Neutrophil extracellular traps (NETs) are formed when neutrophils expel their DNA, histones and intracellular proteins into the extracellular space or circulation. NET formation is dependent on autophagy and is mediated by citrullination of histones to allow for the unwinding and subsequent expulsion of DNA. NETs have an important role in the pathogenesis of several sterile inflammatory diseases, including malignancy, therefore we investigated the role of NETs in the setting of pancreatic ductal adenocarcinoma (PDA). Neutrophils isolated from two distinct animal models of PDA had an increased propensity to form NETs following stimulation with platelet activating factor (PAF). Serum DNA, a marker of circulating NET formation, was elevated in tumor bearing animals as well as in patients with PDA. Citrullinated histone H3 expression, a marker of NET formation, was observed in pancreatic tumors obtained from murine models and patients with PDA. Inhibition of autophagy with chloroquine or genetic ablation of receptor for advanced glycation end products (RAGE) resulted in decreased propensity for NET formation, decreased serum DNA and decreased citrullinated histone H3 expression in the pancreatic tumor microenvironment. We conclude that NETs are upregulated in pancreatic cancer through RAGE-dependent/autophagy mediated pathways.

INTRODUCTION
Classically, neutrophils kill bacteria intracellularly following phagocytosis. However, neutrophils are also involved in a process known as neutrophil extracellular trap formation (NET), which occurs when activated neutrophils expel their DNA and other intracellular contents into the circulation or extracellular space. Following initial studies in sepsis, NETs have also been implicated in setting of sterile inflammation, and in murine models of mammary and lung carcinoma. NET formation requires expression of the enzyme peptidyl arginine dimethylase 4 (PAD4), which is responsible for the post-translational modification of histones, converting arginine residues to the amino acid citrulline in a process known as citrullination. The loss of positively charged arginine exchanged for neutral citrulline allows for chromatin decondensation and subsequent unfolding and unwinding of DNA for expulsion from the cell during NET formation.

The process of autophagy, in which damaged organelles and proteins are degraded and recycled, is also critical for NET formation. Autophagy is an important regulator of cancer cell survival in pancreatic cancer, allowing cells to survive the hypoxic, nutrient-deprived tumor microenvironment. Heightened autophagy correlates with poor prognosis in patients with pancreatic adenocarcinoma. The receptor for advanced glycation end products (RAGE) is a Class III MHC protein receptor that mediates autophagy in the pancreatic tumor microenvironment and promotes carcinogenesis. Because RAGE-mediated autophagy is critical in the pancreatic tumor microenvironment, we hypothesized that RAGE and autophagy would also have a role in circulating neutrophils in pancreatic adenocarcinoma. Therefore, we evaluated the role of neutrophil autophagy in promoting NET formation in both orthotopically injected and Kras-driven genetically engineered models of pancreatic adenocarcinoma and investigated RAGE as a key mediator driving NET formation.

METHODS
Murine models and treatments
C57/BL6 wild-type mice (10–12-week female) were purchased from Taconic Farms (Hudson, NY, USA). RAGE knockout mice (RAGE−/−, SVE129xC57/BL6) were utilized to study the role of RAGE in NET formation. For our orthotopic pancreatic cancer model, the murine pancreatic cancer cell line Panc02 cells (NCI repository, 2008) were cultured in RPMI 1640 media (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum, and PenStrep antibiotic solution (Gemini, West Sacramento, CA, USA) in a humidified incubator with 5% CO2. Wild type and RAGE−/− mice were injected with 1 × 10⁶ Panc02 cells into tail of pancreas through a limited laparotomy. Animals were killed after 4 weeks at which time palpable abdominal tumors were present. We also utilized a genetically engineered model of Kras-driven pancreatic cancer (Pdx1-Cre:KrasG12D) which were purchased from the National Cancer Institute Mouse Repository. Kras-driven pancreatic cancer mice and RAGE−/− mice were crossed to generate Kras-driven pancreatic adenocarcinoma lacking RAGE mice. Animals were treated with oral chloroquine (0.5 mg ml⁻¹) administered in the drinking water ad libitum. Neutrophils were depleted by using treatment with anti-mouse Ly6G antibody (RB6-8C5, EBiocience, San Diego, CA, USA, #16-5931) injected intra-peritoneal at a dose of 5 mg kg⁻¹, 60 h prior to sacrifice. Neutrophil depletion was confirmed by using a couler counter to analyze neutrophil count of whole blood.

