment regime and plasma source

Neutrophils (1×10^6/ml in HBSS) were added in 1 ml aliquots to a tissue culture-treated, 24-well plate. Plasma treatment was carried out for the indicated times at room temperature and depending on the experiment, with or without the presence of 1 of the following: catalase (20 μg/ml), calcium (0.19 mg/ml), DPI (10 mM), NAC (2 mM), 4-ABAH (100 μM), GSH (10 mM), or uric acid (100 μM). Alternatively, a treatment-time, concentration-matched concentration of H_2O_2 (3 min plasma yields ~180 μM) was added as a bolus or as 36 additions of 5 μM each over 3 min to mimic the H_2O_2 generation by plasma. HBSS (1 ml) was also plasma treated for 6 min in the absence of cells, and 500 μl of this solution was added to 500 μl neutrophils in HBSS (at 2×10^6/ml). Some samples were also centrifuged immediately following plasma treatment and resuspended in fresh HBSS. As a plasma source, an atmospheric pressure argon plasma jet (kINPen 11; Neoplas Tools, Greifswald, Germany) was used. During treatment, the jet was set over the center position of each well. When treatment was finished, the jet automatically moved to the center of the adjacent well via a computer-driven xyz table (CNC-Step, Geldern, Germany). This procedure assured a fixed distance of the jet to the treated suspensions. The jet was operated at 1 MHz frequency and by using 3 standard liters/min of argon gas (Air Liquide, Paris Cedex, France). Gas flow rate was calibrated and adjusted with a precision of ±0.1 liter via a gas-flow controller (MKS Instruments, Munchen, Germany). To compensate for any evaporation of liquid as a result of the gas flux, ~33 μl sterile water was added for each minute of plasma treatment. To investigate any effect of pure argon gas alone, plasma was switched off for treatment.

Assessment of cellular redox changes

To assess responses of redox-sensitive probes, neutrophils were loaded with CM-H_2DCF-DA (10 μM), DHR123 (2 μM), or APF (5 μM) for 30 min at 37°C. Subsequently, cells were washed, resuspended in HBSS (1×10^6/ml), treated with plasma, and washed again, and fluorescence was acquired immediately by flow cytometry (Gallios; Beckman Coulter, Brea, CA, USA). Median fluorescence intensities were calculated using Kaluza software (Beckman Coulter). Dead cells were excluded from the analysis by staining with DAPI.

Assessment of cell viability

To assess metabolic activity by means of the generation of reduction equivalents, neutrophils were plasma treated and divided in aliquots immediately (9 replicates/sample) into clear, flat-bottom, tissue culture-treated, 96-well plates. Resazurin (20 μl) was added after 2, 4, and 6 h to 3 replicates, each containing neutrophils or HBSS alone for a blank value. Resazurin freely diffuses into the cells, where it is reduced via NADH/NADPH-dependent reductase activity to its fluorescent form resorufin. Fluorescence (λ_{ex} 530 nm; λ_{em} 590 nm) was recorded continuously >30 min at 37°C using a plate reader (F200 Pro; Tecan, Switzerland). Background (resazurin alone) was subtracted and the slope for each sample calculated. For apoptosis, neutrophils were plasma treated and divided in aliquots immediately in black, 96-well plates, and caspase 3/7 detection reagent, which is cleaved specifically by these caspases to unmask a fluorescent DNA-binding dye, was added. Cells were incubated at 37°C, and fluorescence (λ_{ex} 485 nm; λ_{em} 535 nm) was recorded after 2, 4, and 6 h using a plate reader (F200 Pro; Tecan).

Evaluation of oxidative burst

Neutrophils (1×10^6/ml in HBSS) were plasma treated in the presence or absence of catalase (20 μg/ml) or treated with H_2O_2 (180 μM bolus), transferred to cuvettes containing cytochrome C (1 mg/ml in HBSS) and catalase (20 μg/ml), and then received PMA stimulation (200 nM). Rates of change in absorbance were measured over 10 min at 550 nm (Hitachi 4500; Hitachi, Tokyo, Japan) and 37°C. SOD (20 μg/ml) was used to control for the specificity of the reaction. Alternatively, absorbance was measured in control and plasma-treated cells that did not receive PMA stimulation. Rates were normalized to control values for untreated neutrophils for each independent experiment.

