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The Receptor for Advanced Glycation End Products (RAGE) Enhances Autophagy and Neutrophil Extracellular Traps in Pancreatic Cancer

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Abstract

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 unwinding and subsequent expulsion of DNA. NETs play 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 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 pathways.

Keywords

RAGE; neutrophil extracellular traps; pancreatic adenocarcinoma; autophagy; chloroquine

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Introduction

Classically, neutrophils kill bacteria intra-cellularly following phagocytosis. However, neutrophils are also involved in a process known as neutrophil extracellular trap formation (NET) [1], which occur when activated neutrophils expel their DNA and other intracellular contents into the circulation or extracellular space. Following initial studies in sepsis as a mechanism of bacterial killing, NETs have also been implicated in setting of sterile inflammation, and in murine models of mammary and lung carcinoma [1-3]. NET formation requires expression of the enzyme peptidyl arginine diminase 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 [4]. 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 [2].

The process of autophagy, in which damaged organelles and proteins are degraded and recycled, is also critical for NET formation [3, 5, 6]. Autophagy is an important regulator of cancer cell survival in pancreatic cancer, allowing cells to survive the hypoxic, nutrient deprived tumor microenvironment [7-10]. Heightened autophagy correlates with poor prognosis in patients with pancreatic adenocarcinoma [11]. 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 [12-21]. Because RAGE mediated autophagy is critical in the pancreatic tumor microenvironment, we hypothesized that RAGE and autophagy would also play 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

10 -12 week Female C57/BL6 wild-type (WT) mice were purchased from Taconic Farms (Hudson, NY). RAGE knockout mice ($RAGE^{-/-}$, SVEV129xC57/BL6) were utilized to study the role of RAGE in NET formation [13]. For our orthotopic pancreatic cancer model, the murine pancreatic cancer cell line Panc02 cells (NCI repository, 2008) were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, and PenStrep antibiotic solution in a humidified incubator with 5% CO_2. WT and $RAGE^{-/-}$were injected with 1×10^6 Panc02 cells into tail of pancreas through a limited laparotomy. Animals were sacrificed after 4 weeks at which time palpable abdominal tumors were present. We also utilized a genetically engineered model of Kras driven pancreatic cancer (KC, Pdx1-Cre:$Kras^{G12D}$) which were purchased from the National Cancer Institute Mouse Repository. KC mice and $RAGE^{-/-}$mice were crossed to generate KCR mice. Animals were treated with oral chloroquine (0.5 mg/ml) administered in the drinking waterad libitum. Neutrophils were depleted using treatment with anti-mouse Ly6G antibody (RB6-8C5, EBioscience, #16-5931) injected intra-peritoneal at a dose of 5 mg/kg, 60 hours prior to

sacrifice. Neutrophil depletion was confirmed using a coulter counter to analyze neutrophil count of whole blood.

Patient Samples

Resected pancreatic tumor specimens and serum were obtained from patients enrolled in a phase I/II clinical trial of pre-operative 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 1500mg/m^2. Blood was collected before and after treatment. Clotted blood was spun at 1000g for 10 minutes. The serum was collected, frozen and stored at −80° C. Pooled human A/B control serum was obtained from Gemini Bio-products (#100-512, West Sacramento, CA)

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, #L8918) and rabbit anti-mouse actin (Sigma, #A2066). Membranes were then incubated with peroxidase-conjugated secondary antibodies for 1 hour at 25° C and developed with the SuperSignal West Pico chemiluminescence kit (Pierce, #34079) and exposed on film.

Ex Vivo NET Assay

Neutrophils were isolated using density gradient centrifugation. Cells were plated in a 96 well plate at approximately 1.5×10^4cells per well in Hank's Balanced Salt Solution (HBSS). Neutrophils were then stimulated with platelet activating factor (PAF, 0-50μm, Millipore, #511075) for 30 minutes. Cells were fixed with 3% paraformaldehyde and then DNA was stained with Hoechst 33342 (Molecular Probes, #H-3570). NETs were visualized using a Zeiss Axiovert 40 microscope under 10x-40xmagnification. Supernatant was collected, spun at 14g × 10 min and the level of DNA were measured using Quant-iT™Picogreen© (Invitrogen, MP07581), a fluorescent nucleic acid stain for quantifying double-stranded DNA.

