Cancer Research

Neutrophil Extracellular Traps Promote the Development and Progression of Liver Metastases after Surgical Stress

Samer Tohme[1], Hamza O. Yazdani[1], Ahmed B. Al-Khafaji[1], Alexis P. Chidi[1], Patricia Loughran[1,2], Kerri Mowen[3], Yanming Wang[4], Richard L. Simmons[1], Hai Huang[1,5], and Allan Tsung[1]

Abstract

Risks of tumor recurrence after surgical resection have been known for decades, but the mechanisms underlying treatment failures remain poorly understood. Neutrophils, first-line responders after surgical stress, may play an important role in linking inflammation to cancer progression. In response to stress, neutrophils can expel their protein-studded chromatin to form local snares known as neutrophil extracellular traps (NET). In this study, we asked whether, as a result of its ability to ensnare moving cells, NET formation might promote metastasis after surgical stress. Consistent with this hypothesis, in a cohort of patients undergoing attempted curative liver resection for metastatic colorectal cancer, we observed that increased postoperative NET formation was associated with a >4-fold reduction in disease-free survival. In like manner, in a murine model of surgical stress employing liver ischemia-reperfusion, we observed an increase in NET formation that correlated with an accelerated development and progression of metastatic disease. These effects were abrogated by inhibiting NET formation in mice through either local treatment with DNAse or inhibition of the enzyme peptidylarginine deaminase, which is essential for NET formation. In growing metastatic tumors, we found that intratumoral hypoxia accentuated NET formation. Mechanistic investigations *in vitro* indicated that mouse neutrophil–derived NET triggered HMGB1 release and activated TLR9-dependent pathways in cancer cells to promote their adhesion, proliferation, migration, and invasion. Taken together, our findings implicate NET in the development of liver metastases after surgical stress, suggesting that their elimination may reduce risks of tumor relapse. *Cancer Res; 76(6); 1367–80. ©2016 AACR.*

Introduction

Colorectal cancer is the third most common cancer and third leading cause of cancer-related deaths in the United States (1). Mortality is most often related to liver metastatic disease. When feasible, resection of hepatic metastases promotes improved survival compared with chemotherapy alone (2). Unfortunately, hepatic recurrence after surgical resection occurs in 50% to 60% of patients and is a major cause of treatment failure (3).

Activation of the immune response following surgery is fundamental for reparative processes but evidence suggests that it may also enhance the risk of tumor recurrence and systemic metastases (4, 5). During hepatic surgery, the liver is routinely subjected to injury due to ischemia resulting from the interruption of the hepatic blood supply that is often necessary to control blood loss. Moreover, further dysfunction and damage occurs from excessive activation of inflammatory pathways following restoration of blood flow. The damaging effects that result, referred to as ischemia and reperfusion (I/R) injury, are an unavoidable consequence of liver resection and a major cause of morbidity and mortality (6). Previous studies have clearly demonstrated that the inflammation caused by liver I/R in animal models can accelerate the outgrowth of hepatic micrometastases, although the exact mechanisms remain unclear (7, 8).

Neutrophils are the front-line defense cells against microbes and have long been implicated as one of the principal cells in the immune response to surgery and hepatic damage associated with I/R (9). Neutrophils also play an important role in linking inflammation, a hallmark of cancer, to nearly every stage of tumor progression including metastasis (10, 11). Recently, Brinkman and colleagues described a novel aspect of neutrophil biology by which neutrophils can combat perceived threats in the circulation by forming neutrophil extracellular traps (NET; ref. 12). NETs consist of expelled DNA studded with various proteins to form web-like structures (12). Although NETs were initially described as an adjunctive mechanism of antimicrobial defense, NETs have a variety of potentially adverse effects as they can also participate in the pathogenesis of autoimmune and other inflammatory sterile diseases (13). We recently demonstrated the novel finding

[1]Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania. [2]Center for Biologic Imaging, Department of Cell Biology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania. [3]Department of Pharmacology and Department of Chemical Physiology, Scripps Research Institute, La Jolla, California. [4]Department of Biochemistry & Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania. [5]Department of Surgery, Union Hospital, Huazhong University of Science and Technology, Wuhan, P.R. China.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Allan Tsung, University of Pittsburgh, 3459 Fifth Avenue, UPMC Montefiore, 7 South, Pittsburgh, PA 15213-2582. Phone: 412-692-2001; Fax: 412-692-2002; E-mail: tsunga@upmc.edu

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that neutrophils can form NETs in response to liver I/R and that targeting NETs ameliorates the hepatic as well as systemic I/R-induced inflammation in mice (14).

The role of NETs in tumor progression is still unclear (15, 16). Recent evidence has linked NETs to tumor-associated thrombosis and tumor progression in the setting of systemic infection (17, 18). Given the well-described but poorly understood association between surgical stress and tumor recurrence, we sought to determine whether NETs play a role in increased tumor metastasis in a model of surgical stress induced by liver ischemia-reperfusion. We hypothesize that, in the context of surgical stress, NETs are formed in the liver and promote the adhesion of circulating tumor cells and growth of existing micrometastatic disease.

Materials and Methods

Patient samples and data

Serum samples were collected on postoperative day 1 from patients who underwent hepatectomy for metastatic colorectal cancer (mCRC) at the University of Pittsburgh (Pittsburgh, PA). Sequential eligible patients were included, between the years 2010–2012, who were disease-free at the end of the operation and had a minimum one-year follow-up. Using ELISA, we quantified HMGB1 levels and MPO–DNA complex levels for each sample and determined the fold change compared with healthy controls. Disease-free survival was calculated as the number of months from surgery to recurrence or death. Patients who did not experience an event or were lost to follow-up were censored at the date of last contact or end of the study period. All human materials used were obtained under an approved Institutional Review Board protocol. Written informed consent was received from all participants prior to inclusion.

Cell lines

MC38 and luciferase-expressing MC38 cells (MC38/Luc), authenticated using genomic profiling (IDEXX Radil Cell Check), were obtained from Dr. Michael Lotze (University of Pittsburgh, Pittsburgh, PA) in June 2014. Cell lines were amplified in our laboratory and stored in liquid nitrogen to ensure that cells used for experiments were passaged for fewer than 6 months. No further genomic authentication was performed but cell lines were tested biannually for identity by appearance and growth curve analysis and validated to be mycoplasma free.

Metastases models, hepatic I/R model, and experimental groups

Animal protocols were approved by the Institutional Animal Care and Use Committee and adhered to the NIH Guidelines. The first set of experiments were designed to evaluate whether I/R increased tumor growth in a model of circulating micrometastases. Colorectal liver metastases were induced in mice as described previously (7, 8). In brief, 5×10^4 MC38 or MC38/Luc cells were injected through a 1-cm midline laparotomy into the spleen of 8- to 10-week-old C57BL/6J WT mice or PAD4$^{-/-}$ mice (19) using a 27-gauge needle. Tumor cells were allowed to circulate for 10 minutes. Mice not receiving I/R underwent an additional 15 minutes of circulation, exposure of the portal triad without hepatic ischemia followed by splenectomy, and closure. Those mice that received I/R were subjected to a nonlethal model of segmental (70%) hepatic warm ischemia (60 minutes) and reperfusion for 15 minutes before splenectomy (20). Splenectomy was done to prevent the formation of splenic tumors. The next set of experiments were designed to evaluate whether I/R increased growth of established micrometastatic disease. In these experiments, tumor cells were injected through a small left lateral flank incision followed by splenectomy. Micrometastases were allowed to develop throughout the liver for 5 days. At that time point, the mice underwent either hepatic I/R or sham procedure.

DNAse 1 and YW4-03 treatment

First dose of intraperitoneal injections of DNAse 1 (50 μg/mouse, Roche) or YW4-03 (10 mg/kg) were given immediately prior to abdominal closure and then daily intraperitoneal doses for DNAse and three times weekly for YW4-03 were administered (14).

Neutrophil isolation

Mouse neutrophils were isolated from bone marrow of tibias and femurs (18). Neutrophils were sorted on a BD-Aria-Plus high-speed sorter after incubation with APC-conjugated anti-mouse-Ly6G antibody (BD Biosciences) and APC-Cy7 CD11b (BD Biosciences; purity >96% and >95% viability by Tryptan blue staining; Supplementary Fig. S1).

In vitro NET formation

Neutrophils were plated to adhere on coated plates for 1 hour before stimulation with Phorbol-12-myristate-13-acetate (PMA, 100 nmol/L, Sigma-Aldrich) for 4 hours in 37°C 5% CO_2. After 4 hours, the media were collected and used in subsequent experiments as outlined in Supplementary Methods.