Neutrophil bacterial killing assay

S. aureus (ATTC: 27217) was obtained from the New Zealand Communicable Disease Centre (Porirua, New Zealand). *P. aeruginosa* was a clinical isolate from an unknown source. To measure bacterial killing, neutrophils (10^7/ml in HBSS containing 10% autologous serum) were plasma treated for 3 min

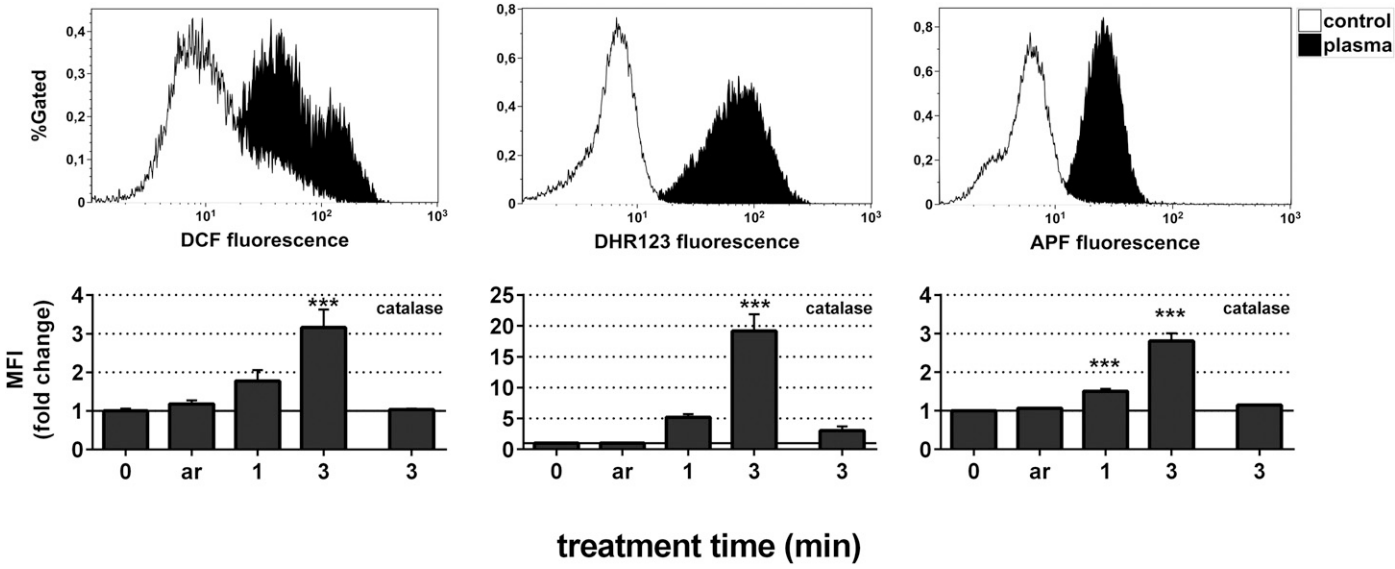

Figure 1. Neutrophils are exposed to oxidants when treated with cold plasma. Neutrophils were loaded with CM-H_2DCF-DA (DCF), DHR123, or APF and then left undisturbed or treated with plasma or argon. Fluorescence intensity was acquired by flow cytometry, and mean fluorescence intensity (MFI) was compared. (Upper) Histograms are of a representative experiment and show the fluorescence increase after 3 min of plasma treatment. (Lower) Probe-loaded neutrophils were incubated in the presence or absence of catalase before plasma treatment. ar, argon gas control. Data are presented as means + SE of 2–3 independent experiments. $***P < 0.001$ indicates statistical significance relative to untreated cells.

before addition of opsonized bacteria (20 min at 37°C) and suspended in HBSS with 10% autologous serum (multiplicity of infection, 10). After incubation (30 min for *S. aureus* and 45 min for *P. aeruginosa*) at 37°C, neutrophils were lysed in 1 M NaOH (pH 11) water, and supernatant was diluted further before plating suspensions onto sheep blood agar plates. These were incubated overnight at 37°C, and the resulting colonies were counted, as in Parker et al. [25]. The number of colonies remaining after incubation with neutrophils was normalized to samples containing bacteria alone.

Fluorometric quantification of NET DNA

DNA was quantified in neutrophil supernatants by collection of extracellular DNA after a brief treatment with DNase and then staining with a DNA-binding dye. Alternatively, the release of DNA was monitored over time in situ with cells and NETs together by adding a cell-impermeable DNA-binding dye immediately after treatment. To quantify DNA in supernatants, neutrophils were plasma treated in HBSS buffer (without serum) and incubated with or without PMA (20 nM) for 3 h at 37°C. To harvest NET DNA, sufficient DNase (10 U/ml), to detach NETs but not fully degrade them, was added for 10 min at 37°C, and the reaction terminated with 5 mM EDTA [18]. Contaminating cells were removed by centrifugation, and the supernatants were divided in aliquots into a black, 96-well plate. Samples were incubated with Sytox Green (1 μM) for 5 min, and then fluorescence (λ_{ex} 485 nm; λ_{em} 520 nm) was measured using a plate reader. To follow the time course of DNA release from cells, neutrophils were exposed to plasma and divided in aliquots to a black, 96-well plate. Triton X-100 (20 μl) was added to several wells to assess the maximal DNA fluorescence. Sytox Orange was added (final concentration, 2 μM) and the plate placed into a prewarmed (37°C) plate reader (M200 Pro; Tecan). Fluorescence was read continuously every 2 min over 4 h (λ_{ex} 530 nm; λ_{em} 575 nm).