Measures of In Vivo NET Formation

After 10x dilution, DNA levels were also measured from mouse and patient serum using QuantiT™ Picogreen©. Murine pancreatic specimens were embedded in Optimal Cutting Temperature (OCT) compound (Sakura), frozen and stored at −80° C. 8 μm sections were cut on cryostat for immunofluorescent staining. Tissue was fixed with 2% paraformaldehyde, permeabilized, blocked with 2% FBS, and incubated with a 1:50 dilution of anti-Histone H3 (citrulline 2+8+17) antibody (Abcam, 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 minutes, once in xylene with 100% ethanol for 3 minutes and 100% ethanol for 3 minutes followed by 95%, 70% and 50% ethanol for 3 minutes. Antigen retrieval was performed by heat-induced

method in 1 mM EDTA, pH 8.0 using a microwave. The slides then were washed two times for 5 minutes in PBS with 0.025% Triton X-100, after which the slides were blocked in 2% BSA for 2 hours. 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) were added for 1 hour. Hoechst (Invitrogen) for nuclear staining was applied for 30 seconds followed by two washes with phosphate buffered saline. Slides were mounted in fluormount-G (Southern Biotech) and confocal images were acquired using Olympus Fluoview 1000 microscope with a Plan Apo N (×60) (Center Valley, PA). Sequential scanning was applied for acquiring individual emission channels when multiple fluorophores were involved. The thickness of the sections were imaged by focusing on the top of the section, setting the Z-axis to 0, and then refocusing to the bottom of the section, an average of 8 sections were acquired. For controls, a sample(s) section was treated excluding the primary antibody ("primary delete"), and all samples within the same sample set were imaged with microscope settings to minimize sample autofluorescences using the primary delete processed sample. All slides were scanned under the same conditions for magnification, exposure time, lamp intensity and camera gain.

Data images were acquired using microscope manufacture software and exported in TIFF format. The TIFF files were then imported into Metamorph (Molecular Devices, Sunnyvale, CA) for maximum projection reconstruction. The final figures were imported and assembled in Adobe Photoshop.

Statistical Analysis

Data are expressed mean +/− SEM of at least two independent experiments performed in duplicate. Statistical analysis was performed using student t-test. p values <0.05 were considered statistically significant.

Results

Pancreatic Cancer Promotes NET Formation

Neutrophils were isolated from mice and stimulated with platelet activating factor (PAF), a known inducer of NET formation. Neutrophils from tumor bearing animals from both an orthotopic and a genetically engineered Kras driven model (KC) had a markedly increased propensity to form NETs compared to controls (**Figure 1A & B**). To confirm that the extracellular DNA visualized 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 & E**).

We 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 & B**). To confirm that clot formation during collection of serum did not confound our results, we measured both serum and 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 microenvironment, 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, citrullinated histone H3 expression was evaluated in resected murine tumor sections from both animal models. Pancreatic citrullinated histone H3 was increased in tumor bearing animals compared with normal pancreas (**Figure 2D & 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 & 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 a reduction in circulating DNA in both animal models (**Figure 3D&E**). To confirm the effect of autophagy inhibition on NET formation in patients, we analyzed serum DNA in patients with pancreatic adenocarcinoma treated with pre-operative 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, $p<0.05$). 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 extra nuclear citrullinated histone H3 expression (**Figure 4 A&B**). Staining was also performed on the resected

specimens of patients treated with pre-operative gemcitabine plus hydroxychloroquine (**Figure 4 C & D**, n=13). Patients who had a decline in CA 19-9 with treatment, suggesting a clinical response, had lower levels of citrullinated histone H3 than those that had no Ca 19-9 response to treatment (**Figure 4 E**).

NET Production is Dependent on RAGE

Because we have previously shown that RAGE mediates autophagy in the pancreatic tumor microenvironment, we suspected that RAGE may play a role in neutrophil autophagy in NET formation. Therefore, we analyzed measures of NET formation in $RAGE^{-/-}$ mice. In orthotopically injected $RAGE^{-/-}$ mice as well as mice with Kras driven pancreatic adenocarcinoma lacking RAGE (KCR), neutrophils from tumor bearing animals had a diminished propensity to form NETs (**Figure 5 A & B**). Serum DNA was decreased in tumor bearing $RAGE^{-/-}$ mice as well as in KCR mice, where DNA returned to the level of WT controls at several time points (**Figure 5C & D**). Additionally, citrullinated histone 3 expression in the pancreas was absent from RAGE null mice from both animal models (**Figure 5 E**).