Statistical analysis

For animal studies, results are expressed either as SEM or mean SD. Group comparisons were performed using one-way ANOVA with *post hoc* Tukey honestly significant difference (HSD) analysis and Student *t* test. For the data analysis of human subjects, we dichotomized MPO–DNA complex levels at the median and compared the baseline characteristics for each group using χ^2 or Fisher exact tests for categorical variables and Student *t* or Wilcoxon rank sum tests for continuous variables. We used the Kaplan–Meier estimator to plot disease-free survival. We used Cox proportional hazards regression to determine the effect of postoperative MPO–DNA and HMGB1 levels on disease-free survival. We evaluated the model's predictive ability using Harrell c-statistic. For all analyses, two-tailed *P* values below 0.05 were considered statistically significant.

Results

Surgical stress induced by liver I/R results in widespread NET formation

Hepatic I/R can generate NETs in mice (14) but the role of NETs in humans and their role in tumor growth is uncertain. We collected serum samples for 50 patients who underwent hepatectomy for mCRC at our center. By measuring serum levels of MPO–DNA complexes, we are certain that the circulating nucleosomes are derived from NETs (14, 21). There was a significant increase in NET formation in patients who underwent major liver resection ($n = 35$, three or more liver segments

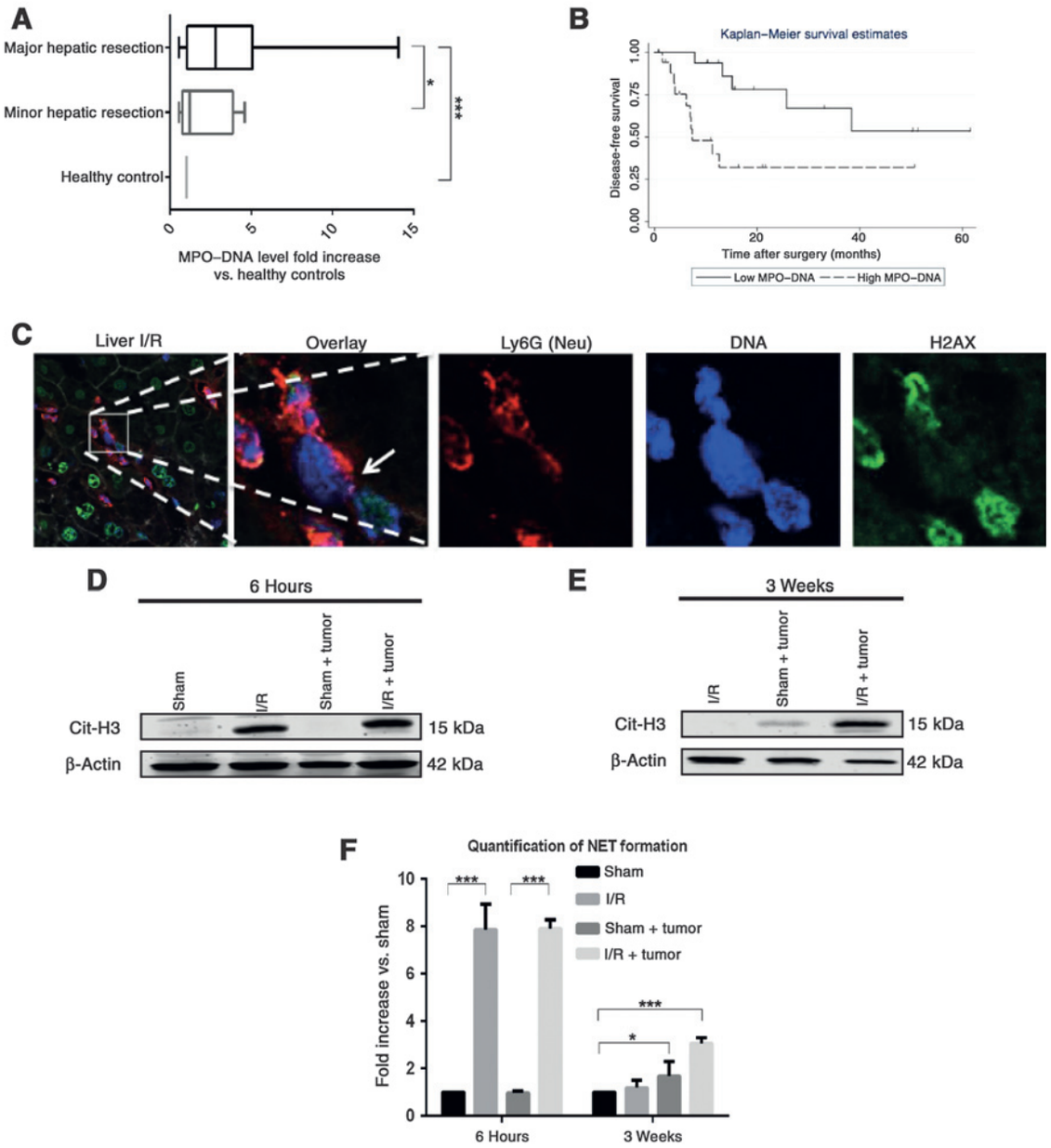

Figure 1.
Surgical stress induced by liver I/R results in widespread deposition of NETs. A, detection of serum MPO–DNA levels in mCRC patients undergoing major ($n = 35$) or minor liver surgery ($n = 15$) or in healthy controls ($n = 20$). Box plots show higher MPO–DNA levels in mCRC patients undergoing major resection. B, Kaplan–Meier disease-free survival curves of mCRC patients who underwent major liver resection based on serum MPO–DNA levels. C, representative immunofluorescence images by confocal microscopy of mice liver sections showing NETs 60 minutes after ischemia/tumor injection and 6-hour reperfusion (magnification, ×40; $n = 6$) with staining for Ly6G (red), nuclei (blue), histone H2AX (green), F-actin (gray). Arrow, released histone and DNA from neutrophils. D and E, cit-H3 protein levels were determined by Western blot analysis in sham, sham + tumor injection, I/R, and I/R + tumor injection mice groups after liver I/R at 6 hours (D) and 3 weeks (E). Hepatic protein lysates from ischemic lobes at 6 hours or tumors at 3 weeks were obtained. The blots shown are representatives of three experiments with similar results. F, NETs acutely form in liver tissue 6 hours after tumor injection and liver I/R, and in tumors 3 weeks later, as assessed by serum levels of MPO–DNA. Results are expressed as the relative folds increase of MPO–DNA levels compared with sham; mean ± SEM ($n = 6$/group). *, $P < 0.05$; ***, $P < 0.001$.

resected), in which liver ischemia-reperfusion is inevitable, compared with patients who underwent minor resections ($n = 15$) or to age/gender-matched healthy volunteers ($n = 20$; Fig. 1A). In the subset of patients who underwent major liver resection, patients were grouped into high ($n = 18$) and low ($n = 17$) categories based on the median MPO–DNA (fold change 1.75). There were no significant differences between groups in variables that have been previously shown to affect long-term outcomes after mCRC resection (Supplementary Table S1; ref. 22). In our analysis of the disease-free survival,