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Patient samples
Resected pancreatic tumor specimens and serum were obtained from patients enrolled in a phase I/II clinical trial of preoperative gemcitabine plus hydroxychloroquine in high-risk pancreatic cancer. Patients were treated with 30 days of 1200 mg oral hydroxychloroquine with two treatments of fixed dose gemcitabine at a dose of 1500 mg m⁻². Blood was collected before and after treatment. Clotted blood was spun at 1000 g for 10 min. The serum was collected, frozen and stored at –80 °C. Pooling of human A/B control serum was obtained from Gemini Bio-products (#100–512, West Sacramento, CA, USA)

Western blot
Whole cell lysates from isolated neutrophils were run on a 4–12% Bis-Tris gel and transferred to a 0.2 mm nitrocellulose membrane. After blocking with 5% milk, membranes were incubated overnight at 4 °C with primary antibodies specific for rabbit anti-mouse LC3 (Sigma, Grand Island, NY, USA, #L8598) and rabbit anti-mouse actin (Sigma, #A2066). Membranes were then incubated with peroxidase-conjugated secondary antibodies for 1 h at 25 °C and developed with the SuperSignal West Pico chemiluminescence kit (Pierce, Grand Island, NY, USA, #34079) and exposed on film.

Ex vivo NET assay
Neutrophils were isolated by using density gradient centrifugation. Cells were plated in a 96-well plate at ~1.5×10⁴ cells well per Hank’s Balanced Salt Solution (Gibco, Grand Island, NY, USA). Neutrophils were then stimulated with platelet activating factor (PAF, 0–50 μm, Millipore, Billerica, MA, USA, #S11075) for 30 min. Cells were fixed with 3% paraformaldehyde and then DNA was stained with Hoechst 33342 (Molecular Probes, Grand Island, NY, USA, #H-3570) and rabbit anti-mouse actin (Sigma, #A2066). Membranes were then incubated with peroxidase-conjugated secondary antibodies for 1 h at 25 °C and developed with the SuperSignal West Pico chemiluminescence kit (Pierce, Grand Island, NY, USA, #34079) and exposed on film.

Measures of in vivo NET formation
After 10x dilution, DNA levels were also measured from mouse and patient serum using Quant-IT Picogreen (Invitrogen, MP07581). Murine pancreatic specimens were embedded in optimal cutting temperature compound (Sakura, Torrance, CA, USA), frozen and stored at –80 °C. Sections measuring 8 μm were cut on cryostat for immunofluorescence staining. Tissue was fixed with 2% paraformaldehyde, permeabilized, blocked with 2% fetal bovine serum, and incubated with a 1:50 dilution of anti-Histone H3 (citrulline 2+8 +17) antibody (Abcam, Cambridge, MA, USA, ab5103) and anti-mouse Ly6G antibody (RB6-8C5, EBioscience, #16-5931) overnight at 4 °C.

For immunolabeling of human pancreatic specimens, resected pancreatic tissue was embedded in formalin. Slides were deparaffinized two times in xylene for 3 min, once in xylene with 100% ethanol for 3 min and 100% ethanol for 3 min followed by 95, 70 and 50% ethanol for 3 min. Antigen retrieval was performed by heat-induced method in 1-ml EDTA, pH 8.0 by using a microwave. The slides were then washed twice for 5 min in PBS with 0.025% Triton X-100 (Fisher, Pittsburgh, PA, USA), after which the slides were blocked in 2% bovine serum albumin for 2 h. Slides were incubated with a 1:50 dilution of anti-Histone H3 (citrulline 2+8+17) antibody (Abcam, ab5103) and anti-neutrophil elastase (1:50, Abcam).