Discussion

After being initially described in the setting of sepsis and infection, neutrophil extracellular trap (NET) formation has also been implicated in sterile inflammation and autoimmune disease, including gout [3], 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 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 preliminary confirmation of our pre-clinical findings in human PDA.

Our data clearly demonstrates that neutrophils harvested from tumor bearing animas 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 citrullinated histone H3, 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 PAD4, the enzyme responsible for citrullination of histones is expressed in tumors [25, 26]. Immunohistochemistry of pancreatic tumors in the current study demonstrates that some citrullinated histone H3 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 [27]. However, evidence from a growing number of studies suggests there is an alternative source to circulating DNA in cancer patients [28, 29]. 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 patients with pancreatic cancer, as not all DNA in the circulation may be tumor derived [30-34]. This finding may also explain the failure of some common genetic markers to be identified in circulating DNA in pancreatic cancer [35]. However, using serum DNA as a surrogate marker of *in vivo* NET formation should be interpreted with caution. It has been suggested that DNA may be elevated in the serum due to the release of DNA from white blood cells during clot formation and that plasma DNA levels may be more appropriate [36]. Interestingly, we did not observe any difference in serum and plasma DNA levels in tumor bearing animals in our models. However, the DNA in serum from wild type control mice was significantly higher than in plasma, consistent with the work by Lee *et al* which studied healthy donors [36]. These findings would suggest that the tumor bearing state may influence the amount of DNA detected in serum and plasma samples. One explanation for this is that in the tumor burdened state, primed neutrophils have already released their DNA and therefore do not release additional DNA during serum formation. To ensure that differences in white blood cell lysis did not confound our results in patient samples, we compared white blood cell counts and serum DNA levels from pancreatic cancer patients. There was no correlation between the number of circulating white blood cells, absolute neutrophil counts and DNA. The presence of citrullinated histone H3 in the plasma would be a more definitive marker to support NET formation in vivo. While a limited number of groups have demonstrated the ability to perform this assay in the setting of sepsis and shock [37, 38], we were unable to consistently obtain adequate results. This may be due to the fact that levels of citrullinated histone H3 and NETosis are much higher in the acute setting of shock than in more chronic malignancy.

Autophagy is a cell survival pathway that is critical for carcinogenesis and promotes resistance to therapy [7-10, 14]. We have previously demonstrated that one key regulator of autophagy in the tumor microenvironment is Damage Associated Molecular 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 inhibited NET formation, as RAGE knockout animals bearing tumor lack the propensity for NET formation, had lower levels of serum DNA and decreased citrullinated histone H3 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 stimulant 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 mediate

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 (sRAGE) which acts as a decoy receptor to antagonize RAGE [45, 46] and nonviral gene delivery vectors [47].

The formation of NETs in pancreatic cancer has tremendous clinical implications. In models of sepsis and trauma, NETs have been closely linked to thrombosis [48, 49]. 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 citrullinated histone H3 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, NCT01128296, NCT01978184, NCT01777477). The current data suggests that the effects of autophagy inhibition may extend beyond the tumor microenvironment. Additionally, an inhibitor of PAD4, which is required for NET formation, Cl-amidine, has been shown to have beneficial effects in murine models of breast and colon cancer [62-64]. The current findings suggest that the role of PAD4 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 PDA as well as in patients. Further research into the clinical implications of NETs in pancreatic cancer and potential inhibitors to target NETs is warranted.

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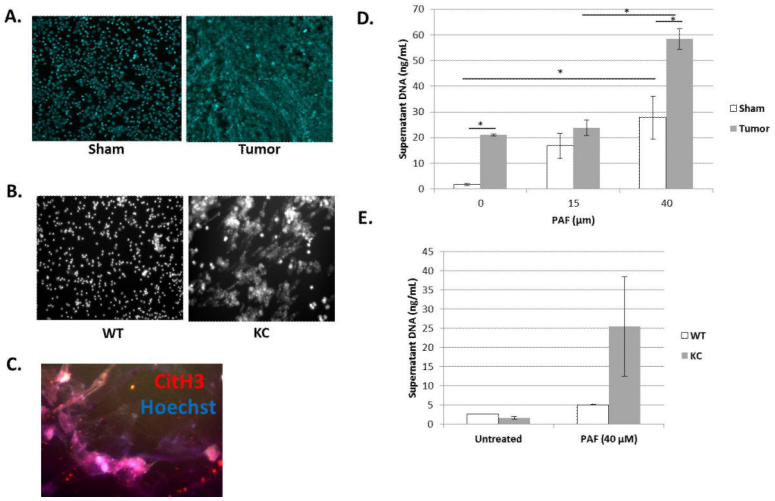