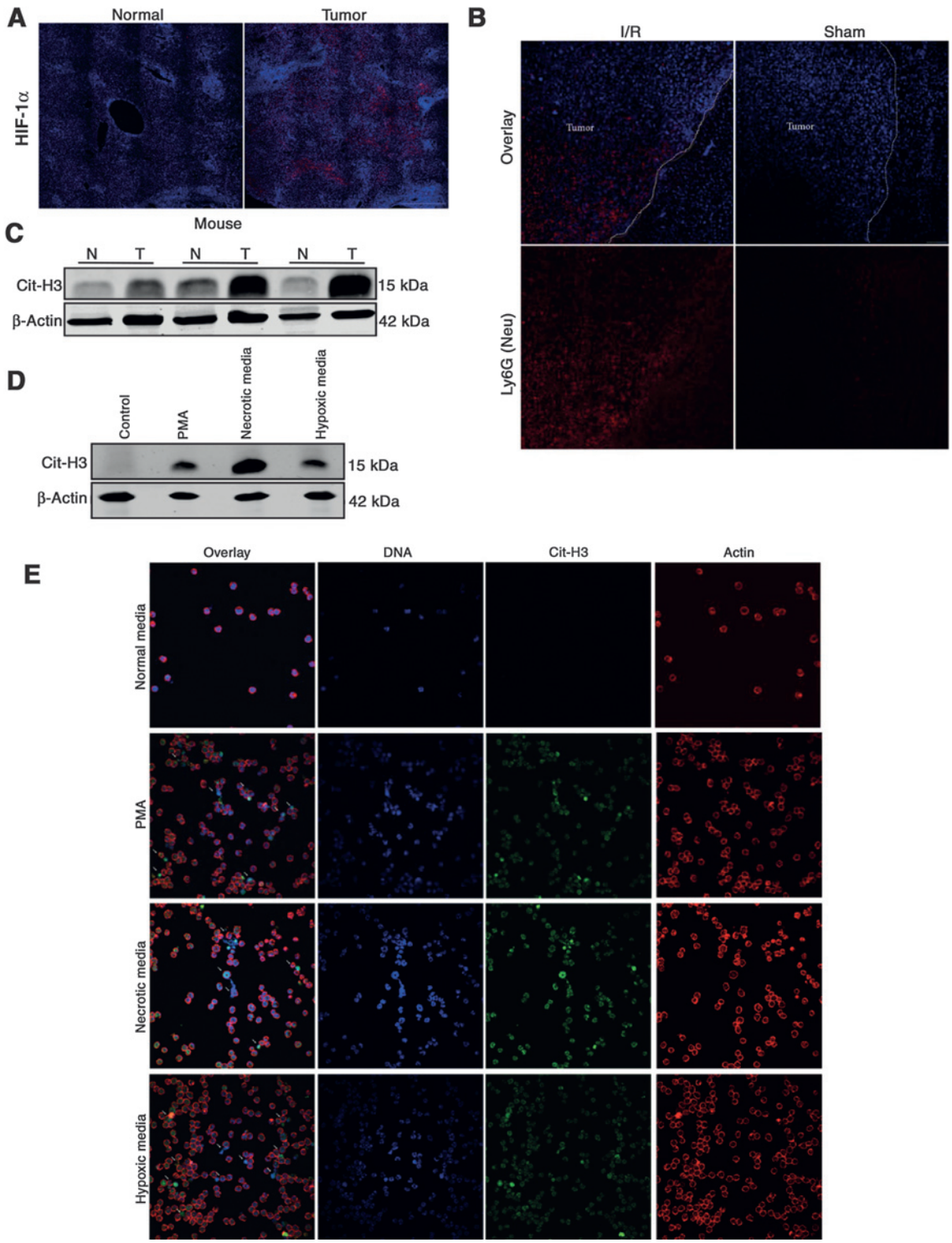

Figure 2.
Growing solid tumors generate a hypoxic environment that potentiated NET formation. A, three weeks after splenic injection of MC38 cells, the liver tumors exhibit significant central hypoxia as evident by increased HIF-1α staining by immunofluorescent imaging compared with background liver. HIF1-α, red; nuclei, blue. B, using confocal microscopy, there is a significant increase in tumor-infiltrating neutrophils 3 weeks after mice were subjected to I/R, compared with mice that only received tumor injection surgery (mean 258 $Ly6G^+$ cells/10^6 μm^2 versus 20 $Ly6G^+$ cells/10^6 μm^2; $P < 0.001$). Ly6G, red; nuclei, blue. Scale bars (A and B), 100 μm. C, cit-H3 levels, marker of NETs, was increased in the hypoxic liver tumors compared with background liver in paired mouse tissues 3 weeks after establishment of the metastatic and liver I/R model. D and E, *in vitro*, there was a significant increase in NET formation after neutrophils isolated from mice were cocultured with media from MC38 cells subjected to hypoxia or thermal necrosis as evident by Western blot analysis for cit-H3 (D) and confocal microscopy (E) at ×40 magnification. Cit-H3, green; nuclei, blue; F-actin, red. Arrows, neutrophils forming NETs. N/T, normal/tumor.

9 deaths and 6 recurrences were identified in 620 person-months of follow-up. As seen in Fig. 1B, the risk of recurrence was 4.22 times as high in patients with higher MPO–DNA as in those with lower MPO–DNA [95% confidence interval (CI), 1.39–12.81; $P = 0.011$]. The Harrell c-statistic for MPO–DNA dichotomized at the median was 0.717.

To better understand these findings, we sought to further characterize this phenomenon in a mouse model of liver metastasis and surgical stress. Briefly, the mice were subjected to liver I/R or sham procedure after intrasplenic injection of either MC38, a murine colorectal cancer cell line, or PBS only. After 60 minutes of ischemia, the livers were reperfused and a splenectomy was performed. In lobes exposed to I/R, Ly6G-positive cells colocalized with extracellular histone H2AX and web-like DNA, indicating NET formation in response to the acute ischemic stress (Fig. 1C). Ischemic lobes also exhibited significantly higher levels of citrullinated-histone H3 (cit-H3; ref. 23), another specific marker of NET formation (Fig. 1D). Furthermore, we found that the serum level of MPO–DNA complexes was significantly increased in mice undergoing liver I/R compared with controls (Fig. 1F). At 3 weeks, there was a sustained increase in NET formation in the livers of mice that received tumor injection plus I/R, whereas the NET response after I/R did not persist in the absence of tumor (Fig. 1E and F). The mice receiving tumor cells without deliberate I/R showed evidence of NET formation in the liver after 3 weeks albeit to a significantly lesser degree than mice that underwent both liver I/R and tumor injection (Fig. 1E and F).

Growing solid tumors generate a hypoxic environment that potentiates NET formation

The mechanisms responsible for sustained elevation of NETs in the rapidly growing tumor, as seen after liver I/R, are unknown. Hypoxia is commonly present in rapidly growing metastatic tumors and has been linked to tumor progression (24). However, the mechanisms by which tumors survive and grow within the hypoxic environment remains unclear. Therefore, we next examined whether chronic hypoxia can have a similar effect to acute hypoxia in inducing NET formation in the tumor environment. We confirmed the presence of tumor hypoxia in the metastatic tumors 3 weeks after the mice underwent liver I/R plus tumor injection by immunofluorescent staining for hypoxia-inducible transcription factor (HIF)-1α (Fig. 2A). In addition, we found a median 12-fold increase in tumor-associated neutrophils within the hypoxic tumor environment in the group of mice that had undergone liver I/R versus sham (Fig. 2B). There was an increase in NET formation among those tumor-infiltrating neutrophils compared with normal background liver (Fig. 2C). To study whether hypoxic cancer cells induce NET formation, we exposed MC38 cells in culture to either hypoxia or induced necrosis by incubation at 60°C. The conditioned media from these cultures was incubated with mouse neutrophils. Similar to the PMA-treated neutrophils (positive control), there was a significant increase in NET formation as shown by increased expression of cit-H3 (Fig. 2D) and by immunofluorescent staining (Fig. 2E). Similarly, we found that, in paired human mCRC tissue samples and their corresponding nontumor liver tissue, the expression of HIF-1α and cit-H3 was higher in the recurrent mCRC tumors (Supplementary Fig. S2A). Taken together, these results indicate an acute increase in NET formation after surgical stress that is chronically sustained in the hypoxic environment present in growing tumors.

Surgical stress promotes the development of gross metastases, which is attenuated by administration of DNAse, a NET inhibitor

Having shown that liver I/R induced surgical stress results in widespread intrahepatic NET formation and deposition, we sought to determine whether NET formation was associated with the development of metastasis. We aimed to mimic the surgical setting in which major resection of livers containing metastases leads to tumor cell shedding and an increased level of circulating tumor cells (25). Luciferase-labeled MC38 cells were injected into the spleen before I/R. Recipients of tumor cells and cohort controls were given DNAse daily (Fig. 3A). DNAse is a known inhibitor of NET formation and was used to explore whether disruption of NETs would alter the course of hepatic tumor progression. Liver tissue from DNAse-treated mice 6 hours after intrasplenic tumor injection had significantly lower levels of cit-H3 compared with vehicle-treated mice undergoing liver I/R (Fig. 3B). Similarly, circulating MPO–DNA complexes were significantly reduced in the DNAse-treated I/R mice (Fig. 3C). Figure 3D shows that livers of mice subjected to I/R without DNAse treatment contained significantly more hepatic metastases compared with mice not receiving I/R at 2 weeks. Daily administration of DNAse after liver I/R resulted in a 68% reduction in tumor nodules compared with untreated I/R mice (Fig. 3D). In addition, as a 70% I/R model was used, we observed significantly more NET formation and tumor growth in the ischemic compared with the nonischemic lobes (data not shown). Bioluminescent imaging permitted *in vivo* tracking of tumor growth and assessment of DNAse effect. Both histologic examination and bioluminescent imaging showed that DNAse treatment significantly slowed the accelerated growth of tumors associated with I/R (Fig. 3E and F). Tumors from mice treated with DNAse after I/R showed a significant decrease in proliferation and a decrease in tumor-associated angiogenesis compared with nontreated mice subjected to I/R (Fig. 3G)

Surgical stress promotes the growth of established liver micrometastases, which is attenuated by administration of DNAse, a NET inhibitor

In addition to inducing circulating tumor cells during surgery, many patients already have microscopic tumor deposits at the time of surgery, which may contribute to postoperative tumor progression (26). We used our model to examine whether NETs affect the growth of these existing micrometastases. MC38 cells were injected into the spleen and micrometastases were allowed to develop, 5 days later, the mice were subjected to sham or I/R surgery with or without daily DNAse treatment (Fig. 4A). Again, there was a significant increase in intrahepatic expression of cit-H3 and circulating levels of MPO–DNA 3 weeks after the tumor injection, both of which were attenuated by the administration of DNAse (Fig. 4B and C). Mice treated with DNAse displayed significantly decreased tumor growth, which was grossly appreciable as smaller and less numerous tumors (Fig. 4D). In addition, DNAse significantly decreased the tumor load after I/R as evidenced by the liver-to-body weight ratio and the tumor hepatic replacement area (Fig. 4E and F). Of note, DNAse alone did not significantly reduce tumor growth in the sham-treated mice.

