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Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis

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Introduction
Cancer remains a devastating cause of mortality worldwide, with the majority of patients dying as a result of metastasis (1–3). Currently, locoregional control in the form of complete oncologic resection remains an essential curative modality for nearly all solid tumors and provides improved overall and disease-free survival (2, 4, 5). Control of distant recurrence is predominantly achieved through systemic chemotherapy, with variable results (6–8). However, standard oncologic interventions can have negative consequences. First, manipulation of the primary tumor during surgery is associated with increased numbers of circulating tumor cells (CTCs) (9). Second, infectious complications occur as a result of cancer progression itself, such as bowel obstruction or pneumonia (10, 11), and due to complications of standard cancer treatments, such as chemotherapy and surgery (12–14).

Postsurgical infections occur with alarming frequency, with an incidence approaching 40% in some series (15–17). Given that the majority of the nearly 2 million patients diagnosed with cancer in 2012 in the United States alone underwent at least one surgical procedure, the tremendous potential burden of infection becomes apparent (18). One disturbing feature of severe infectious complications in patients with cancer is their association with adverse oncologic outcomes independent of the morbidity associated with the infectious insult (14, 19–21). This phenomenon has been observed across a broad range of malignancies, including lung, esophageal, breast, ovarian, and colorectal cancer, whereby severe postoperative infectious complications, such as pneumonia, pneumonia, and sepsis, are significantly associated with an increased rate of death from metastatic disease (21–25).

The cellular players underlying this observation are only beginning to be elucidated. What is becoming apparent is that tumor-neutrophil interactions play an important role in linking infection, inflammation, and metastasis (3, 26–31). Neutrophils are the predominant circulating granulocyte in humans and comprise 50%–75% of circulating leukocytes (32). They function as the first line of defense against infections, such as those seen in the postsurgical period, and are being increasingly recognized as important actors in cancer progression (3, 27, 32). While antitumor effects have been demonstrated, neutrophils also appear to support the development of metastatic disease under certain conditions (27, 28, 30, 31).

Clinical studies suggest that elevated numbers of circulating neutrophils are an independent marker of adverse prognosis in patients with cancer (26). This has been demonstrated in several malignancies, including lung and gastric cancers (26, 33, 34). Experimental evidence supports some of these findings. Systemic inflammation induced by lipopolysaccharide administration results in increased adhesion of CTCs within hepatic sinusoids. Neutrophil depletion abolishes this response, which translates into a decrease in overall gross hepatic metastasis formation. This is reversed by polymorphonuclear leukocyte (PMN) reinfusion, providing strong evidence for the involvement of neutrophils in this process. Similarly, neutrophils have been shown to stabilize adhesion of CTCs to pulmonary microvasculature and facilitate gross pulmonary metastasis formation (3, 30).

The basic mechanisms by which neutrophils may act to promote the development of metastasis are diverse. Contact-dependent mechanisms, whereby neutrophils act as a bridge, tether-
ing CTCs to end-organ endothelium, have been described (19). This interaction is mediated by the interaction of β2 integrins on neutrophils and ICAM-1 on tumor cells (31, 35–37). Additional contact-independent mechanisms have also been proposed. Neutrophils are able to secrete soluble factors that can activate endothelium and parenchymal cells, enhancing CTC adhesion in distant sites (27, 38–40).

In addition to the mechanisms proposed thus far, novel aspects of neutrophil biology may contribute to cancer progression and metastasis. Neutrophil extracellular traps (NETs) are neutrophil-derived structures composed of extruded DNA, decorated with antimicrobial proteins (41–45). NETs are formed in response to infectious stimuli and serve as a host defense against pathogens, trapping and killing bacterial, fungal, and protozoan invaders in vitro (41, 46, 47). In addition, NETs have been shown to effectively trap circulating bacteria within the liver in experimental models of sepsis (48). In contrast to their postulated beneficial role, exaggerated NET formation is implicated in a number of pathologies, including the multiorgan dysfunction that may accompany sepsis (12, 49, 50). Given the potential association between severe infec-

Figure 1
CLP results in widespread deposition of extracellular DNA, which colocalizes with neutrophils and expresses neutrophil-derived granules. (A) Under anesthesia, murine livers were externalized and imaged using SD-IVM, permitting real-time visualization of neutrophil trafficking and DNA extrusion. Intravascular administration of E-fluor 660 anti-GR1, Sytox Green, Alexa Fluor 555 anti-H2AX, or anti-NE was used to visualize polymorphonuclear neutrophils (PMN, blue), DNA (green), H2AX (red), and NE (yellow), respectively. DNA is visualized adjacent to PMN within hepatic sinusoids. DNA stains positive for histone H2AX and NE, in keeping with what has been described for NETs (48). Scale bars: 40 μm. Extracellular DNA was quantified within (B) hepatic sinusoids and (C) pulmonary capillaries by measuring total area of fluorescence per hpf over 5 hpf. Data are represented as relative area of fluorescence compared with sham. CLP was associated with increased amounts of extracellular DNA compared with sham. Neutrophil depletion or systemic administration of DNase 1 or NEi after CLP results in decreased extracellular DNA staining compared with CLP alone. Data are presented as mean ± SEM from n = 3–5 mice per group. ***P < 0.001, *P < 0.05 versus sham as determined by 1-way ANOVA with Tukey’s HSD post-hoc analysis. See also Supplemental Figures 1 and 2.
Sepsis induced by cecal ligation and puncture results in widespread deposition of NETs. In the setting of disseminated infection, bacteremia, and sepsis, neutrophils demonstrate NET formation in vivo (48). Cecal ligation and puncture (CLP) invariably results in polymicrobial abdominal sepsis and systemic sepsis, which are characteristic features of postoperative infections following gastrointestinal surgery in humans. Accordingly, it was selected in lieu of systemic administration of individual bacterial components, such as LPS, to induce systemic infection (51, 52). In order to determine whether CLP induces NET formation, multichannel spinning disk confocal intravital microscopy (SD-IVM) was used to visualize hepatic and pulmonary microvasculature within mice subjected to CLP (Figure 1 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI67484DS1).