After three washes, goat anti-rabbit Alexa 596, and anti-mouse Alexa 488 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were added for 1 h. Hoechst (Invitrogen) for nuclear staining was applied for 30 s followed by two washes with phosphate-buffered saline. Slides were mounted in fluoromount-G (Southern Biotech, Birmingham, AL, USA) and confocal images were acquired using Olympus Fluoview 1000 microscope with a Plan Apo N objective (x60) (Olympus, Center Valley, PA, USA).

Sequential scanning was applied for acquiring individual emission fluorescent nucleic acid stain for quantifying double-stranded DNA.

RESULTS

Pancreatic cancer promotes NET formation
Neutrophils were isolated from mice and stimulated with PAF, a known inducer of NET formation. Neutrophils from tumor bearing animals from both an orthotopic and a genetically engineered Kras-driver model (Kras-driven pancreatic cancer) had a markedly increased propensity to form NETs compared with controls (Figure 1a and b). To confirm that the extracellular DNA visualization was the result of NET formation, we also stained for citrullinated histone H3 (CitH3), which has been implicated NETs. CitH3 was highly expressed following PAF stimulation and co-stained with DNA, confirming that NET formation was being visualized (Figure 1c). To more objectively quantify NET formation, supernatant levels of DNA were measured as a marker of NETs. PAF treatment led to a dose-dependent increase in supernatant DNA in both tumor bearing and control animals with greater levels of supernatant DNA in tumor bearing animals from both models (Figure 1d and e).

Next assessed whether NET formation was occurring in vivo in mice with pancreatic cancer. Serum levels of DNA were analyzed as a marker of in vivo NET formation. Serum DNA was elevated in tumor bearing animals from both the orthotopic model as well as the genetic model (Figure 2a and b). To confirm that clotted serum during collection of serum did not confound our results, we also measured plasma DNA from orthotopic and control mice. There was no significant difference between DNA in serum and plasma from tumor burdened animals; however, in control mice there was more DNA in the serum compared with plasma (data not shown).

To ascertain if the DNA in the serum was released from neutrophils rather than from necrosis of cancer cells within the tumor micro-environment, neutrophils were depleted in orthotopic mice with anti-Gr1 antibody. Neutrophil depletion led to a significant reduction in serum DNA in tumor bearing animals down to the level of sham control (Figure 2c). This suggests that circulating DNA in the serum of tumor bearing animals can be considered a surrogate marker for in vivo NET formation in the circulation in tumor bearing animals.

To determine if neutrophils infiltrating the pancreatic tumor microenvironment form NETs, CitH3 expression was evaluated in resected murine tumor sections from both animal models. Pancreatic CitH3 was increased in tumor bearing animals compared with normal pancreas (Figure 2d and e). CitH3 staining co-localized with Gr1, a marker on neutrophils, suggesting that it was released from infiltrating neutrophils.

Neutrophil autophagy promotes NET formation in pancreatic cancer
To identify the potential mechanism promoting upregulated NETs in pancreatic cancer, neutrophils were isolated from tumor bearing mice and sham controls and analyzed by western blot for the autophagy marker LC3-II. In both murine models of pancreatic cancer, neutrophils had elevated levels of LC3-II expression when compared with controls, consistent with an upregulation of autophagy in tumor bearing animals (Figure 3a and b).

To confirm whether NET formation in pancreatic adenocarcinoma is an autophagy-mediated process, we assessed whether treatment with the autophagy inhibitor chloroquine (CQ) could...
inhibit NET production. Neutrophils isolated from mice treated with CQ had a significant reduction in the propensity to form NETs after stimulation with PAF (Figure 3c). Treatment with CQ also led to the reduction in circulating DNA in both animal models (Figure 3d and e). To confirm the effect of autophagy inhibition on NET formation in patients, we analyzed serum DNA in patients with pancreatic adenocarcinoma treated with preoperative gemcitabine plus hydroxychloroquine. 80% of patients (12/15) had a decline in serum DNA levels with treatment (Figure 3f). There was a significant reduction in average serum DNA in response to treatment (560 vs 451 ng ml\(^{-1}\), \(P < 0.05\)). There were no correlations between white blood cell count or absolute neutrophil count and serum DNA levels.