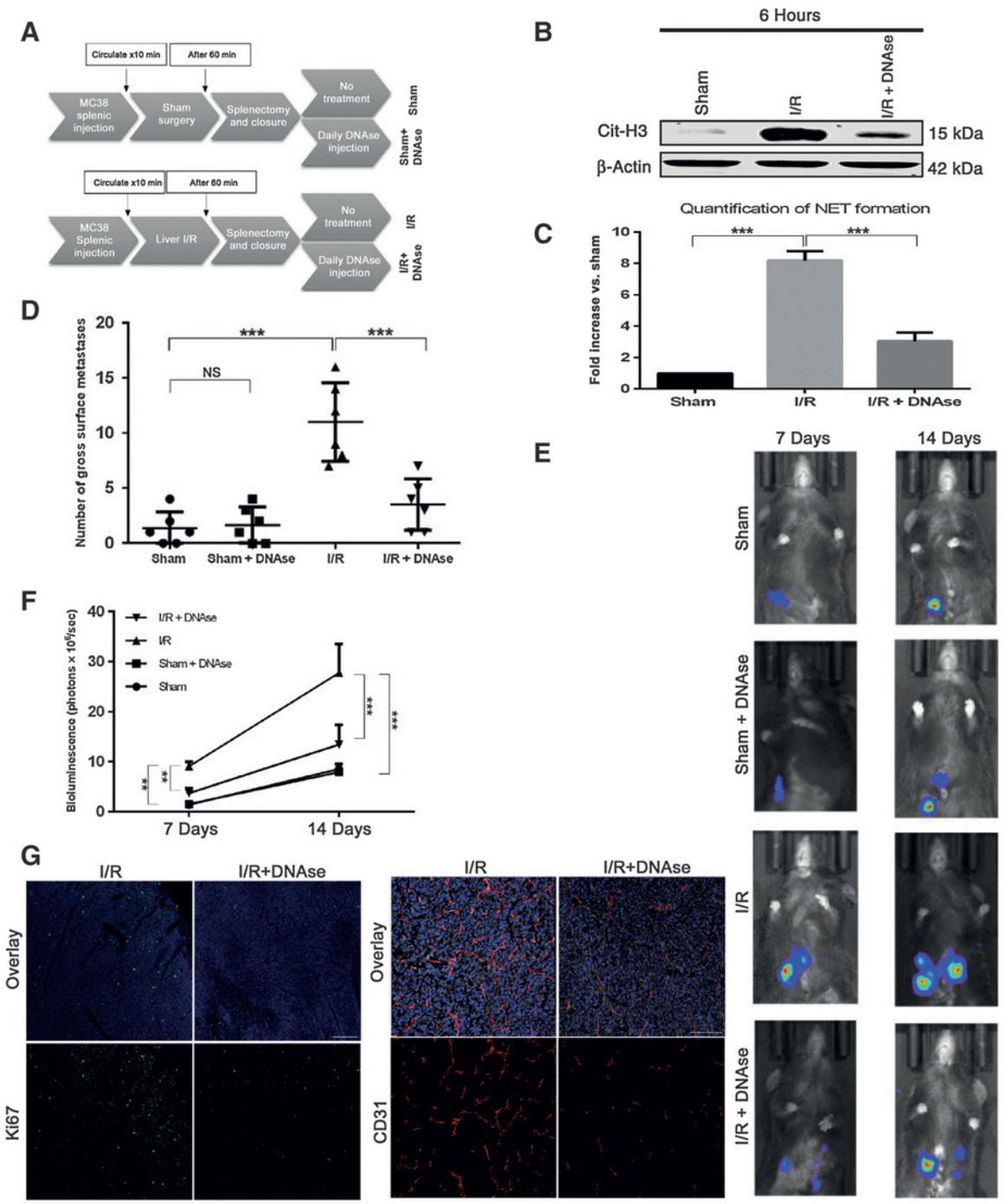

Figure 3.
Surgical stress promotes the development of gross metastases, which is attenuated by administration of DNAse, a NET inhibitor. A, a schematic representation of the experimental design is depicted. Mice were subjected to liver I/R to induce surgical stress. Intrasplenic injection of MC38 colorectal cancer cell lines was performed at the same time. Daily DNAse intraperitoneally was started at the time of procedure. B, cit-H3 levels were significantly decreased after administration of DNAse after 1 hour of ischemia and 6 hours of reperfusion. C, treatment with DNAse after liver I/R resulted in a significant decrease in the levels of serum MPO–DNA at 6 hours. D, at 14 days, I/R resulted in a significant increase in gross surface metastatic nodules compared with the sham groups weeks (mean 11 nodules in I/R versus 2 nodules in sham; $P < 0.0001$). Treatment with DNAse resulted in a significant decrease in the number of gross metastases. E and F, the use of luciferase-labeled MC38 cells allowed weekly *in vivo* tracking of tumor growth with bioluminescence imaging. DNase does not affect tumor growth in the unstressed tumors, the sham group. In the I/R group, we again demonstrate the effect of surgical stress on the acceleration of tumor growth and this is significantly inhibited at day 7 and 14 by the daily administration of DNAse. Data represent mean ± SEM; $n = 6$ mice/group. The above data are each representative of three experiments with similar results. G, daily treatment with DNAse after I/R significantly attenuated tumor cell proliferation weeks (mean 22 $Ki67^+$ cells/10^6 μm^2 I/R and DNase group versus 96 $Ki67^+$ cells/10^6 μm^2 in I/R group; $P < 0.001$) and tumor-associated tumorigenesis (mean $CD31^+$ area 6×10^3 μm^2 versus 18×10^3 μm^2/10^6 μm^2; $P < 0.001$) in liver metastatic tumors at three. Nuclei, blue; Ki67, green; Cd31, red; scale bar, 500 μm. NS, not significant; **, $P < 0.01$; ***, $P < 0.001$.

DNAse and PAD4 targeting inhibit the protumorigenic effects of NETs

The results obtained from the above two models demonstrate that hepatic I/R has a stimulatory effect on NET formation and that, by using DNAse to inhibit NETs, significantly inhibited the development and growth of metastatic disease. We next evaluated different strategies to inhibit NET formation. Peptidyl arginine deiminase type IV or PAD4 is an essential enzyme for NET formation that catalyzes the citrullination of histones-H3, a critical step for chromatin decondensation and expulsion. We used the protocol in Fig. 4A and treated mice with direct PAD4 inhibitor YW4-03 or performed our model in PAD4$^{-/-}$ mice. There was a significant decrease in the tumor load after I/R in mice given YW4-03 or in PAD4$^{-/-}$ mice (Fig. 5A and B). Of note, in sham mice, YW4-03 caused a 32% decrease in tumor growth compared with sham-untreated mice, which trended towards statistical significance; this is in comparison to close to 73% decrease in tumor growth in WT mice who received YW4-3 and liver I/R compared with mice who underwent I/R without YW4-03 ($P < 0.001$). Levels of cit-H3 in the tumors after three weeks were increased in mice who underwent I/R and were absent in PAD4$^{-/-}$ mice and mice receiving YW4-03 (Fig. 5C).

The cumulative effect of NETs on tumor growth is demonstrated, but the precise mechanism is not clear. In order for circulating cancer cells to effectively form micrometastatic foci in the liver, adhesion, migration and invasion through the endothelium, and proliferation are required. In order to support the *in vivo* data, we sought to examine the above findings *in vitro* in a system using only tumor cells and neutrophils. In order to study the role of NETs in cancer cell adhesion, PMA (100 nM) was used to stimulate NET formation in neutrophil monolayers. Adhesion of CFSE-labeled MC38 cells to neutrophil monolayers was significantly increased with PMA stimulation compared with control (Fig. 5D). However, inhibition of NETs by the addition of DNAse or pretreatment with YW4-03 or using PAD4$^{-/-}$ neutrophils significantly decreased tumor adhesion after PMA treatment (Fig. 5D). We next sought to study the direct role of NETs on cancer cell proliferation *in vitro*. Conditioned media was collected after WT or PAD4$^{-/-}$ neutrophil monolayers were stimulated with PMA. Cultured MC38 cancer cells with conditioned WT neutrophil media increased tumor cell proliferation by MTT assay by 60% and 97% at 24 and 48 hours, respectively; $P < 0.0001$ (Fig. 5F and G). Adding DNAse or YW4-03 to the conditioned neutrophil media or using conditioned media from PAD4$^{-/-}$ neutrophils significantly reduced the tumor cell accelerated growth; $P < 0.0001$ (Fig. 5F and G). Furthermore, the presence of conditioned neutrophil media resulted in a 3.5–fold increase in MC38 cell migration and a 7–fold increase in MC38 cell invasion compared with control (Fig. 5E). Migration and invasion of MC38 cells in the presence of conditioned neutrophil media was almost abrogated by the different NET inhibition strategies (Figure 5E). The above protumorigenic effects of NETs were also observed in different cell lines, arguing against a cell-specific phenomenon (representative cell line in Supplementary Fig. 2).