Results

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NETs are composed of extracellular DNA decorated with neutrophil-derived granules and modified histones (48). Visualization of extracellular DNA was performed by intravascular injection of the cell impermeant dye, Sytox Green. This revealed web-like DNA within hepatic sinusoidal spaces and pulmonary capillaries (Figure 1 and Supplemental Figure 1). To further support the claim that these extracellular webs represent NETs, we demonstrated colocalization of DNA with neutrophil-derived granules. This was achieved by intravascular administration of fluorescently labeled antibodies against polymorphonuclear neutrophils (PMN efluor 660 anti-GR1), histone H2AX (Alexa Fluor 555 anti-H2AX), neutrophil elastase (NE) (Alexa Fluor 555 anti-NE), and Sytox Green (Figure 1A and Supplemental Figure 1).

Quantification of microvascular DNA was subsequently performed in mice subjected to CLP, in mice subjected to neutrophil depletion 24 hours prior to CLP, and in mice administered DNase 1 or NE inhibitor (NEi) after CLP. Relative area of fluorescence was determined in each high-power field (hpf) of view, expressed relative to mice subjected to sham surgery alone (relative area of fluorescence = 1; Figure 1, B and C). Significantly more microvascular Sytox Green staining was observed in mice subjected to CLP compared with mice subjected to sham surgery (10.3 and 5.7 relative area of fluorescence in liver and lungs, respectively, \(P < 0.001 \) compared with sham). Neutrophil depletion (0.74 and 0.2 relative area of fluorescence in liver and lungs, respectively; \(P < 0.001 \) compared with sham). Neutrophil depletion (0.74 and 0.2 relative area of fluorescence in liver and lungs, respectively; \(P < 0.001 \) compared with sham) also reduced metastatic nodules (1.52 and 0.74 relative area of fluorescence in liver and lungs, respectively; \(P = NS \) compared with sham) (Figure 2A and C). This was associated with levels of NET deposition comparable to those after sham surgery (Figure 2B).

Systemic sepsis promotes the development of gross metastasis, which is attenuated by systemic administration of inhibitors of NET formation. Having shown that CLP-induced sepsis results in widespread microvascular NET deposition, we sought to establish an association between NET formation and metastasis. Twenty-four hours after performing CLP or sham surgery, H59 Lewis lung carcinoma cells (referred to as H59 cells hereafter) were injected via the intrasplenic route. CLP animals were either left untreated or received DNase 1 (2.5 mg/kg) intramuscularly or a NEi per os daily (2.2 mg/kg). Both of these reagents are known inhibitors of NET formation and were used to determine whether disrupting...
NET formation would alter the course of hepatic tumor progression (53, 54). Quantification of gross hepatic metastases was performed at necropsy at 2 weeks (Figure 2). Significantly more gross hepatic metastases were observed in CLP mice compared with mice after sham surgery (mean 400 nodules versus 2 nodules; \(P < 0.0001\)) (Figure 2B). Systemic administration of DNase 1 or NEi after CLP attenuated the development of hepatic metastases at 2 weeks compared with administration of CLP alone (mean 234 and 132 nodules, respectively; \(P < 0.05\) and \(P < 0.01\)) (Figure 2B).

**Figure 3**

Tumor cell adhesion after CLP is augmented by trapping within neutrophil-derived extracellular DNA. In order to demonstrate that tumor cells become embedded within NETs, SD-IVM was performed permitting visualization of (A) hepatic sinusoids in living mice and (B) pulmonary capillaries ex vivo within 10 minutes. Images shown in A and B represent a single 3-dimensional reconstruction of confocal z-stacks (10- to 20-μm thickness, 1-μm intervals), rotated 180° from an inferior to a superior perspective. In both liver and lungs, tumor cells (red) were found to arrest within extracellular chromatin (Alexa Fluor 555 anti-histone H2AX [green]) adjacent to neutrophils (E-fluor 660 anti-GR1 [blue]). Scale bars: 20 μm.

See also Supplemental Video 1 and Supplemental Figure 3. (C) Quantification of adhesion of H59 cells within hepatic sinusoids. \(3 \times 10^4\) cells were injected via the spleen 24 hours after CLP or sham surgery. Adhesion was increased after CLP compared with sham. Systemic administration of DNase 1 or NEi starting 1 day prior to CLP abolished this increase. (D) Quantification of B16 melanoma cells within pulmonary capillaries. \(1 \times 10^5\) cells were injected via tail vein 24 hours after CLP or sham. Adhesion was increased after CLP compared with sham and was abolished by administration of DNase 1 or NEi starting 1 day prior to CLP. Quantification was performed by counting the number of cells per hpf in 8 to 10 hpf (×20) per experiment. Data are presented as mean ± SEM from \(n = 5\) mice per group. ***\(P < 0.001\) versus sham, DNase, and NEi as determined by 1-way ANOVA with Tukey's HSD post-hoc analysis.