In addition to assessing circulating DNA levels, NET formation within the tumor microenvironment after treatment with autophagy inhibition was also analyzed. Mice from both the orthotopically injected and the genetically engineered model of pancreatic adenocarcinoma had a substantial reduction in the amount of extranuclear CitH3 expression (Figure 4a and b). Staining was also performed on the resected specimens of patients treated with preoperative gemcitabine plus hydroxychloroquine (Figure 4c and d, \(n = 13\)). Patients who had a decline in CA 19-9 with treatment, suggesting a clinical response, had lower levels of CitH3 than those that had no CA 19-9 response to treatment (Figure 4e).

**DISCUSSION**

After being initially described in the setting of sepsis and infection, NET formation has also been implicated in sterile inflammation and autoimmune disease, including gout, systemic lupus erythematosus\(^{22,23}\) and vasculitis.\(^{24}\) A single study has demonstrated this process in malignancy, reporting NETs in murine models of mammary and lung carcinoma.\(^{2}\) The current study

**Figure 1.** Neutrophils in murine pancreatic adenocarcinoma are more prone to neutrophil extracellular trap formation. Neutrophils isolated from sham and tumor bearing mice (a) and wild-type and Kras-driven pancreatic cancer mice (b) upon stimulation with 40-μM platelet activating factor, demonstrating a substantial increase neutrophil extracellular trap formation in tumor bearing animals from both models. Extra nuclear DNA structures (Hoechst, Blue) were also positive for citrullinated histone H3 (red), confirming that neutrophil extracellular traps were being visualized (c). Platelet activating factor treatment of isolated neutrophils resulted in a dose-dependent increase in DNA in the supernatant (d), with tumor bearing animals have more DNA released in the supernatant compared to controls (d and e), consistent with increased neutrophil extracellular trap formation. \(* P < 0.05.\)
Figure 2. Neutrophils form neutrophil extracellular traps in vivo in murine pancreatic cancer. Serum DNA is elevated in tumor bearing orthotopic mice compared with sham controls (n = 15) (a) and in Kras-driven pancreatic cancer mice compared with age matched wild type (n = 6) (b). Depletion of circulating neutrophils with anti-Gr1 antibody reduced the serum DNA (n = 5), demonstrating that a significant portion of the DNA measured in circulation is originating from neutrophils (c). Citrullinated histone H3 expression (red) is elevated in tumor bearing animals from the orthotopic model (d) and the genetic Kras-driven model (e). Citrullinated histone H3 expression co-localizes with neutrophils (Gr-1, green, white arrows). These are representative images from at least three independent analyses. *P < 0.05.
supports a role for NETs in both murine models and human pancreatic ductal adenocarcinoma. We observed that pancreatic cancer-burdened mice and patients demonstrated evidence both in vitro and in vivo of increased NET formation. These important patient correlates serve as the preliminary confirmation of our preclinical findings in human pancreatic ductal adenocarcinoma. Our data clearly demonstrates that neutrophils harvested from tumor bearing animals have an increased propensity to form NETS when stimulated ex vivo with PAF. Moreover, we provide several pieces of evidence that suggest that NET formation is occurring in vivo in the setting of pancreatic cancer. First, we demonstrate evidence NET formation within the tumor microenvironment. This study is the first to demonstrate that CitH3, which is formed and deposited during the process of NET formation, is present in both human and murine pancreatic tumors, suggesting that infiltrating neutrophils are releasing NETs into the tumor microenvironment. It is also interesting to note that several studies have also demonstrated that peptidyl arginine dimethylase 4, the enzyme responsible for citrullination of histones is expressed in tumors. Immuno-histochemistry of pancreatic tumors in the current study demonstrates that some CitH3 expression is present without co-localization to Gr-1, suggesting that a portion of citrullinated histones may be localized to the tumor cells. The potential expression of citrullinated histone H3 in pancreatic tumor cells is an active area of exploration and will be further evaluated in future studies. Second, we provide data suggesting that serum DNA can be used a surrogate maker of NET formation. It has long been recognized that cancer patients have elevations in circulating DNA, however the prevailing hypothesis was that this was the result of release of DNA by necrotic cells in the tumor microenvironment. Evidence from a growing number of studies suggests there is an alternative source to circulating DNA in cancer patients. Our data suggests that a significant portion of DNA in the circulation of tumor bearing animals is derived from neutrophils. This may have implications for diagnostic studies evaluating the genomics of circulating DNA in patients with...