IL-8 measurements

Neutrophils were plasma treated and incubated for 3, 6, and 24 h. Supernatants were harvested, centrifuged to remove residual cells and debris, and stored at −80°C until analysis. IL-8 concentrations were determined using an anti-human IL-8 ELISA, according to the manufacturer's instructions.

Microscopic quantification of NET DNA

Neutrophils were added to a tissue culture-treated, 24-well plate, in duplicate for each condition, and exposed to plasma. Sytox Orange (final concentration, 200 nM) was added, and image acquisition was set to acquire a mosaic set of pictures in a meandering fashion (3 × 3 images/well). This was done every 30 min, while incubating the cells in the heated (37°C) microscope stage (Observer; Zeiss, Oberkochen, Germany). Self-written algorithms in ImageJ were applied to batch-stitch and crop-mosaic images to a predefined size. To obtain total NET area, this algorithm then applied a threshold to exclude low fluorescence (background) as well as high fluorescence (nuclei) from the images to perform quantitative fluorescence image analysis.

NET verification by elastase and citrullinated histone immunofluorescence

After plasma treatment, neutrophils were transferred to 12-well plates containing glass coverslips and incubated for 3 h at 37°C. Plates were centrifuged (100 *g* for 5 min), media were gently removed, and cells were fixed in fixation buffer (BioLegend) for 20 min. After permeabilization (0.5% Triton X-100), coverslips were blocked with Dako protein block. Cells were then incubated with 1:50 polyclonal goat anti-human neutrophil elastase and rabbit anti-human citrullinated histone H3 antibodies in protein block for 1 h at 37°C. After washing, secondary antibodies (donkey anti-rabbit Alexa Fluor 488 and chicken anti-goat Alexa Fluor 594, 1:300, in Dako protein block) were added, and samples were incubated for 1 h at 37°C. As a control, some samples were incubated with secondary antibodies only. Coverslips were washed and mounted in Vectashield containing DAPI. Slides were stored at 4°C in the dark until analysis by confocal laser-scanning microscopy (DMI6000B; Leica, Wetzlar, Germany).

Statistical analysis

Data were analyzed by Student's *t* test for comparison between 2 groups. One-way ANOVA with Dunnett post-testing, referring to untreated control (or plasma in the presence of Ca^{2+}; see Fig. 4), was used for comparison of 2 or more groups. Significance levels are indicated in figure legends.

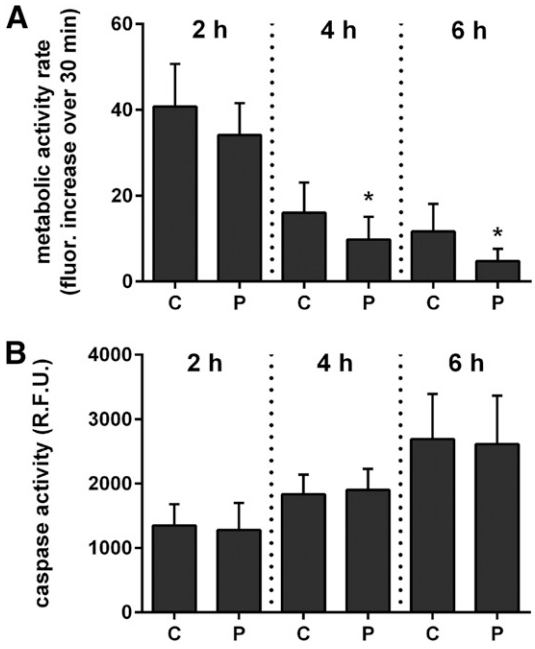

Figure 2. Plasma treatment reduced neutrophil metabolic activity but did not induce early apoptosis. (A) To assess metabolic activity, the rate of resazurin to resorufin transformation was recorded over 30 min at different time points in control and plasma-treated cells. (B) To determine the relative amount of apoptotic cells, control and plasma-treated neutrophils were incubated at different time points with CellEvent caspase reagent for 30 min, and fluorescence was determined. All incubations were carried out at 37°C. C, Control untreated cells; P, plasma-treated; fluor., fluorescence; R.F.U., relative fluorescence units. Data are presented as means + SE of 3–4 independent experiments. $*P < 0.05$ indicates statistical significance relative to time-matched, untreated cells. Refer to Materials and Methods for more specific details.

RESULTS

Plasma treatment exposed neutrophils to reactive oxidants and reduced their metabolic activity but did not induce apoptosis

Cold physical plasma generates a variety of reactive species. To test whether these species caused oxidation within human neutrophils, cells were loaded with 3 different fluorescent redox probes (CM-H_2DCF-DA, DHR123, and APF). The purpose of this experiment was to assess general intracellular oxidation. Therefore, the lack of specificity of these probes was not of concern [14, 26]. The fluorescence intensity of each of the probes increased significantly after 3 min of plasma treatment with a detectable increase after 1 min (**Fig. 1**). Treatment with argon gas alone had no effect on fluorescence. The presence of catalase during plasma exposure largely abrogated the fluorescence increase. These results show that the H_2O_2 generated by the plasma enters the neutrophils and is largely responsible for oxidation of the probes.