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CLP is a potent inflammatory stimulus, which may exert numerous systemic effects extending beyond NET formation (51, 52). Accordingly, we sought to determine whether systemic LPS administration, an established model of microvascular NET deposition in vivo, could produce comparable levels of tumor cell adhesion to those after CLP administration (43). Quantification of H59 cells within hepatic sinusoids 4 hours after intraperitoneal LPS administration yielded comparable results. LPS injection resulted in significantly increased tumor cell adhesion to hepatic sinusoids compared with controls (mean 2.34 cells per hpf versus 0.42 cells per hpf, respectively; \( P < 0.001 \)). Systemic administration of DNase or NEi in mice subjected to LPS stimulation resulted in levels of tumor cell adhesion comparable to those of mice after sham surgery (mean 0.37 cells per hpf and 0.55 cells per hpf; \( P < 0.001 \)), suggesting that the stimulatory effects of PMA alone were not responsible for the observed adhesive phenotype. Thus, while intact NETs do not appear to be critical for tumor cell adhesion to take place, their presence appears to significantly augment this process.

Figure 4

NET production by neutrophils is sufficient to increase tumor cell adhesion within hepatic sinusoids. (A) A schematic representation of the experimental design is depicted. All mice used were subjected to neutrophil depletion via intraperitoneal injection of anti-GR1 (150 \( \mu \)g). Twenty-four hours later, \( 1 \times 10^8 \) bone marrow–derived neutrophils (or control buffer) from syngeneic mice were reinfused via the intrasplenic route. This was followed by injection of \( 3 \times 10^3 \) H59-GFP cells into the spleen 20 minutes later. Ten minutes later, epifluorescence microscopy was used for quantification of adherent cells. IVM, intravital microscopy. (B) Quantification of arrested tumor cells within hepatic sinusoids 10 minutes following intrasplenic injection of H59-GFP cells. Injection of 500 nM PMA without neutrophils prior to tumor cell injection resulted in low levels of tumor cell adhesion. Infusion of unstimulated neutrophils increased tumor cell adhesion above control, but this was significantly lower than when neutrophils were pretreated with 500 nM PMA. This increase is abrogated if PMA treatment of neutrophils occurs in the presence of NEi (10 \( \mu \)M) or DNase 1 (1,000 U) prior to reinfusion. Adherent cells were quantified by counting the number of cells per hpf in 8 to 10 hpf (\( \times \)20) per experiment. Data are presented as mean ± SEM from \( n = 4–5 \) mice per group. Significance was determined using 1-way ANOVA with Tukey’s HSD post-hoc analysis. ***\( P < 0.0001 \), #\( P < 0.05 \) compared with control.
In order to more closely approximate physiologic conditions, we sought to determine whether tumor cell adhesion to NETs is maintained under conditions of flow. This was achieved using a parallel-plate flow chamber, in which H59 or A549 cells were perfused over neutrophil monolayers at a rate of 1 dyne/cm/s⁻¹. Addition of PMA (800 nM) to the tumor cell perfusate resulted in rapid formation of NETs, resulting in a significant increase in tumor cell adhesion compared with controls (mean 75.6 H59 and 56.9 A549 cells per hpf versus 5.4 H59 and 5.7 A549 cells per hpf; P < 0.001) (Figure 5B). Adhesion was significantly reduced by addition of DNAse 1 (1,000 U) to the perfusate or by pretreatment of neutrophils with NEi (5 μM; P < 0.001 versus PMA alone) (Figure 5B).

Real-time video acquisition of H59 cells perfused over neutrophil monolayers was performed in order to visualize tumor cell entrapment within NETs. Addition of 800 nM PMA to the tumor cell perfusate resulted in the rapid extrusion of NETs, as visualized by Sytox staining. Extrusion of extracellular DNA was accompanied by the rapid arrest of tumor cells. Addition of 1,000 U DNase 1 to the perfusate resulted in a dramatic detachment of adherent cells in conjunction with a loss of extracellular DNA (data not shown).