NETs exert their protumorigenic effects through activation of TLR9 pathways

Our results support the hypothesis that DNA release during NET formation can play an important role in tumor growth. Toll-like receptors (TLR) play a key role in the innate immune response to stress and TLR expression seems to play a role in tumor progression (24, 27, 28). TLR9 is a cellular DNA receptor that is widely expressed in different cancers and promotes tumor growth by activation of a cascade of intracellular growth signaling pathways (24, 27, 28). We therefore evaluated the role of surgical stress in our model of liver I/R in the activation of TLR9-dependent pathways. Phosphorylation of p38, Stat3, JNK, and the p65 subunit of NF-κB were each amplified in the tumors of mice 3 weeks after I/R (Fig. 6A). When we treated the mice with DNAse, there was a significant inhibition of these pathways and this correlated with decreased tumor growth (Figs. 4D and 6A). *In vitro*, TLR9 expression is significantly increased after MC38 cancer cells are cultured in media derived from PMA-stimulated neutrophils (Fig. 6B). When we cultured MC38 cells *in vitro* with conditioned neutrophil media (media collected from WT or PAD4$^{-/-}$ PMA-stimulated neutrophils), TLR9-dependent pathways were activated and this effect was abolished by the addition of DNAse to the media or pretreatment of neutrophils with YW4-03 or when MC38 where cultured with PMA-stimulated neutrophils from PAD4$^{-/-}$ neutrophils (Fig. 6C and D). The NET-driven activation was similar to the activation of MC38 cells seen when adding a known TLR9 agonist. Knocking down TLR9 using shRNA significantly decreased the activation of those pathways even if the cancer cell was cultured with conditioned media (Fig. 6C). These results paralleled the responses for tumor cell proliferation, migration, and invasion (Fig. 6E and F). Similar findings were seen using Hep1–6 cells treated under the same conditions (data not shown). Furthermore, studies have shown that the tumorigenic effects of TLR9 depend on NF-κB–mediated upregulation of IL6 expression (29). Indeed, we found that IL6 was significantly elevated in the MC38 cells cultured in conditioned neutrophil media and decreased in the groups treated with DNAse or in TLR9 knocked-down cells (Supplementary Fig. S2).

In vivo, mice injected with MC38 cells deficient in TLR9 had a significant decrease in tumor growth in response to I/R at 3 weeks compared with mice injected with WT MC38 cells (Fig. 6G and H). As expected, there was not an increase in MAPK pathway activation after I/R in TLR9 knocked down MC38 cells (Fig. 6I).

HMGB1 released from NETs promote tumor progression and TLR9 activation

The mechanism by which NET components, mainly DNA, activates intracellular TLR9 signaling remains elusive. A prime suspect is High Mobility Box (HMGB)-1 protein because previous studies have shown that extracellular HMGB1, a highly conserved DNA-binding protein, plays a critical role in regulating the process of TLR9 activation by circulating DNA complexes (30, 31). We confirmed by immunofluorescence that HMGB1 is a component of NETs (Fig. 7A). Furthermore, there was almost a 4-fold increase in HMGB1 released from neutrophils after stimulation with PMA (Fig. 7B). After culturing MC38 cells with conditioned neutrophil media, activation of the MAPK signaling pathways was inhibited by the addition of neutralizing HMGB1 antibodies to the conditioned neutrophil media (Fig. 7C). Similarly, the NET-mediated increase in proliferation, invasion, and migration of tumor cells was significantly decreased with the addition of anti-HMGB1 antibodies (Fig. 7D and E). Furthermore, there was a significant increase in preoperative HMGB1 levels in patients with mCRC compared with healthy controls (median, 41.1 vs. 1.4 ng/mL) and almost a 3-fold further increase in HMGB1 levels postoperatively

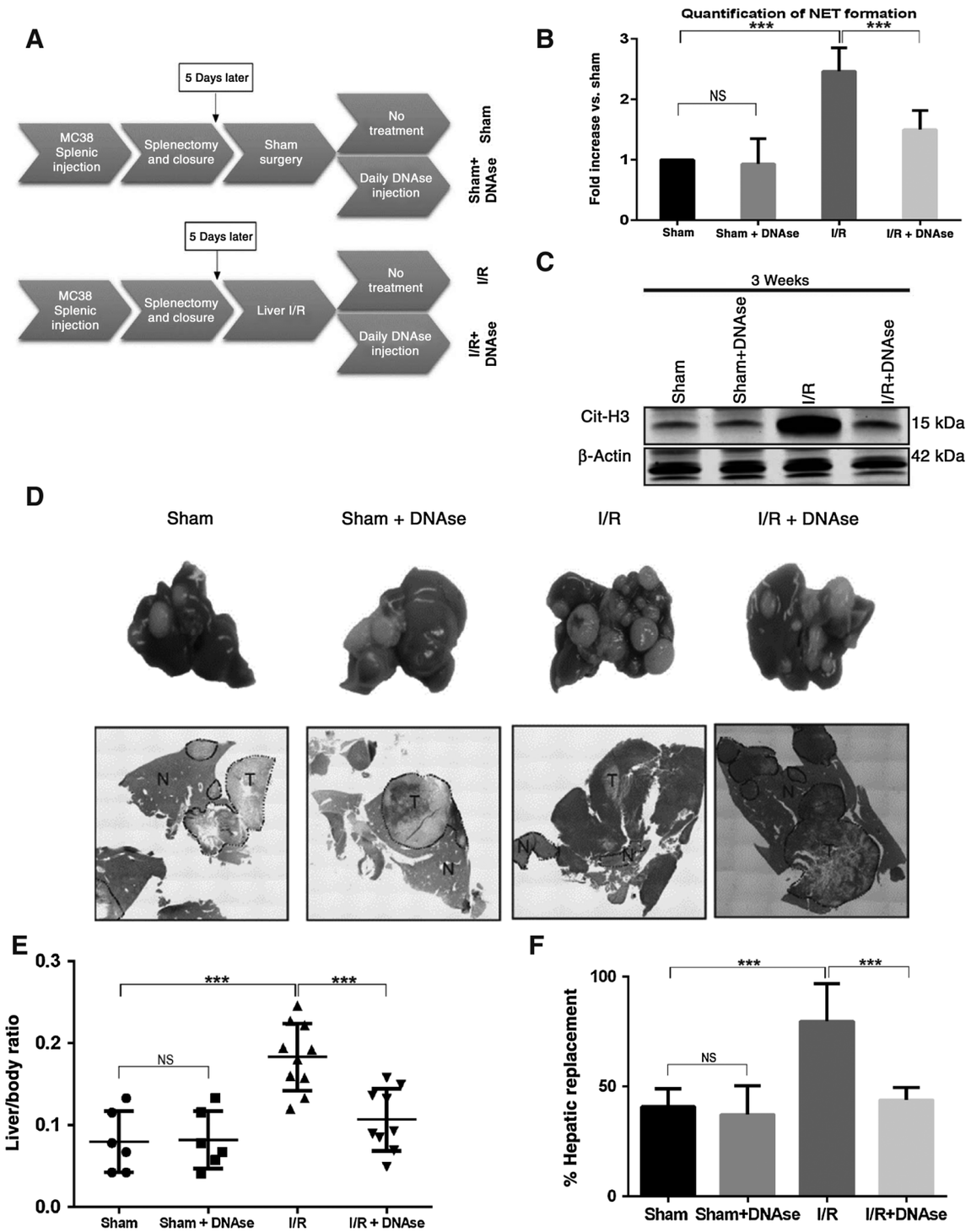

Figure 4.

Surgical stress promotes the growth of established liver micrometastases, which is attenuated by administration of DNAse. A, schematic representation of the experimental design is depicted. Intrasplenic injection of MC38 cells was performed and metastatic tumor was allowed to grow for 5 days before the mice were subjected to liver I/R. Daily DNAse treatment was started at the time of liver I/R. At 3 weeks, the mice were sacrificed and tumor growth was assessed. B and C, there was a significant increase in NET formation in the tumors after I/R, as evident by increase in tumor cit-H3 levels (B) and serum levels of MPO–DNA (C). Daily DNAse treatment resulted in a significant inhibition NET formation. D, representative images of hepatic nodules and hematoxylin and eosin staining of liver section (N, normal; T, tumor) after necropsy in mice subjected to sham or I/R with or without daily DNAse treatment. E and F, liver I/R resulted in a significant increase in tumor burden at 3 weeks as seen by liver-to-body ratio (E; 45% decrease with DNAse treatment after I/R; $P < 0.001$) and percentage hepatic replacement by metastatic tumor (F; mean percentage replacement 78% in I/R vs. 44% in I/R plus DNAse; $P = 0.02$). Treatment with DNAse after I/R resulted in a significant decrease in growth of already established micrometastases. Data represent mean ± SEM; $n = 6$ mice/group. The above data are each representative of three experiments with similar results. NS, not significant; ***, $P < 0.001$.