We next assessed whether plasma treatment compromised cell metabolism or viability. The former was assessed via quantifying the conversion of intracellular resazurin to fluorescent resorufin by NADH/NADPH-reducing equivalents. Four and 6 h after plasma challenge, metabolic activity was decreased in controls and treated cells, with the latter significantly decreased compared with its respective time-matched control (Fig. 2A). Apoptosis was assessed by examination of caspase activity using a detection reagent that becomes fluorescent by binding to intracellular DNA after cleavage by caspase 3 or 7. No difference in caspase activity in plasma-treated cells compared with controls was observable, 2, 4, or 6 h after exposure to plasma (Fig. 2B).

NOX activity and bactericidal activity were not severely altered by plasma treatment

Exposure to cold physical plasma alone increased the rate of superoxide production modestly but significantly compared with untreated, unstimulated cells (**Table 1**). PMA induced a substantial increase in superoxide production by plasma-treated and untreated cells. However, there was a subtle but significant decrease in the rate of superoxide production after plasma treatment. The presence of catalase during plasma treatment partially protected against the decline in superoxide generation after PMA stimulation. However, addition of an equivalent concentration of H_2O_2 (180 μM) to that generated in the 3 min plasma exposure had no effect on the respiratory burst in response to further stimulation. Cell counts were similar in all samples (data not shown), excluding the possibility that during plasma treatment, neutrophils became increasingly adherent, which would have affected the number of cells transferred to the cuvettes for analysis of superoxide production.

Next, we tested whether plasma treatment alters the ability of neutrophils to phagocytose and kill engulfed bacteria. No large reduction in the ability of neutrophils to kill *P. aeruginosa* or *S. aureus* was observed in plasma-treated samples (**Table 2**). It should be noted though that in contrast to our other experiments, plasma treatment was done in the presence of human serum, which may have altered the concentrations of oxidants being deposited by the plasma. This was done to ensure constant opsonization of the bacteria once they were added.

TABLE 1. Effect of plasma treatment on the neutrophil respiratory burst following PMA stimulation

Treatment	Superoxide release (rate normalized to PMA control)	*P* compared with respective control
Control (+PMA)	100.0 ± 2.4	–
Control + SOD (+PMA)	0.2 ± 0.1	0.001
Plasma (+PMA)	83.7 ± 2.5	0.049
Plasma + catalase (+PMA)	93.2 ± 1.6	0.519
H_2O_2 (+PMA)	103.1 ± 1.1	0.945
Control (−PMA)	2.5 ± 0.7	–
Plasma (−PMA)	7.8 ± 1.3	0.011

Neutrophils were treated with plasma (3 min) in the presence or absence of catalase or given a bolus of H_2O_2 (180 μM). Subsequently, the respiratory burst was assessed by measuring the rate of reduction of cytochrome at 550 nm, either without adding a stimulant or after addition of PMA. PMA stimulation, in the presence of SOD, was used to determine the specificity of the assay. Data are presented as means ± SE of 3–5 independent experiments.

TABLE 2. Effect of plasma treatment on neutrophil bactericidal activity

Bacterial species	CFU/ml (% of control bacteria incubated without neutrophils)		*P* compared with control
	Control	Plasma	
P. aeruginosa	5 ± 3	5 ± 3	0.919
S. aureus	45 ± 7	57 ± 8	0.311

Neutrophils were treated with plasma (3 min), and then bactericidal activity was assessed by incubating control (untreated) or plasma-treated neutrophils with *S. aureus* or *P. aeruginosa* for 30 and 45 min, respectively. Results were normalized to controls of bacteria incubated without neutrophils. Data presented are means plus range from 2 independent experiments (*P. aeruginosa*) or means ± SE from 3 experiments (*S. aureus*).

Exposure to plasma evoked extracellular trap formation in human neutrophils

NET release was examined by several methods: harvesting NET fragments from neutrophil supernatants; fluorescence measurement of cells and NETs in situ using a cell-impermeant nucleic acid dye; and live cell microscopy. Plasma treatment of the neutrophils for 3 min was compared with addition of PMA, a strong inducer of NETs [27]. After 3 h, a low concentration of DNase was added to harvest NET fragments [18], which were stained with the nucleic acid dye Sytox Green. Compared with untreated cells, there was a significant increase in extracellular DNA after plasma treatment (Fig. 3A). The amount of DNA was similar to that released from untreated cells stimulated with PMA. Plasma treatment, followed by PMA stimulation, did not have a synergistic effect. DNA accessibility was also measured over 4 h in a plate reader after exposure to plasma (Fig. 3B). Plasma-treated neutrophils showed enhanced DNA fluorescence compared with untreated cells. DNA fluorescence was greater with the longer plasma exposure time and increased progressively over 1–4 h. This process was not induced by exposure to argon gas alone and was fully inhibited when catalase was present during plasma treatment. However, neither a single dose nor repetitive addition of an equivalent concentration of H_2O_2 caused significant DNA release (**Fig. 4A**). Neutrophils that were suspended in fresh HBSS immediately following plasma treatment and addition of plasma-treated HBSS to unexposed cells both reduced DNA release compared with the standard treatment. These results suggest the involvement of short- and longer-lived species that depend on H_2O_2 but are not solely H_2O_2 itself.