Tumor cell–NET interactions in vitro were visualized using confocal microscopy (Figure 5C). After PMA stimulation of neutrophil monolayers, clusters of A549 cells were visualized within webs of extracellular DNA (green) in proximity to neutrophils (blue). (D) Scanning electron microscopy of A549- and PMA-stimulated neutrophils (800 nM) (original magnification, ×3,000). After PMA stimulation, neutrophils flatten (white arrow) and extrude strands consistent with NETs. Strands encompass a cluster of adherent tumor cells (black arrow). (E) Scanning electron microscopy demonstrates that NETs are in direct contact with adherent A549 tumor cells (original magnification, ×3,500). Scale bars: 40 μm (confocal microscopy); 5 μm (electron microscopy). See also Supplemental Figures 2 and 3.
extracellular DNA or inhibition of NET extrusion, as previously described (Supplemental Figure 6, A and B, and refs. 53, 54). To ensure that the differences in adhesion were not due to differences in neutrophil numbers after treatment with DNAse 1 or NEi, this was quantified directly. PMA stimulation increased adhesion of neutrophils to coverslips. However, numbers were unaffected after treatment with DNAse 1 or NEi (Supplemental Figure 6C). Tumor cell trapping within NETs is associated with increased micrometastases at 48 hours. We have previously shown that early adhesive events are important in the development of gross metastatic disease (30). In the context of this study, we sought to determine whether the increased adhesion observed in animals with CLP-induced sepsis translated into a greater number of micrometastases compared with CLP alone. (B) Representative images of tumor cells trapped within hepatic sinusoidal spaces in mice subjected to sham surgery, CLP, and NEi after CLP at 24 and 48 hours following intrasplenic injection of \(5 \times 10^5\) H59 cells. Scale bars: 40 μm. (C) To further demonstrate that adherent tumor cells persist and are able to grow in this model of sepsis, individual tumor cells (as depicted in B) were quantified within the liver at 24 and 48 hours after intrasplenic injection. Quantification of individual tumor cells within hepatic sinusoids reveals increased numbers at both 24 and 48 hours after intrasplenic injection in mice subjected to CLP compared with sham. Systemic administration of NEi after CLP resulted in decreased numbers of tumor cells at both 24 and 48 hours compared with CLP alone. Data were acquired using \(n = 3–5\) mice per group. Quantification was performed by counting the number of cells per 5–10 hpf per experiment (×20). *\(P < 0.05\), ***\(P < 0.0001\) compared with control, DNAse, and sham. Significance was determined using 1-way ANOVA with Tukey’s HSD post-hoc analysis.

In order for micrometastatic foci to form, adherent cells must persist within the microvasculature. In an attempt to demonstrate this, SD-IVM was used to quantify the number of individual adherent H59 cells within the liver at 24 and 48 hours after intrasplenic injection (Figure 6B and C). In mice subjected to sham surgery, few entrapped cells were visualized at 24 hours and no increased number in adherent cells was observed at 48 hours (mean 0.76 cells per hpf at 24 hours, 0.82 cells per hpf at 48 hours; \(P = NS\)). Taken together, these results suggest that tumor cell entrapment within NETs promotes stable retention, permitting subsequent growth within the liver (Figure 6A).
Liver at 24 and 48 hours was observed compared with that in mice after sham surgery (mean 2 cells per hpf at 24 hours and 1.5 cells per hpf at 48 hours; P = NS) (Figure 6C). NETs stimulate migration and invasion of human lung carcinoma cell lines in vitro. The demonstration that entrapment within NETs is associated with tumor cell adhesion and subsequent micrometastasis and macrometastasis formation implies that NETs may stimulate migration and invasion. We sought to test this directly in vitro (Figure 7). In the presence of neutrophils alone, a slight but non-significant increase in migration of A549 cells through PET membranes was appreciated compared with A549 cells alone (mean 3.6 cells per hpf versus 7.5 cells per hpf; P = NS) (Figure 7A). Addition of 25 nM PMA resulted in a greater than 3-fold increase in migration (mean 17.8 cells per hpf; P < 0.01 versus neutrophil plus A549) (Figure 7A). However, addition of PMA to A549 cells in the absence of neutrophils failed to induce migration to the same extent (mean 4.8 cells per hpf versus A549 alone; P = NS) (Figure 7A). Migration of A549 cells in the presence of PMA and neutrophils was abrogated by the addition of 1,000 U DNAse 1 or pretreatment of neutrophils with 5 μM NEi (mean 3.6 and 3.9 cells per hpf, respectively, versus A549 alone; P = NS) (Figure 7A).

Similarly, in the presence of neutrophils alone, there was no appreciable increase in invasion of A549 cells through endothelial monolayers and PET membranes compared with A549 cells alone (mean 4.4 versus 3.3 cells per hpf; P = NS) (Figure 7B). Conversely, addition of 25 nM PMA resulted in a greater than 4-fold increase in invasion (mean 15.1 cells per hpf versus control; P < 0.001) (Figure 7B). However, addition of PMA to A549 cells in the absence of neutrophils failed to induce invasion at levels above control (mean 5.2 cells per hpf; P = NS versus control) (Figure 7B). Invasion of A549 cells in the presence of PMA and neutrophils was abrogated by the addition of 1,000 U DNAse 1 or pretreatment of neutrophils with 5 μM NEi (mean 4.8 and 2.4 cells per hpf, respectively; P = NS versus control) (Figure 7B). Taken together, these results support the role of intact NETs as the agents responsible for enhanced tumor cell migration and invasion in vitro.

**Discussion**

Surgical resection of solid tumors confers improved survival in appropriately selected patients. However, patients who suffer severe postoperative infections are more likely to die of metastasis than those who do not (20, 22, 24, 25). Mitigating the impact of severe infectious complications is not feasible in current clinical practice, in part because of the limited understanding of the biological processes linking infection and tumor progression. Clinical and experimental evidence suggests that activated neutrophils may facilitate metastatic progression in the context of systemic infection (26, 30, 33, 34). Accordingly, therapeutic strategies aimed at modifying tumor neutrophil interactions may one day maximize the therapeutic benefit of surgery in this important cohort of patients.

NETs are formed in response to infectious stimuli and constitute a recently described addition to the antimicrobial armamentarium of neutrophils (41). They have been observed to trap pathogens in vitro and in vivo (44, 46, 48). In this study, we provide evidence suggesting that NETs are able to trap CTCs in an analogous manner in the context of severe postoperative sepsis, thus promoting early adhesion of tumor cells to distant organ sites.