Figure 1. Neutrophils in murine pancreatic adenocarcinoma are more prone to neutrophil extracellular trap (NET) formation

Neutrophils isolated from sham and tumor bearing mice (A) and WT and KC mice (B) upon stimulation with 40 μM PAF, demonstrating a substantial increase NET formation in tumor bearing animals from both models. Extra nuclear DNA structures (Hoechst, Blue) were also positive for citrullinated histone H3 (CitH3, red), confirming that NETs were being visualized (C). PAF 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 & E), consistent with increased NET formation. *$p<0.05$.

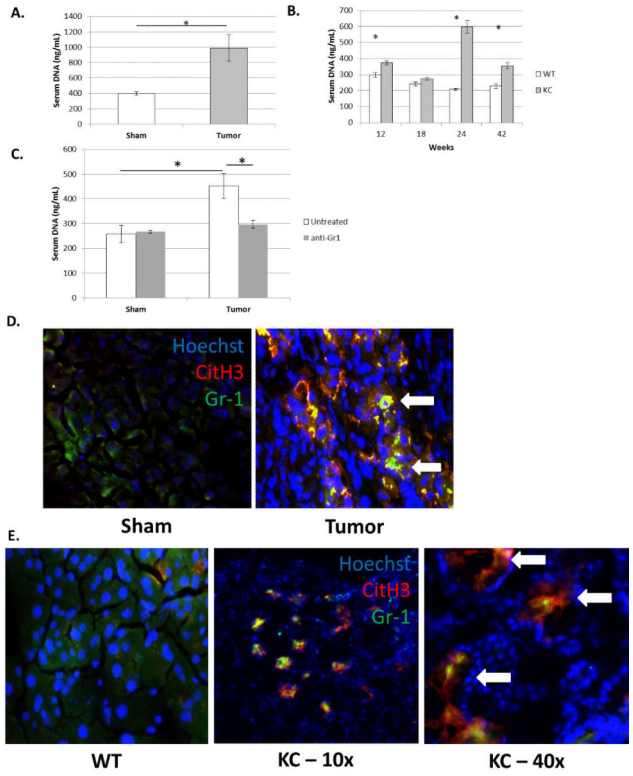

Figure 2. Neutrophils form NETs *in vivo* in murine pancreatic cancer

Serum DNA is elevated in tumor bearing orthotopic mice compared with sham controls (n=15), (A) and in KC mice compared with age matched WT (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 (CitH3, Red) is elevated in tumor bearing animals from the orthotopic model (D) and the genetic Kras driven model (E). CitH3 expression co-localizes with neutrophils (Gr-1, Green, White arrows). These are representative images from at least 3 independent analyses. *p<0.05