NET production with other stimuli is frequently associated with NOX activation and often involves MPO activity [27–29]. To test for this, we exposed neutrophils to plasma in the presence of the NOX inhibitor DPI or the MPO inhibitor 4-ABAH. Neither decreased DNA release (Fig. 4B). Omission of calcium in the media had no effect, excluding the possibility that plasma could activate NET formation in a similar manner to calcium ionophores [27, 30]. The presence of the thiol compounds GSH and NAC did not affect NET formation, but it was decreased in the presence of uric acid.

We investigated whether plasma treatment could initiate release of IL-8, which has been suggested to promote NET formation [17, 31]. Release of IL-8 from plasma-treated neutrophils was observed but not until 6 h or more after exposure, which is too late to influence NET production (**Fig. 5**). High IL-8 concentrations at 24 h in plasma-treated samples may have been released by dying cells [32].

Fluorescence of Sytox Orange, as measured in the plate reader, does not discriminate between nuclear DNA from nonviable cells and extracellular DNA. Therefore, neutrophils were evaluated using live cell microscopy. Diffuse structures were observed around cells, 3 h after plasma treatment (**Fig. 6A**). With the use of ImageJ software, we calculated NET area by excluding cell nuclei from the analysis. Plasma treatment of neutrophils led to a significant increase in total NET area, which

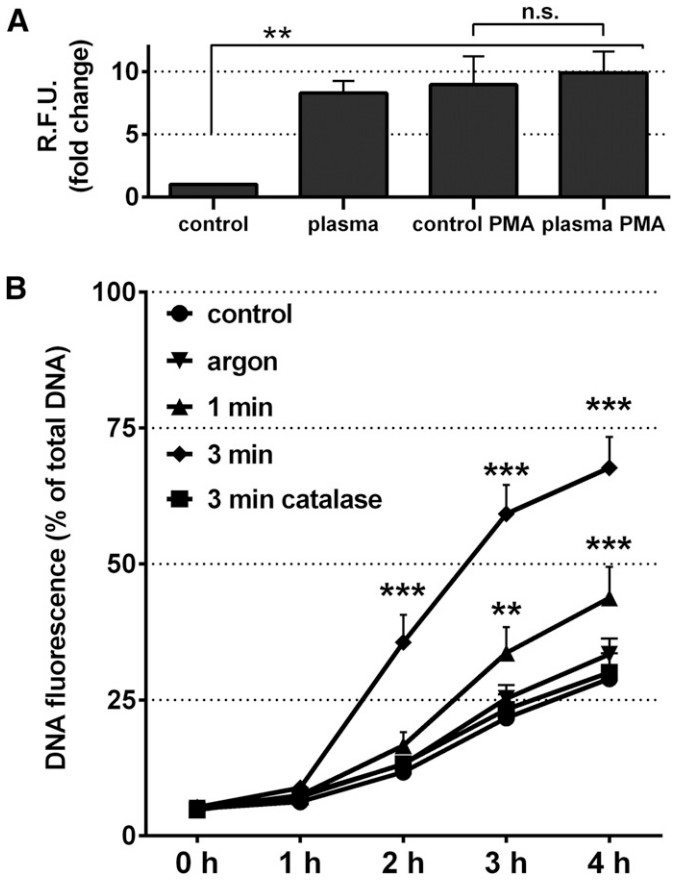

Figure 3. The presence of extracellular DNA was increased significantly after plasma treatment of neutrophils. (A) Neutrophils were exposed to plasma or PMA (20 nM) or both and incubated for 3 h at 37°C. Following brief digestion with DNase I, supernatants were collected and DNA quantified using Sytox Green. control, No plasma-treatment. (B) For real-time monitoring of NET formation, DNA fluorescence was followed after plasma (with or without the presence of catalase) or argon gas treatment using Sytox Orange by measuring the fluorescence every 2 min on a plate reader. Fluorescence intensity was normalized to the maximum DNA fluorescence for each sample (Triton X-100). Data are presented as means + SE of 4–8 independent experiments. (A) $^{**}P < 0.01$ indicates statistical significance relative to untreated cells. (B) $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate statistical significance relative to time-matched, untreated cells.