Severe postoperative infections are characterized by a sustained disseminated polymicrobial infection with representative flora derived from the skin or operative site (55). For example, following gastrointestinal surgery complicated by leakage of enteric...

**Figure 7**

Migration and invasion of A549 cells in vitro is promoted in the presence of intact NETs. In the presence of neutrophils exposed to PMA, (A) A549 cell migration through 8-μm PET membranes and (B) invasion through endothelial monolayers was increased compared with A549 cells alone, A549 cells in the presence of unstimulated neutrophils, and A549 cells stimulated with PMA (25 nM) in the absence of neutrophils. This phenotype was reversed back to control levels after addition of DNase 1 (1,000 U) or pretreatment of neutrophils with NEi (5 μM). Data are presented as mean ± SEM from n = 2–4 separate experiments. **P < 0.01, ***P < 0.001 versus A549 alone. *P < 0.05 versus A549 plus neutrophils and PMA. Significance was determined using 1-way ANOVA with post-hoc multiple comparisons with Tukey’s HSD post-hoc analysis.
Despite the significant clinical benefits of neutrophils, patients may manifest clinical sequelae ranging from intra-abdominal abscess to fulminant peritonitis, with cultured organism reflecting an intestinal origin. Because neoplastic disease progression in humans appears to be influenced predominantly by severe, as opposed to any septic, complication, selecting an experimental model that reproduces these features was considered imperative. CLP closely approximates the severe septic complications that can occur after a number of gastrointestinal surgeries, including colorectal, gastric, and esophageal oncolytic resections (13, 20, 56). By definition, CLP results in the rapid onset of polymicrobial sepsis with endogenous flora. Furthermore, CLP has been validated as a robust and reproducible septic stimulus (51, 52, 57, 58). Consequently, it was selected in lieu of experimental models of sepsis relying on a single microorganism or bacterial component (e.g., LPS).

Although the mechanism of bacterial dissemination following CLP is susceptible to exogenous pharmacologic inhibition with DNase and NEi. Both DNase and inhibition of NE have been shown to abrogate NET formation both in vitro and in vivo (48, 53, 59, 61–63). DNase is thought to act via degradative pathways, which promote microvascular coagulation. The possibility of NETs as a novel neutrophil-dependent adhesive mechanism is widely applicable to various cancer sites and target organs (liver and lung), suggesting that this novel metastatic mechanism is applicable to various cancer sites and target organs. Early adhesive events after tumor dissemination are an important precursor to the development of metastatic disease (3, 30, 35, 65). Consistent with our hypothesis that NETs are able to trap CTCs, we visualized tumor cell arrest within areas of histone staining, which was consistent with that in NETs adjacent to neutrophils (48). These findings were consistent across cell lines and host metastatic organs (liver and lung), suggesting that this novel metastatic mechanism is applicable to various cancer sites and target organs.

Sepsis in vivo is associated with the deposition of cytotoxic histones, which promote microvascular coagulation. The possibility that tumor cell trapping in this context is due to passive retention thus arises. Video analysis demonstrates unimpeded trafficking of neutrophils through sinusoidal spaces after tumor cell retention. In addition, red blood cells were visualized passing through vascular spaces, which were stained densely for NETs. These observations argue against passive tumor cell retention as the sole adhesive mechanism. While a detailed examination is beyond the scope of this study, these observations raise the possibility that NET-mediated tumor cell trapping is specific.

Neutrophils support early adhesion through a number of mechanisms (35, 65). We have previously demonstrated that neutrophil depletion abrogates both early adhesion of malignant cells within the liver and the development of microscopic hepatic metastases (30). However, our study is the first to implicate NETs as a novel neutrophil-dependent adhesive mechanism. This is highlighted by the fact that hepatic tumor cell arrest in neutrophil-depleted mice is greatly enhanced if tumor cell administration is preceded by reinjection of neutrophils stimulated with PMA. At the doses used in this study, PMA has been shown to result in NET formation by neutrophils (41, 53). This phenotype is reversed by presumed NET disruption with either DNase 1 or NEi prior to neutrophil reinjection. Because mice in all groups differed only in the manner in which the reinjected neutrophils were treated, the observed phenotype can more confidently be attributed to the adhesive action of NETs on tumor cells rather than the systemic effects of inflammation or the pharmacologic agents used.

In order to further support an adhesive interaction between NETs and neoplastic cells, an in vitro adhesion assay comprising only neutrophils and either murine (H59) or human (A549) lung cancer cell lines was used. Stimulation of neutrophils with PMA produced a significant increase in tumor cell adhesion compared with that of controls. This adhesion was robust and persisted at physiologically relevant shear stresses. However, as in the in vivo experiments, this was abolished by the addition of DNase 1 or pretreatment of neutrophils with NEi. This effect was identical in assays using both human and murine cancer cells, arguing against a cell-specific phenomenon.

Confocal imaging in vitro demonstrated clusters of cells embedded within DNA webs adjacent to but not in direct contact with neutrophils. This finding was corroborated by scanning electron microscopy images demonstrating intimate contact between NETs and adherent tumor cells. After adhesion in distant organ sites, tumor cells must be able to survive and grow in order to form metastases (31). In this study, adhesion in vivo was persistent, with mice subjected to CLP exhibiting significantly greater numbers of hepatic micrometastases at 24 and 48 hours compared with controls. In keeping with the
results thus far, this phenotype was abolished by treatment with DNAsase or NEi after CLP, again implicating NETs as players in this process. The demonstration that trapped tumor cells are able to survive and ultimately go on to form gross metastatic nodules contrasts with reports demonstrating that NETs are cytotoxic to epithelial and endothelial cells (49, 66). Treatment of endothelial and epithelial cells with isolated NETs resulted in 40% to 80% cytototoxicity. However, cell death was not complete, with a substantial proportion (20%) remaining viable and able to form colonies. Furthermore, NET disruption with DNAsase 1 or NEi failed to rescue cells from these cytotoxic effects, which were attributed predominantly to histones (49).