Figure 3. Autophagy promotes neutrophil extracellular trap formation in pancreatic cancer. Neutrophils from tumor bearing orthotopic mice have increased LC3-II expression by western blot compared with sham (a) and in Kras-driven pancreatic cancer mice compared with wild type (b), suggesting upregulated autophagy in neutrophils from tumor bearing mice. Inhibiting autophagy in vitro with chloroquine decrease neutrophil extracellular trap formation after platelet activating factor stimulation in a dose-dependent fashion, as measured by supernatant DNA (c). Treatment of mice with chloroquine decreases serum DNA levels in tumor bearing orthotopic animals but not sham (n = 15) (d) and in Kras-driven pancreatic cancer but not wild-type mice (n = 7) (e). Eighty percent of pancreatic cancer patients treated with autophagy inhibition with preoperative gemcitabine and hydroxychloroquine had a decrease in serum DNA with treatment, with the waterfall plot demonstrating the change in serum DNA for individual patients. *P < 0.05, **P < 0.10.
pancreatic cancer, as not all DNA in the circulation may be tumor derived. This finding may also explain the failure of some common genetic markers to be identified in circulating DNA in pancreatic cancer. Chloroquine treated mice have a reduction in extra nuclear citrullinated histone H3 expression compared with untreated mice seen in Figure 2. Representative images of staining for citrullinated histone H3 (red) and neutrophil elastase (green) from patients who did not demonstrate a Ca 19-9 response to preoperative Gemcitabine+ HCQ (c, n = 3) compared with patients who had decline in Ca 19-9 following the treatment (d, n = 7). Patients with a Ca 19-9 response had significantly lower amounts of staining for citrullinated histone H3 and neutrophil elastase co-localization, compared with those that did not (E, 0.49 ± 0.1 vs 0.26 ± 0.04).