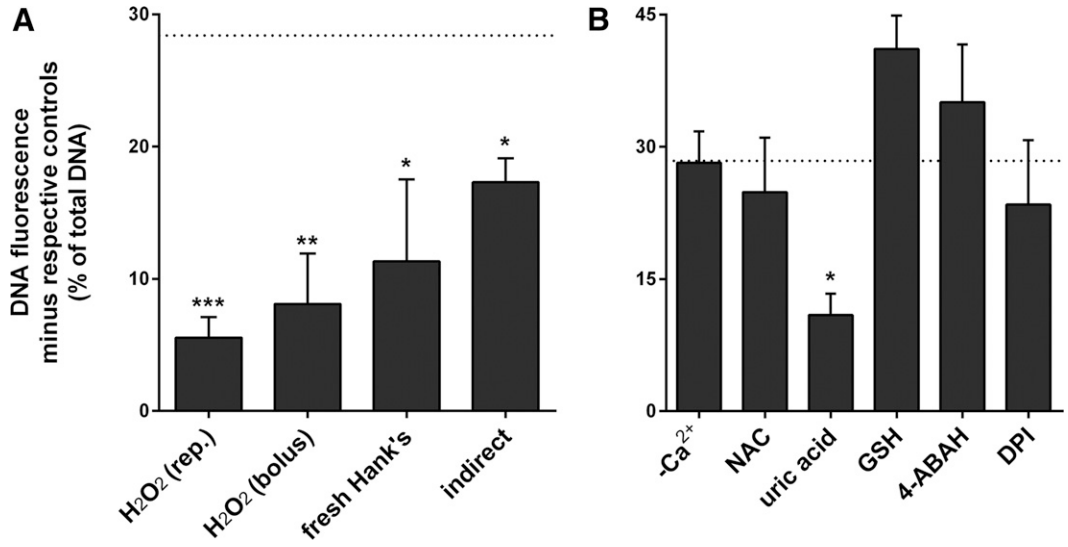

Figure 4. Plasma-induced NETosis was reduced in the presence of uric acid, but neither elicited in response to H_2O_2 nor reduced by inhibition of NOX or MPO. (A) To neutrophils (in HBSS with Ca^{2+}), H_2O_2 (180 μM) was added either as a bolus or in 36 repetitive additions (rep.) of 5 μM each over 3 min. Alternatively, HBSS medium was plasma treated and then added to nontreated neutrophils (indirect), or plasma-treated cells were centrifuged to remove plasma-treated medium and then were suspended in fresh medium (fresh HBSS). (B) Neutrophils (in HBSS with Ca^{2+}) were preincubated with DPI, 4-ABAH, NAC, uric acid, or GSH before exposure to plasma (3 min). Furthermore, to examine the requirement for calcium, neutrophils were suspended in HBSS without Ca^{2+} before plasma treatment. DNA fluorescence was followed using Sytox Orange by measuring the fluorescence every 2 min on a plate reader. Results show the fluorescence after 4 h. Fluorescence intensity was normalized to the maximum DNA fluorescence (Triton X-100), and the fluorescence of each respective untreated control condition was subtracted. The dashed lines represent the DNA fluorescence of neutrophils exposed to the standard treatment (3 min of plasma in the presence of Ca^{2+}) minus DNA fluorescence of its untreated control. Data are presented as means + SE of 3–6 independent experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ indicate statistical significance relative to directly plasma-treated cells (dashed lines).

became progressively more apparent after 90 min. (Fig. 6B). Catalase abrogated this effect, but addition of exogenous H_2O_2 (180 μM) did not initiate it. To verify that DNA was not expelled as a result of necrosis but represented active NET release, samples were counterstained for DNA and the known NET constituents [33], citrullinated histone H3 and neutrophil elastase. String-like DNA (observed when preparations are subject to disturbance when fixing and staining) was associated with both markers, verifying that the structures released upon plasma treatment were NETs (Fig. 6C).

DISCUSSION

It has been proposed that treatment with cold physical plasma may have therapeutic benefits for treating nonhealing wounds [5–10]. As neutrophils have been identified as principal players in this pathology [3], it is important to understand how neutrophils are affected by exposure to plasma. We found cold plasma to induce NET formation oxidant dependently, whereas other cell functions, such as phagocytosis and oxidative burst, were only modestly affected.

We used a plasma source typically used for wound treatment, which generates a variety of ROS and nitrogen species, including H_2O_2, superoxide, hydroxyl radicals, and NO [34]. Thus, plasma treatment should expose cells to these ROS and nitrogen species. Similar to our findings with PBMCs [14], our results here show that the neutrophil cytosol is also exposed to these reactive species and/or their byproducts. Inhibition of the fluorescence of oxidation-sensitive intracellular probes in the presence of catalase indicates that plasma-produced H_2O_2—presumably through an interaction with MPO, as DCF and DHR are oxidized by radical mechanisms that are generally initiated in neutrophils by H_2O_2 and MPO—was responsible for the fluorescence increase.