With regard to our study, it appears that some trapped tumor cells do survive NET sequestration. In addition, because our interventions (DNAsase 1 and NEi) only affect NET quantity but not cytototoxicity, the differences in the numbers of micrometastases observed likely reflect differences in initial adhesion and not cell viability.

In order for adherent cells to effectively grow in distant organ sites, migration and invasion through endothelium is required (31, 67). The in vivo data presented suggest that NETs may facilitate these processes during sepsis, as adherent cells ultimately go on to form micrometastases and macrometastases. This hypothesis is supported by the in vitro demonstration of increased migration and invasion of A549 lung cancer cells in the presence of intact NETs. These results are in keeping with reports that NET-associated proteins are involved in tumor progression. For example, NE is active in elaborated NETs and has been shown to promote extracellular matrix degradation and tumor cell shedding, thereby favoring the development of metastasis (67). These laboratory observations have a clinical corollary, with elevated serum levels of NE conferring a poor prognosis in patients with lung cancer (67).

Along these lines, NETs contain high concentrations of MPP-9. This enzyme is associated with increased cell migration, invasion, tumor dissemination, and metastasis (41, 68, 69).

In conclusion, this study is the first to implicate NETs as potential facilitators of tumor progression in the context of postoperative sepsis. We directly visualized tumor cell trapping within NETs in murine liver and lung in real time. This was associated with a drastic increase in hepatic metastatic disease burden compared with nonseptic controls. Furthermore, we demonstrated that NET formation in vivo can be inhibited by exogenous administration of DNAsase 1 and NEi with favorable oncologic outcomes in this experimental model. This raises the possibility that mitigating the adverse oncologic consequences of severe sepsis in patients with cancer is at least plausible. While additional studies are required to further validate these results, we propose that the NET represents a novel potential therapeutic target in selected patients with cancer.

Methods

Cells culture and cell lines

The A549 human lung carcinoma cell line was purchased from Cedarlane. Murine Lewis lung carcinoma cell subline H59, which stably expresses GFP after plasmid transfection, was obtained and cultured as previously described (19). HUVECs were purchased from Cell Systems. Cells were maintained in DMEM F12 containing 10% FBS, RPMI containing 10% FBS, and DMEM containing 20% FBS, respectively, and incubated at 37°C, 5% CO2. Human neutrophils were isolated from healthy subjects using Ficoll Hypaque density centrifugation as previously described (70). Only isolates with >98% purity and viability, as determined by Tryck’s staining and Trypan Blue exclusion, were used.

Animals

Seven- to ten-week-old C57BL/6 mice (25 g; Charles River) were used for all experiments. Peritonitis was induced by CLP as previously described (57, 58). Briefly, a midline laparotomy was performed under inhaled anesthesia, and the cecum was exteriorized. The distal 1 cm of the cecum was ligated with a 4-0 silk suture, and the tip of the cecum was perforated on the antimesenteric aspect with a 25-gauge needle. The cecum was returned to the abdominal cavity, and the abdomen was closed in 2 layers with a 4-0 silk suture. Animals received 0.2 mg/kg buprenorphine subcutaneously every 12 to 24 hours or as required. Sham animals had their cecums exteriorized without CLP. Mice were monitored postoperatively for signs of systemic sepsis, including changes in posture or activity, abdominal tenderness, rubor, and calor as described previously (58). Mice were treated with daily administration of DNAsase 1 (2.5 mg/kg [2,000 U/mg] intramuscularly Roche) or NEi GW311616A (2.2 mg/kg by gavage; Sigma-Aldrich), starting 24 hours prior to CLP until the termination of the experiment. These agents were selected given their demonstrated safety profile in previous animal and human experiments (32, 71, 72).

For PMN depletion, mice received intraperitoneal injection of PMN-depleting antibodies (150 μg anti-GR1 RB6-8C5; Cedarlane) as previously described (73). For PMN reinfusion experiments, neutrophils were isolated from bone marrow of syngeneic mice as described by Mocsai et al. (74). Briefly, tibias and femurs were removed and stripped of their muscles. The bone marrow was flushed using Hanks’ balanced salt solution supplemented with 0.5% FBS, and cell aggregates were disrupted via filtration through 70-μm cell strainer (Falcon). Erythrocytes were removed by hypotonic lysis. Neutrophils were separated by density centrifugation over a Percoll (62% vol/vol in HBSS; Sigma-Aldrich) gradient at 1,000 g for 30 minutes at 4°C. Neutrophils were recovered as a pellet at the bottom of the 62% Percoll and were >90% pure and 95% viable as determined by methylene blue and Trypan blue staining. Incubation of isolated cells with FITC-conjugated anti-mouse Gr-1 (Ly6G) antibody (0.1 μg; Cedarlane) followed by analysis with a flow cytometer FACSscan and analysis with CellQuest Software similarly demonstrated >90% purity (Supplemental Figure 7).