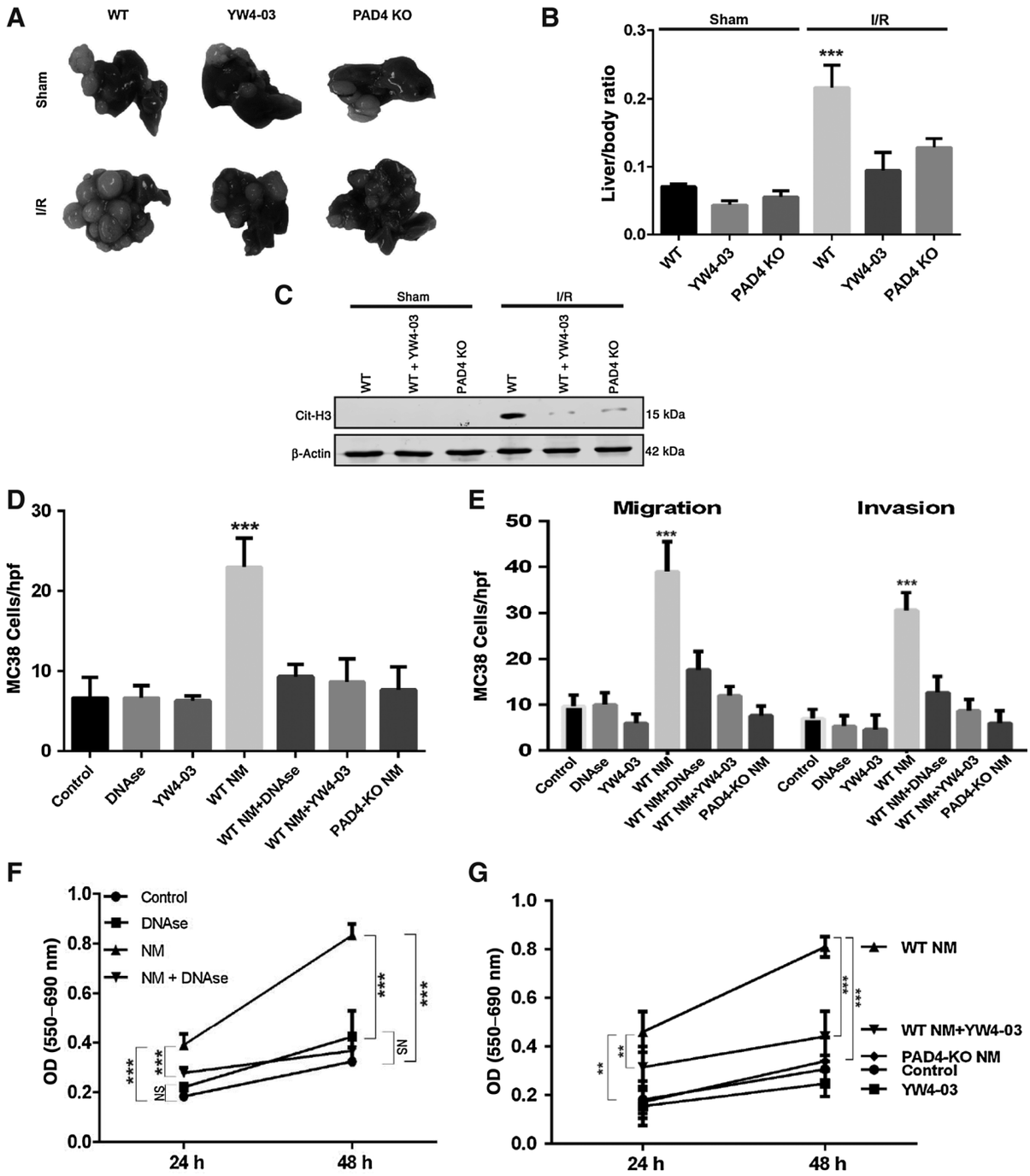

Figure 5.

DNAse and PAD4 targeting inhibit the protumorigenic effects of NETs. MC38 cancer cells were injected in the spleen of C57BL/6 wild-type (WT) mice or PAD4 knockout (KO) mice with or without liver I/R. In addition, a subgroup of WT mice was injected intraperitoneally three times/week with PAD4 inhibitor YW04-03. Mice deficient in PAD4 or WT mice treated with YW4-03 had a significant decrease in tumor burden at 3 weeks as demonstrated by the representative images (A) and the liver-to-body ratio (B) compared with WT mice after I/R. C, there was a significant decrease in NET formation in the tumors after I/R in PAD4-deficient mice and in mice treated with YW4-03 as evident by decrease in tumor cit-H3 levels. D, *in vitro*, CFSE-labeled MC38 cells demonstrated increased adhesion to neutrophil monolayers stimulated with PMA (100 nmol/L) compared with unstimulated neutrophils or neutrophils from PAD4-deficient mice (mean 19.1 MC38 cells/hpf vs. 5.7 MC38 cells/hpf vs. 6.2 MC38 cells/hpf; $P < 0.0001$). Addition of DNAse or YW4-03 results in adhesion levels comparable with control (mean 8.1 and 7.2 MC38 cells/hpf, respectively; $P < 0.0001$ compared with PMA treatment). E, MC38 cell migration through 8-μm PET membranes and invasion through Matrigel was significantly increased in the presence of media from PMA-stimulated neutrophils (NM) compared with MC38 alone. This was reversed back to control values with the addition of DNAse or YW3-04 to the stimulated media or when using stimulated media from PAD4-deficient mice. F and G, MTT assay show increased tumor proliferation at 48 hours after culturing MC38 cells with media from PMA-stimulated neutrophils but not from PMA-stimulated neutrophils from PAD4-deficient mice. Addition of DNAse or YW4-03 result in proliferation levels similar to control. Data are presented as mean ± SEM from $n = 3$ separate experiments. NS, not significant; ***, $P < 0.001$.

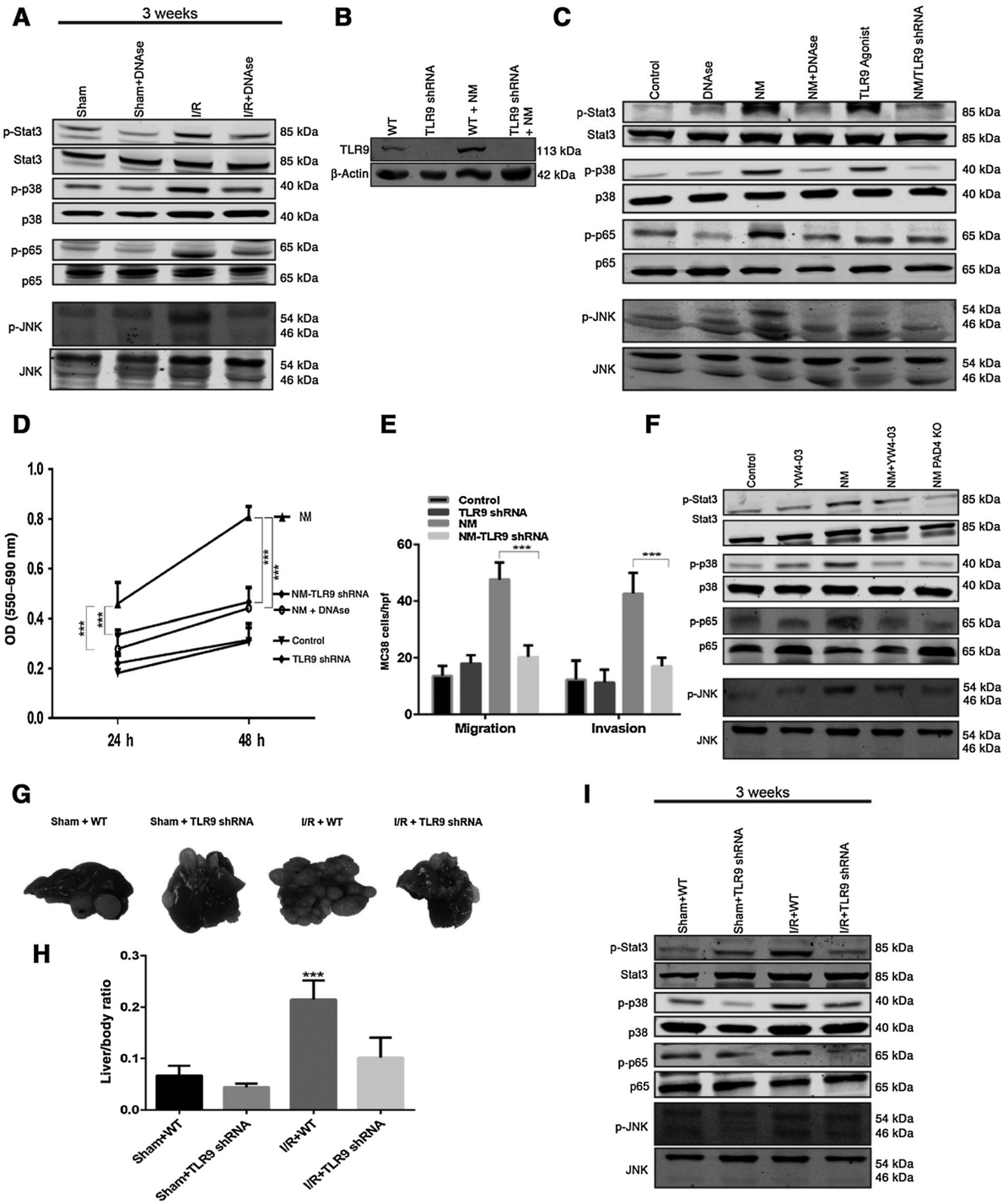

Figure 6.