With the use of a similar plasma source and treatment regime, as in this study, we have previously shown that 20 s of plasma treatment induces apoptosis but not necrosis in ~50% of lymphocytes [35], with monocytes more resistant [36]. With neutrophils, no increase in caspase activation was found, up to 6 h after exposure to plasma, even though activation can be observed within 3 h in response to some stimuli [37]. The lack of response with neutrophils may be because caspase activation in the other cell types was largely H_2O_2 dependent [14], and the high concentration of catalase in neutrophils may make them more resistant to H_2O_2 [38]. Oxidative inactivation of neutrophil caspases [39] could also be a factor. Plasma treatment caused a greater loss of metabolic activity than in untreated cells. Control cells showed a gradual decline, which may, in part, be related to priming after attachment to the high binding plates used in the assay and not necessarily represent loss of viability. However, the decline was more rapid with plasma-treated cells and may be linked to NETosis, which is an active form of cell death [40].

Plasma profoundly induced NET formation. This conclusion is supported by several pieces of evidence, including the following: 1)

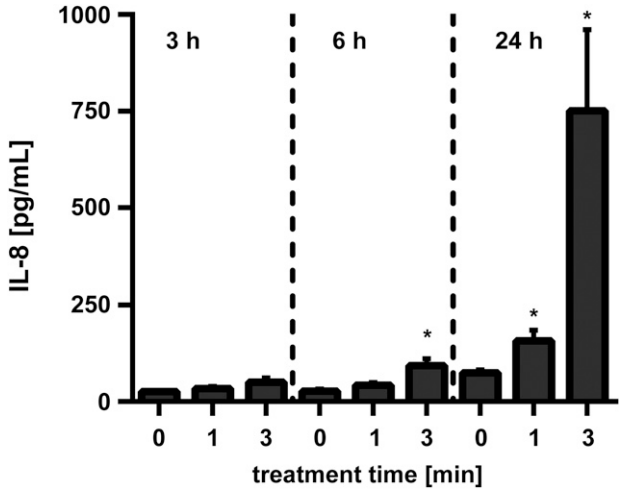

Figure 5. Neutrophils released IL-8 after plasma treatment but not in a time frame consistent with the induction of NETs. Neutrophils were exposed to plasma, and supernatants were collected after 3, 6, or 24 h of incubation. IL-8 concentrations were quantified via ELISA. Data presented are means + SE of 4 donors. $*P < 0.05$ indicated statistical significance relative to time-matched, nonplasma-treated samples.

measurement of extracellular DNA in supernatants of plasma-treated cells; 2) measurement of DNA release over time in situ by plasma-treated neutrophils; 3) quantification of NET DNA in live cell images using a software protocol that measures released NET area and excludes brightly stained cell nuclei; and 4) showing colocalization of elastase and citrullinated histones with the released DNA. The amount of DNA released in response to plasma was similar to that with PMA, a robust NET inducer, and NET formation occurred over a time course similar to PMA [27, 40]. Catalase prevented NET formation, implicating a requirement for H_2O_2. However, an equivalent bolus or flux of H_2O_2 had no effect, implying that interaction of H_2O_2 with an additional plasma component is required for NET induction.

Several groups have investigated whether exposure of neutrophils to H_2O_2 alone induces NET formation. Our present findings that NETs are not induced by a bolus or incremental addition of low-dose H_2O_2 are consistent with the lack of induction observed previously at low concentrations [40] and with studies showing that exposure to millimolar H_2O_2 [41] or a flux of ~100 μM/min, generated over hours by glucose oxidase [40], is required. However, there is some confusion in the literature, as NET formation at low H_2O_2 exposure has been reported [42, 43], whereas higher concentrations have been shown to induce apoptosis and necrosis [44]. For plasma, our results imply that H_2O_2 is required but must either act synergistically with another constituent or mediate the formation of another active species. The partial decrease in NET formation when neutrophils are exposed to pretreated media suggests the involvement of a species present only during plasma exposure. However, the decrease seen when treated neutrophils were resuspended in fresh media implies a contribution from a longer-lived species. NET formation was not affected by the thiol reagents, NAC and GSH, and although uric acid was partially inhibitory, its ability to react via complex mechanisms with numerous oxidants, including peroxynitrite, complicates interpretation of this result.

With many stimulants, NET formation is dependent on NOX activation [28, 40]. Despite our observation that plasma treatment led to a small but significant activation of the NOX, this activity was not required to induce NETs, as it was not