For neutrophil reinfusion, 1 × 106 cells were injected into the spleen. In some experiments, isolated neutrophils were stimulated with 500 nM PMA for 1 hour prior to reinfusion. Similarly, in some experiments, PMA stimulation took place in the presence of 10 μM NEi or 1,000 U DNAsase 1. Tumor cell infection (HS9) followed neutrophil reinfusion after 20 minutes in all cases. For intravital microscopy, mice were anesthetized with a mixture of 30 mg/kg ketamine (Wyeth Ayerst Canada) and 5 mg/kg xylazine (Bayer) injected intraperitoneally and maintained throughout the course of the experiments with doses via jugular vein cannula.

All experiments were performed in accordance with the guidelines of and with the approval of the McGill University Animal Care Committee.

Gross metastasis assay

Gross liver metastases were quantified 2 weeks after systemic tumor cell administration. All mice received intrasplenic injection of 3 × 105 HS9 cells followed by splenectomy on postoperative day 2 after CLP or sham surgery. Mice were sacrificed 2 weeks later, and the livers were harvested in toto for analysis. Gross metastases were quantified with a maximum quantifiable number of 400 gross metastases per liver. Representative images were captured using a digital camera (Nikon).
**Fluorescence intravital microscopy**

After induction of anesthesia, the right jugular vein and left carotid artery were cannulated for additional administration of anesthesia or reagents and tumor injection, respectively, as indicated. The anterior abdominal wall was removed from the costal margin to the midaxillary line bilaterally, and the falciform ligament was dissected away from the liver and gallbladder. In some experiments, the spleen was identified for intrasplenic tumor cell injection. The left lobe of the liver was gently positioned on a glass coverslip over the microscope objective. Animals were maintained at 37°C throughout the experiment with a heating pad or heated stage.

For epifluorescence microscopy, mice were visualized with an inverted microscope equipped with a ×20 objective and digital camera for image acquisition (Nikon TE300). For SD-IVM, the exposed liver or lung lobe was visualized with an Olympus IX81 microscope equipped with a confocal light path (Wave-Fx; Quorum) based on a modified Yokogawa CSU-10 head (Yokogawa Electric Corporation) using a UPLSAPO ×10/0.40 or UPLSAPO ×20/0.70 air objectives. Three laser excitation wavelengths (488, 561, and 635 nm; Cobalt) were used in rapid succession and visualized with the appropriate long-pass filters (Semrock). Exposure times for excitation wavelengths were 303 ms (488 nm), 303 ms (561 nm), and 300 ms (635 nm). A back-thinned EMCCD S12 × 512 pixel camera (C9100-13, Hamamatsu) was used for fluorescence detection. Velocity acquisition software (Improvision) was used to drive the microscope.

**Visualization and quantification of NETs**

NETs were visualized by fluorescence imaging of their components. Extracellular DNA was labeled with Sytox Green (5 μM), histone H2AX was labeled with Alexa Fluor 555 anti-mouse H2AX antibody (5 μg; Cedarlane), and NE was labeled with Alexa Fluor 555 anti-mouse NE antibody (0.6 μg) (Santa Cruz Biotechnology Inc.). Neutrophils were visualized with E-fluor 660 anti-mouse Gr-1 (Ly6G) antibody (3 μg; Cedarlane). Endothelium was imaged with PE anti-mouse PECAM-1 antibody (3 μg). All antibodies and dyes were visualized 15 minutes after intravenous administration.

NETs were quantified within hepatic and pulmonary vasculature. Briefly, images were acquired as z-stacks (1-μm intervals) and saved as extended focus images in tiff format. Images in the green channel were exported to ImageJ (NIH) for analysis. Contrast was adjusted in each image to minimize background and autofluorescence in order to try and minimize the variation in background fluorescence between experiments. A minimum brightness threshold was then set to yield only positive staining, and the same threshold and contrast settings were applied to each image within a given experiment. Threshold images were then converted to binary black-and-white images, and the area of positive staining was calculated as described previously (48). Data are expressed as relative area of fluorescence compared with sham surgery.

**In vivo adhesion assays**

**Sinusoidal arrest.** Ten minutes following intrasplenic injection of 5 × 10^4 H59-GFP cells or 48 hours following the intrasplenic injection of 1 × 10^6 H59-GFP cells into CLP, DNAse, NEi, and sham mice, the liver was visualized. GFP-expressing tumor cells were imaged using epifluorescence, and cells arrested within unoccluded sinusoids were considered adherent. Cells or tumor islands were quantified as the number of cells per micrometastatic foci in 8 to 10 hpf, and representative images were recorded.

**Tumor cell entrapment within NETs.** Neutrophils and H2AX were labeled using confocal microscopy. Neutrophils were plated on 40-mm glass coverslips as above. Concurrently, tumor cell perfusates were created as described above with the addition of 20 mM Sytox orange to the solution. Cell suspensions were perfused over neutrophil monolayers for 10 minutes at shear rates of 1 dye/cm^2/s. To quantify coelenterate, coverslips were fixed with 4% PFA for 10 minutes, washed with PBS, and mounted on glass slides. Cells were quantified in 5 to 10 hpf.