NETs exert their protumorigenic effects through activation of TLR9 pathways. A, at 3 weeks, MAPK pathways were increasingly activated in the tumors obtained from mice that underwent I/R and tumor injection. B, *in vitro*, TLR9 expression was significantly increased after MC38 cancer cells were cultured in media derived from PMA-stimulated neutrophils (NM). C, the addition of PMA-stimulated neutrophils significantly increased the activation of the TLR9-associated MAPKs compared with control; this was similar to the addition of a TLR9 agonist (ODN 1668). The addition of DNAse to PMA-stimulated neutrophils resulted in phosphorylation levels similar to control. Furthermore, the addition of PMA-stimulated neutrophils had no effect in TLR9-knockdown MC38 cells. D and E, similarly, the addition of PMA-stimulated neutrophils did not affect tumor cell proliferation (D), migration (E), or invasion in TLR9 knocked down MC38 cells when compared with the effect of PMA-stimulated neutrophils in wild-type MC38 cells. F, the addition of PMA-stimulated neutrophils failed to activate TLR9 pathways when neutrophils were pretreated with YW4-03 or when using PAD4-deficient neutrophils. G and H, *in vivo*, mice injected with MC38 cells deficient in TLR9 had a significant decrease in tumor growth in response to I/R at 3 weeks as demonstrated by the representative images (H) and the liver-to-body ratio (I) compared with mice injected with WT MC38 cells. I, as expected, there was not an increase in MAPK pathway activation after I/R in TLR9 knocked down MC38 cells. *, $P < 0.0001$ versus control; **, $P < 0.001$; ***, $P < 0.01$ versus PMA-stimulated neutrophils. Data in C–E are presented as mean ± SEM from $n = 3$ separate experiments; *in vivo* experiments ($n = 6$/group; tumor protein lysate were obtained; each lane represents a separate animal). NS, not significant; **, $P < 0.01$; ***, $P < 0.001$.

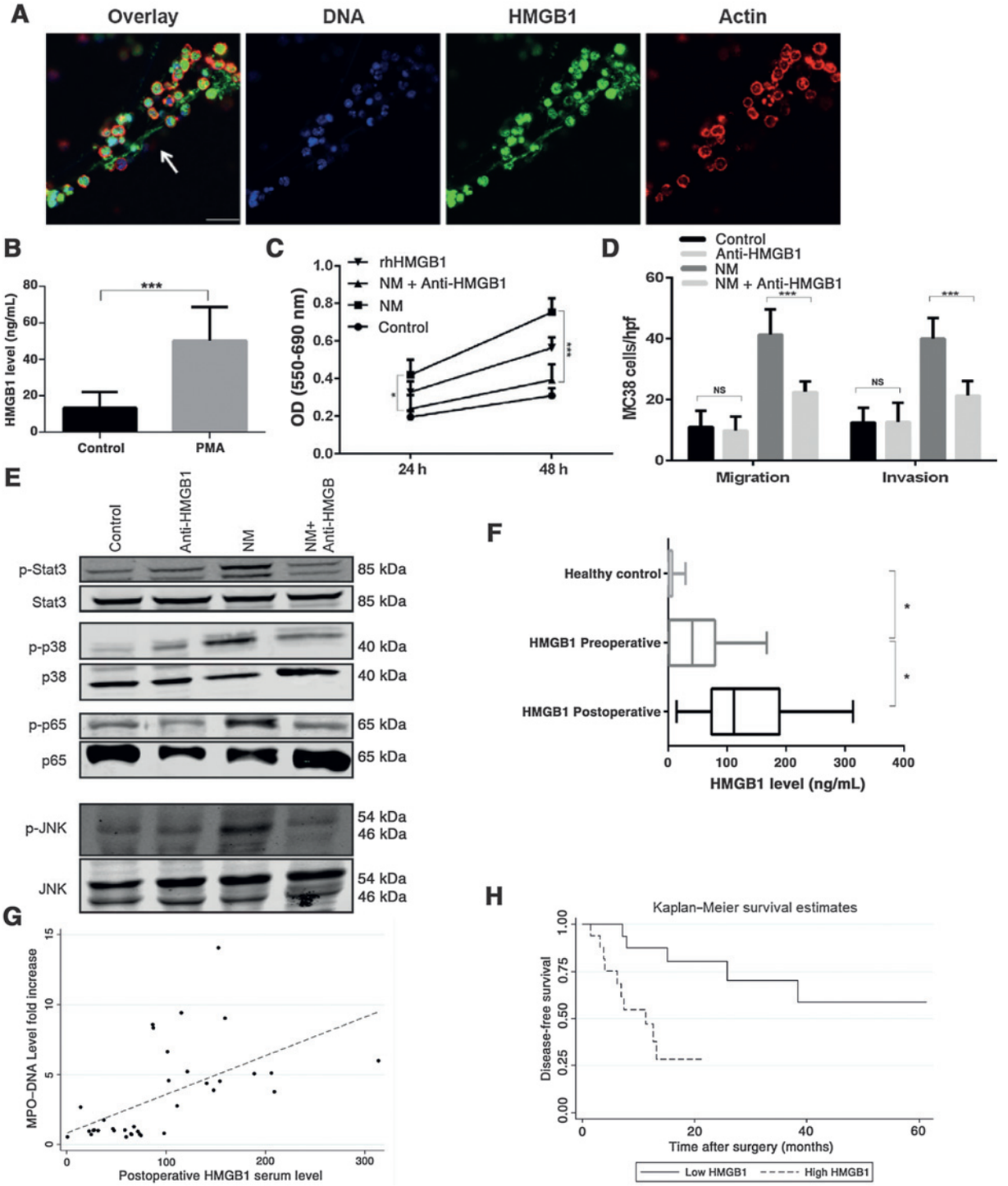

Figure 7.

HMGB1 released from NETs promote tumor progression and TLR9 activation. A, after PMA stimulation for 4 hours, confocal microscopy images of NETs revealed that HMGB1 is released and colocalizes with extruded DNA. Ly6G, red; nuclei, blue; HMGB1, green. B, using ELISA, HMGB1 levels were increased in the supernatant from PMA-stimulated neutrophils (NM) compared with unstimulated neutrophils. C and D, the addition of neutralizing HMGB1 antibodies substantially reversed the effect of PMA-stimulated neutrophils of MC38 cell proliferation (C), migration, and invasion (D). E, MC38 cells were cultured in media collected from PMA-stimulated neutrophils. The addition of neutralizing HMGB1 antibodies to the PMA-stimulated neutrophils reversed the activation of the MAPKs back to control level. Data are presented as mean ± SEM from $n = 3$ separate experiments. *, $P < 0.05$; ***, $P < 0.001$. F, detection of preoperative and postoperative serum HMGB1 levels in mCRC patients undergoing minor or major surgery ($n = 50$) or in healthy controls ($n = 20$). Box plots show higher HMGB1 levels in mCRC patients compared with healthy controls and higher HMGB1 levels postoperatively compared with preoperatively. G, postoperative HMGB1 levels correlated with increases in serum MPO–DNA levels. H, Kaplan–Meier disease-free survival curves of mCRC patients who underwent major liver resection based on serum HMGB1 levels.

(median, 11.3 ng/mL; $P < 0.001$; Fig. 7F). Similar to MPO–DNA levels, there was a significant increase in HMGB1 levels after major resection compared with minor liver resection (median, 99.8 vs. 46.1 ng/mL; $P < 0.001$; Supplementary Fig. S2). There was a significant strong correlation between postoperative MPO–DNA levels and HMGB1 levels (Spearman coefficient 0.7, $P < 0.001$; Fig. 7G). Finally, similar to MPO–DNA, high postoperative HMGB1 levels were associated with decreased disease-free survival ($P < 0.01$; Fig. 7H).