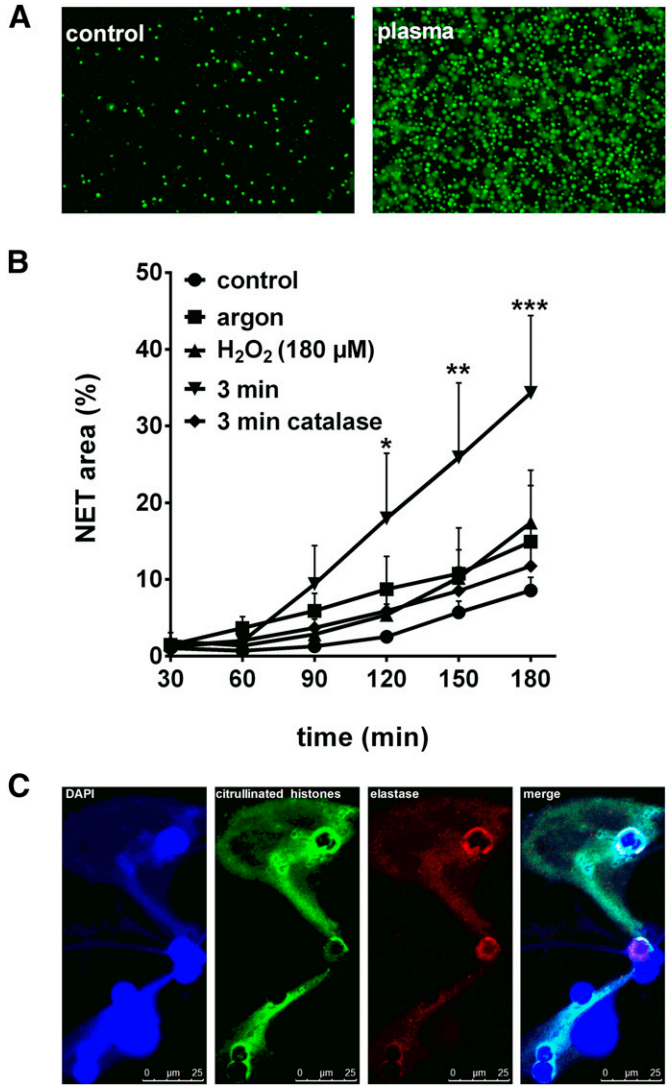

Figure 6. Quantitative image analysis and verification of extracellular traps. (A) Live cell images of untreated neutrophils (left) and neutrophils that had been exposed to plasma for 3 min (right). The cells were then incubated for 3 h and stained with Sytox Green. (B) Quantitative image analysis at different times after plasma treatment, carried out as described in Materials and Methods. Percent describes the total area of fluorescence per image related to the total image area. (C) Fluorescent images of neutrophils that were plasma treated and incubated for 3 h before fixing and staining with DAPI, anti-elastase, and anti-citrullinated histone H3 antibodies. Samples incubated with secondary antibodies alone showed no nonspecific binding (not shown). Data presented are 1 representative (A and C) or means + SE (B) of 4–6 independent experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ indicate statistical significance relative to time-matched, untreated controls.

inhibited by DPI. Induction by some stimuli, including PMA, involves MPO [27, 29], but this was not the case with plasma-induced NETs, as the MPO inhibitor ABAH had no effect. IL-8 (CXCL8) has been reported to induce NETs [31], but the lateness of IL-8 release makes its involvement unlikely.

Plasma treatment induced a subtle yet significant decrease in the rate of superoxide production when the neutrophils were subsequently stimulated with PMA. The effect was partially inhibited if catalase were present during plasma exposure but was not replicated by H_2O_2. This suggests that there might be an interaction between H_2O_2 and another plasma constituent. The modest effect on the respiratory burst after stimulation did not appear to affect the killing of 2 pathogens, both of which are commonly found in wounds [45], although slight inhibition by plasma cannot be excluded.

In wounds, neutrophils are the first inflammatory cells to arrive. They are also the first to leave and are virtually nonexistent in normal-healing wounds, 48–72 h after injury [46]. In contrast, nonhealing ulcers are consistently populated with neutrophils, but these tissues display a low-grade inflammatory profile [47]. A prerequisite to healing and onset of the proliferative phase is profound inflammation. It can thus be hypothesized that nonhealing wounds lack a timed and appropriate inflammatory response, and 1 mechanism whereby cold plasma treatment could be beneficial is by partially restoring this response. This is supported by the notion that numerous aspects of wound healing are subject to redox control [48]. The most dramatic response of neutrophils to plasma was the release of NETs, reported to be proinflammatory [49, 50], which in chronic wounds, may help to reset the normal healing response. However, in other pathologies, in vivo evidence shows NETs to be detrimental [51, 52]. A recent study found NETs to inhibit wound healing in diabetes [53], but their general role is not very clear, as they can be proinflammatory [54] as well as limit inflammation by degrading cytokines and chemokines [55]. Therefore, whether NETs may be beneficial or harmful in the healing of chronic wounds by plasma requires further examination in an animal model. Release of the chemokine IL-8, possibly as a result of accelerated cell death, could also enhance neutrophil migration to the wound site [56]. Arndt and colleagues [5] have found increased neutrophil influx in plasma-treated wounds in mice, was associated with an improved healing response. Clearly, further study is needed to understand the relevance of neutrophil interactions in wound responses to plasma treatment and also to elucidate how H_2O_2 and other plasma components cause NET formation and other changes in plasma-treated neutrophils. Additionally, whether NETs are beneficial or detrimental for plasma treatment of wounds needs to be tested in vivo.

AUTHORSHIP

S.B. and H.A.P. designed and performed the experimental work and contributed to the interpretation and preparation of the manuscript. K.M., S.H., B.M.B., J.K., and C.C.W. contributed to the preparation of the manuscript. C.C.W. and J.K. contributed to experimental design.

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DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:
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