**In vitro adhesion assays**

**Static adhesion assays.** 5 × 10^5 neutrophils were plated in 24-well tissue culture plates and allowed to incubate for 1 hour at 37°C 5% CO_2 in RPMI alone or RPMI containing 5 μM NEi. 1 × 10^4 H59-GFP cells were added to wells with 800 nM PMA or 800 nM PMA plus 1,000 U DNase1. Unstimulated neutrophils and untreated tumor cells in RPMI served as controls. Following incubation for 4 hours at 37°C 5% CO_2, wells were washed with PBS and fixed in 4% PFA. Adhesion was quantified as the number of cells in 4 random hpf at ×20 using a Nikon TE300 microscope.

**Neutrophil adhesion and quantification**

Neutrophils were quantified by prestaining with carboxyfluorescein succinimidyl ester (CFSE) (Cedarlane). 5 × 10^6 neutrophils were plated in 24-well tissue culture plates and allowed to incubate for 1 hour at 37°C 5% CO_2 in RPMI alone or RPMI containing 5 μM NEi. 800 nM PMA or 800 nM PMA plus 1,000 U DNase1 was subsequently added to wells. Following incubation for 4 hours at 37°C 5% CO_2, wells were washed with PBS and fixed in 4% PFA. Images in the green channel were exported to ImageJ (NIH) for analysis. Contrast was adjusted in each image to minimize background and autofluorescence in order to try and minimize the variation in background fluorescence between experiments. A minimum brightness threshold was then set to yield only positive staining, and the same threshold and contrast settings were applied to each image within a given experiment. Threshold images were then converted to binary black-and-white images, and the area of positive staining was calculated as described previously (48). Data are expressed as relative area of fluorescence compared with sham surgery.

**Dynamic adhesion assays**

Untreated neutrophils or neutrophils preincubated with 5 μM NEi for 1 hour at 37°C 5% CO_2 were placed on 40-mm coverslips and allowed to adhere at 37°C 5% CO_2 for 1 hour. Concurrently, solutions of 5 × 10^5 cells/ml H59 cells were prepared in RPMI alone, RPMI plus 800 nM PMA, or RPMI plus 800 nM PMA and 1,000 U DNase1. Following incubation, 40-mm coverslips with above treated neutrophils were placed in a parallel flow chamber (Biotechcs). Cell suspensions were perfused over neutrophil monolayers for 10 minutes at shear rates of 1 dye/cm^2/s. To quantify coelenterate, coverslips were fixed with 4% PFA for 10 minutes, washed with PBS, and mounted on glass slides. Cells were quantified in 5 to 10 hpf.

For video acquisition, neutrophils were stained with E-fluor 660 anti-mouse Gr-1 antibody (3 μg) for 1 hour at 37°C 5% CO_2. Neutrophils were plated on 40-mm glass coverslips as above. Concurrently, tumor cell perfusates were created as described above with the addition of 20 mM Sytox orange to the solution. Cell suspensions were perfused over neutrophil monolayers as described above. Cell interactions under flow conditions were visualized using an Olympus IX81 microscope equipped with a confocal light path (Wave-Fx; Quorum) (Calgary Camera) at ×10 magnification.

**Confocal microscopy**

NET formation at the termination of static adhesion was confirmed using confocal microscopy. Neutrophils were plated on 13-mm coverslips (Fisher Scientific) and treated as in the static adhesion assay. Neutrophils were stained with CellTracker Red (Invitrogen), A549 cells were stained with CellTracker Orange (Invitrogen), and extracellular DNA was stained with Sytox Green (Molecular Probes). Representative images were captured using a Zeiss LSM 780 laser scanning confocal microscope using a ×40/1.20 W corr C-Apochromat air objective. Three laser excitation wavelengths (514, 543, and 633 nm; Argon, DPSS, HeNe) were used.
Scanning electron microscopy

Neutrophils were plated on 13-mm coverslips (Fisher Scientific) and treated as in the static adhesion assay. Cells were fixed in 2.5% glutaraldehyde and then post-fixed using repeat incubations with 1% osmium tetroxide/1% tannic acid. Samples were dehydrated with graded increasing concentrations of ethanol, critical point dried using a Leica EM CPD30 critical point drying device (Leica Microsystems, Austria), and coated with 5 nm carbon. Imaging was done with the Hitachi S-4700 FE-SEM.

Functional assays

Twenty-four–well Boyden chambers with 5-μm PET membranes (Fisher Scientific) were used for migration and invasion assays. Invasion assays differed in that HUVECs were placed in the upper chamber and allowed to proliferate for 72 hours. After 72 hours, monolayer integrity was confirmed by verifying impermeability of the upper chamber to culture medium. 2.5 × 10⁵ untreated neutrophils or neutrophils pretreated with 5 μM NEI for 1 hour were placed in the upper chamber. A549 cells from 80% confluent cultures were detached using 0.5% trypsin (Wisent). Cells were adjusted to a concentration of 2.5 × 10⁴ cells/ml and stained for 10 minutes with CPSE (Molecular Probes). 2.5 × 10⁴ cells were added to the upper wells. Granulocyte–tumor cell suspensions were treated with 25 nM PMA alone, 25 nM PMA plus 1,000 U DNAse I, or media alone. Isolated suspensions of A549 and AS49 plus 25 nM PMA served as controls. The cell suspensions were incubated for 24 hours at 37°C ± 5°C. After incubation, the contents of the upper chambers were aspirated, washed with PBS, and wiped with a sterile cotton swab. Cells adherent to the undersurface of the membrane were quantified in 4 random hpf.

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