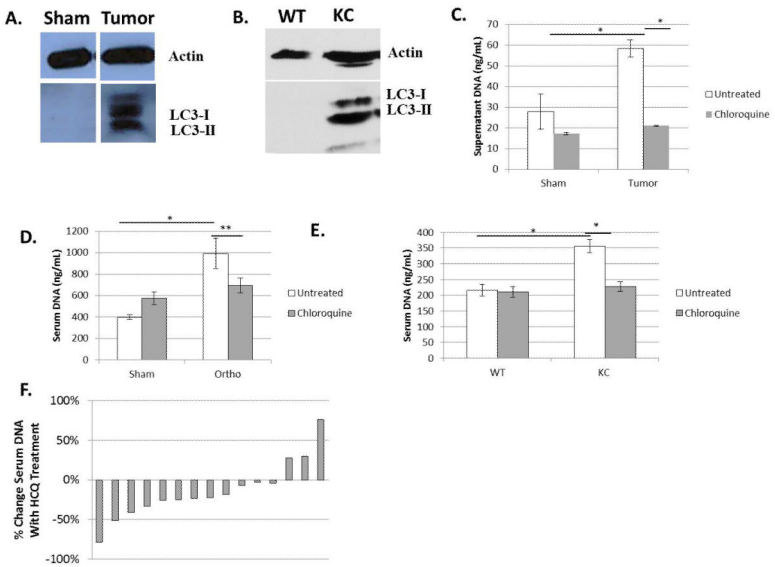

Figure 3. Autophagy promotes NET formation in pancreatic cancer

Neutrophils from tumor bearing orthotopic mice have increased LC3-II by western blot compared with sham (A) and in KC mice compared with WT (B), suggesting up regulated autophagy in neutrophils from tumor bearing mice. Inhibiting autophagy *in vitro* with chloroquine (CQ) decrease NET formation after PAF stimulation in a dose dependent fashion, as measured by supernatant DNA (C). Treatment of mice with CQ decreases serum DNA levels in tumor bearing orthotopic animals but not sham (n=15) (D) and in KC but not WT mice (n=7) (E). 80% 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.

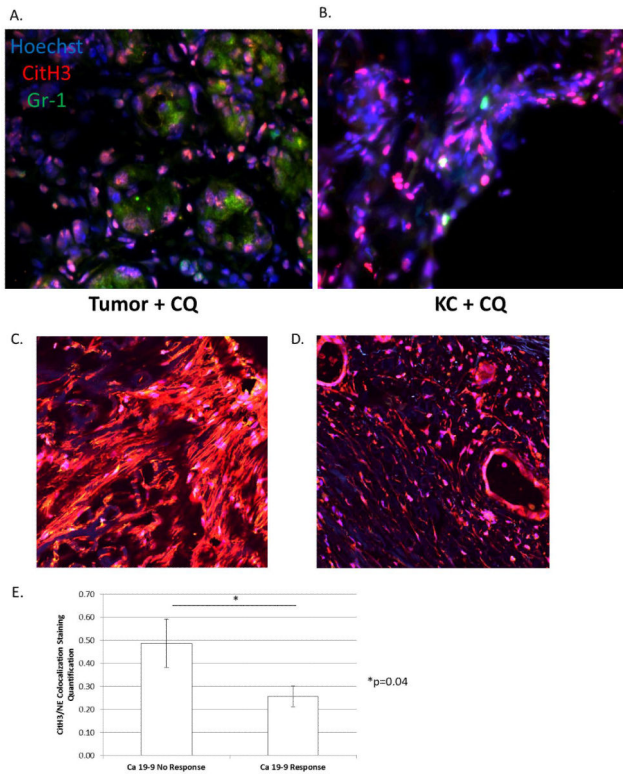

Figure 4. Autophagy promotes NET formation in the pancreatic tumor microenvironment

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. CQ 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 pre-operative Gemcitabine+ HCQ (C, n=3) compared with patients who had decline in Ca 19-9 following 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).

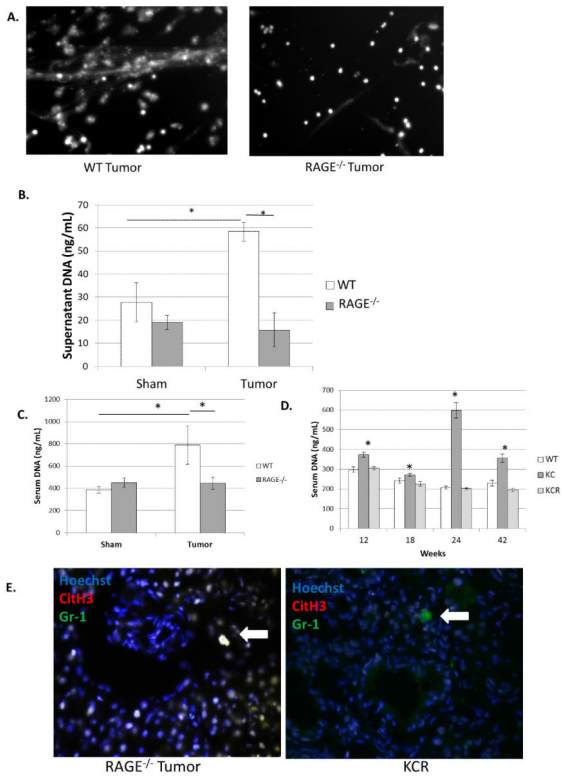

Figure 5. RAGE promotes propensity to form NETs in pancreatic adenocarcinoma

Neutrophils harvested from RAGE$^{-/-}$ tumor bearing animals are less prone to form NETs upon stimulation with PAF (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 (CitH3) expression in tumor bearing animals (E). Infiltrating neutrophils stained with Gr-1 are shown by the white arrows. *p<0.05.