Figure 4. Autophagy promotes neutrophil extracellular trap formation in the pancreatic tumor microenvironment. Representative images of staining for citrullinated histone H3 (red) and Gr-1 (green) in both the orthotopic (a) and genetically engineered (b) models of murine pancreatic cancer. Chloroquine treated mice have a reduction in extra nuclear citrullinated histone H3 expression compared with untreated mice seen in Figure 2. Representative images of staining for citrullinated histone H3 (red) and neutrophil elastase (green) from patients who did not demonstrate a Ca 19-9 response to preoperative Gemcitabine+ HCQ (c, n = 3) compared with patients who had decline in Ca 19-9 following the treatment (d, n = 7). Patients with a Ca 19-9 response had significantly lower amounts of staining for citrullinated histone H3 and neutrophil elastase co-localization, compared with those that did not (E, 0.49 ± 0.1 vs 0.26 ± 0.04).
Figure 5. Receptor for advanced glycation end products (RAGE) promotes propensity to form neutrophil extracellulars in pancreatic adenocarcinoma. Neutrophils harvested from RAGE−/− tumor bearing animals are less prone to form neutrophil extracellular traps upon stimulation with platelet activating factor (a). Supernatant DNA does not increase in RAGE−/− tumor bearing animals after neutrophil stimulation (b). RAGE−/− mice have decreased serum DNA in both the orthotopic model (n=7) (c) and the genetically engineered Kras-driven model lacking RAGE (KCR, n=6) (d). RAGE−/− mice have no citrullinated histone H3 expression in tumor bearing animals (e). Infiltrating neutrophils stained with Gr-1 are shown by the white arrows. *P < 0.05.
pattern molecules or DAMPs; specifically the DAMP High Mobility Group Box 1 (HMGB1) and its receptor RAGE.14,16 The current study demonstrates that the role of RAGE-mediated autophagic flux in pancreatic adenocarcinoma is not limited to the tumor microenvironment. Genetic ablation of RAGE-mediated NET formation, as RAGE knockout animals bearing tumor lack the propensity for NET formation, had lower levels of serum DNA and decreased Cith3 expression in the tumor microenvironment. Thus, these findings suggest that RAGE also promotes neutrophil autophagy, thereby promoting NET formation in pancreatic cancer. When considering RAGE ligands that drive NET development, it is likely that HMGB1 is the primary stimulus of NET formation in pancreatic cancer. Circulating HMGB1 is elevated in pancreatic cancer, is known to induce autophagy intrinsically and extrinsically by binding to RAGE, and has been shown to stimulate NET formation.39–41 Further investigation is required to identify the proximal signals and ligands that promote NET formation in pancreatic cancer. The implication of RAGE mediated autophagy being critical to NET formation in pancreatic adenocarcinoma outlined in the current study encourages further research into the use of inhibitors targeting these pathways. Current strategies to target RAGE include treatment with small molecule inhibitors,42–44 soluble RAGE that acts as a decoy receptor to antagonize RAGE45,46 and nonviral gene-delivery vectors.57 The formation of NETs in pancreatic cancer has tremendous clinical implications. In models of sepsis and trauma, NETs have been closely linked to thrombosis58,59. Pancreatic cancer is associated with a hypercoagulable state,50,51 therefore it is possible that NETs may be promoting hypercoagulability in pancreatic cancer patients.52 Additionally, there are several mechanisms by which NETs may promote tumor growth and metastases. NETs release extracellular DNA, which promotes pancreatic cancer cell invasiveness.53 The release of elastase from neutrophils degrades components of the tumor extracellular matrix and promotes the dyshesion of tumor cells, suggesting a role for NETs in the epithelial to mesenchymal transition that results in metastases.54,55 Elastase also directly increases proliferation in human and mouse lung adenocarcinomas and therefore may also contribute to pancreatic tumor growth.56 Additionally, the increase in circulating DNA and histones may allow for sequestration of circulating cancer cells and thus promote metastasis.57 NETs may also promote inflammation in the tumor microenvironment, inducing changes in the phenotype of infiltrating immune cells or stromal fibroblasts that subsequently promote tumor growth.58–60 These potential mechanisms by which NETs may promote carcinogenesis suggest a novel approach to NET inhibition in pancreatic cancer. In the current study, inhibiting autophagy by treatment with chloroquine reversed the propensity to form NETs in vitro. Both murine models and patients treated with autophagy inhibition had a reduction in NET formation in circulating neutrophils and decreased NET production in the tumor microenvironment. Furthermore, patients who had a Ca 19-9 response to treatment with autophagy inhibition and chemotherapy had lower levels of Cith3 staining, suggesting that a greater response to treatment resulted in greater inhibition of NETs in the tumor microenvironment. Autophagy inhibition with chloroquine or hydroxychloroquine is currently being evaluated in a number of clinical trials for treatment of pancreatic cancer, particularly to limit autophagic-mediated survival of tumor cells.61 (Active clinical trials reported on clinicaltrials.gov: NCT01494155, NCT01506973, NCT01122896, NCT01978184, NCT01777477). The current data suggests that the effects of autophagy inhibition may extend beyond the tumor microenvironment. Additionally, an inhibitor of peptidyl arginine diminase 4 , which is required for NET formation, Ci-amidine, has been shown to have beneficial effects in murine models of breast and colon cancer.52–54 The current findings suggest that the role of peptidyl arginine diminase 4 inhibitors in the treatment of pancreatic cancer should also be explored.

In conclusion, we demonstrate that neutrophils in murine models of pancreatic cancer are more prone to NET formation. Additionally, we observed evidence for NET formation in vivo in both murine models of pancreatic ductal adenocarcinoma as well as in patients. Further research into the clinical implications of NETs in pancreatic cancer and potential inhibitors to target NETs is warranted.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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