Discussion

For patients with mCRC, liver resection is the only available treatment modality that offers a significant chance of cure. However, it has long been recognized that surgical removal of malignancies may enhance the risk of tumor recurrence (5). There are few clinically applicable interventions to counteract this phenomenon as the exact mechanisms behind it remain poorly understood. This study is the first to implicate NETs as potential contributors to metastatic cancer progression in the context of surgical stress. We visualized NET formation in the liver after I/R, a model of surgical stress, and found that this was associated with a drastic increase in hepatic metastatic disease burden compared with nonsurgical controls. In addition, hypoxic tumor cells at the center of growing tumors further induced NET formation. Furthermore, we demonstrated that inhibition of NET formation using DNAse or PAD4 inhibitors had favorable oncologic outcomes in mice *in vivo*. Similar results were obtained when carrying out these experiments in PAD4-deficient mice. As liver I/R results in NET formation and NET inhibition reduces the establishment and growth of metastatic tumors, these results support a role for intrahepatic NETs in tumor progression. *In vitro*, NETs exerted their protumorigenic effect by activation of TLR9 growth signaling pathways in cancer cells, an effect partially related to released HMGB1 (Supplementary Fig. S3).

In this study, we demonstrated that NET formation was accelerated immediately after major liver resection in patients with mCRC. Thus, increased NET formation, as measured by circulating MPO–DNA levels, was associated with a significant increase in early metastatic recurrence. This is the first study to show evidence of NET formation in response to hepatic surgery and provide support for the idea that the environment generated after tumor removal can affect long-term cancer-related outcomes. Our results are in line with Zychlinsky and colleagues who assessed surgical resection specimens from eight pediatric patients with Ewing sarcoma. Of these patients, two had NET deposition in their resected tumor and those patients went on to develop early recurrence (16). There is also indirect evidence that elevated levels of circulating-free DNA and nucleosomes in the serum of patients with solid tumors are associated with adverse outcomes such as reduced disease-free and overall survival (32–34). However, in most of the studies tumor cells were thought to be the predominant source of the circulating DNA and nucleosomes. Our study reveals that a substantial portion of the circulating DNA is neutrophil-derived and consistent with increased NET formation after surgical stress. This suggests that MPO–DNA levels may serve as a prognostic marker for long-term outcomes in patients undergoing oncologic resections or as a measure of surgical stress.

Tumor growth itself induces a state of hypoxia, leading to a vicious cycle of NET formation and tumor progression. We have previously shown that hypoxia is one mechanism by which hepatocytes can sustain NET formation (14). Similarly, we show here, that hypoxic cancer cells are able to induce NET formation likely through the release of several inflammatory signals. This effect was more pronounced when neutrophils were treated with media from necrotic cancer cells, which could be the result of the higher release of NET-inducing signals from necrotic cancer cells compared with hypoxic cancer cells. Demers and colleagues have also demonstrated that hematologic and solid tumors can predispose circulating neutrophils to form NETs. The effect was attributed to the release of granulocyte-colony stimulating factor, a known inducer of NETs, by the tumor cells (17).

In the current study, we show that NETs can contribute to metastatic tumor growth by either enhancing establishment of metastatic foci or promoting the growth of existing micrometastatic disease. We cannot yet distinguish between the many mechanisms involved as NETs seem to facilitate cancer cell adhesion as a first step toward the development of metastatic disease and also facilitate migration, invasion, and proliferation during the establishment of metastatic foci. NETs can enhance all these processes and DNAse and PAD4 inhibition abolished all of these effects. Because NETs are composed of neutrophil-derived DNA studded with proteins, it was of no surprise that TLR9 in the cancer cells orchestrated tumor progression in response to NETs. TLR9 promotes tumor growth by activation of a cascade of intracellular growth signaling pathways including MAPK pathways that control fundamental cellular processes such as growth, proliferation, differentiation, and migration. The JNK family, Stat family, and p38 isoforms are strongly activated by environmental stresses and inflammatory cytokines, which are encountered in the setting of surgical stress and contribute to cancer cell growth (35, 36). In addition, NF-κB signaling can lead to suppression of apoptosis in response to stress and continue to proliferate (37).

In addition to directly interacting with cancer cells, NETs can cause changes in the microenvironment that promote tumor establishment and growth. We have previously shown that NETs play an important role in exacerbating liver I/R injury and DNAse and inhibition of PAD4 significantly reduces inflammation (14). Furthermore, NETs can induce Kupffer cells, in the setting of surgical stress, to release multiple inflammatory cytokines and chemokines including IL6 and TNFα, and CXCL-10 (14). All of these factors have been shown to promote cancer progression. There is abundant evidence to suggest that IL6 is associated with tumor progression through inhibition of tumor cell apoptosis, stimulation of angiogenesis, and drug resistance (38). The effects of IL6 are mainly mediated through Stat3, which we show is overexpressed in cancer cells in response to NETs. Similarly, TNFα and CXCL10 possess a wide array of protumorigenic effects (39, 40). Therefore, DNAse and PAD4 inhibition also alleviates metastasis by inhibiting the NET-induced inflammatory mediator storms provoked by surgical stress.

The data presented thus far provide strong evidence that NETs promote metastatic tumor growth in the liver after surgical stress. The mechanisms underlying these observations deserve study. We show here that HMGB1 is released by neutrophils during degranulation and that HMGB1 is postulated to assist in the recognition of extracellular DNA by intracellular TLR9 to activate the protumorigenic pathways (30, 31). In addition, we, among others, have shown that HMGB1 is implicated in nearly every step of tumor progression (24, 41, 42). In addition to HMGB1, other peptides released during NETosis may play a role in tumor progression. These proteins may include MMP-9, cathelicidins, cathespin G,

and neutrophil elastase, which have all been shown to be released during NET formation and have been extensively studied in the field of tumor progression albeit independent of NETs. MMP-9 has been implicated in tumor angiogenesis and invasion (43) as has NET formation in our study. Nicoud and colleagues have also demonstrated that MMP-9 promotes outgrowth of colorectal carcinoma micrometastases after liver I/R injury, although the source of MMP-9 was not examined in that study (7). Cathelicidins are upregulated in several types of human tumor tissues and have recently emerged as novel modulators of tumor growth, metastasis, and carcinogenesis of various types of cancers (44). Furthermore, both neutrophil elastase and cathespin G have been shown to directly enhance tumor cell proliferation and migration (45). Therefore, with the release of all the protumorigenic proteins, NETs act as potent "fertilizers" enriching the liver microenvironment to foster metastatic disease growth. Because DNAse reverses many of the protumorigenic effects of NETs, one may speculate that the effect of DNA is both a direct stimulatory effect through TLR9 signaling and DNA also acting as a scaffold, allowing the attachment of protumorigenic proteins to DNA–protein complexes, which may be necessary for their protumorigenic functions.

As surgery remains the appropriate and only potential cure for patients with metastatic disease, we aimed to provide better understanding of the mechanisms of surgery-enhanced tumor recurrence. New therapeutic strategies that prevent tumor recurrence after surgery need to be explored because the perioperative period is a window of opportunity to counteract the protumorigenic inflammatory storm induced by surgery and ultimately improve long-term outcomes. Such putative therapy should be designed not to affect healing or reduce anti-infective host defense. The use of DNAse to inhibit NETs is a promising approach for potential clinical application. Interestingly, recombinant human DNAse (rhDNAse) has been previously used in a randomized, placebo-controlled trial in patients with autoimmune systemic lupus erythematosus disease (46). rhDNAse administration was well tolerated without significant adverse events. Our findings provide preliminary evidence that perioperative administration of DNAse may reduce the risk of recurrence in patients with metastatic cancer undergoing resection and will hopefully pave the way for future clinical trials.

Disclosure of Potential Conflicts of Interest

K. Mowen is a Director of Biology in Padlock Therapeutics; reports receiving a commercial research grant from Janssen and other commercial research support from Padlock Therapeutics; and is a consultant/advisory board for Padlock Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S. Tohme, Y. Wang, A. Tsung
Development of methodology: S. Tohme, H.O. Yazadani, P. Lougharn, H. Huang, A. Tsung
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Tohme, A.B. Al-Khafaji, P. Lougharn, K. Mowen, H. Huang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Tohme, H.O. Yazadani, A.B. Al-Khafaji, A.P. Chidi, P. Lougharn, Y. Wang, R.L. Simmons, H. Huang, A. Tsung
Writing, review, and/or revision of the manuscript: S. Tohme, A.B. Al-Khafaji, A.P. Chidi, Y. Wang, R.L. Simmons, H. Huang, A. Tsung
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.O. Yazadani, R.L. Simmons
Study supervision: A. Tsung
Other (offered key reagents): Y. Wang

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