Cancer Research

Neutrophils Promote Liver Metastasis via Mac-1–Mediated Interactions with Circulating Tumor Cells

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Abstract

Although circulating neutrophils are associated with distant metastasis and poor outcome in a number of epithelial malignancies, it remains unclear whether neutrophils play an active causal role in the metastatic cascade. Using *in vivo* models of metastasis, we found that neutrophils promote cancer cell adhesion within liver sinusoids and, thereby, influence metastasis. Neutrophil depletion before cancer cell inoculation resulted in a decreased number of gross metastases in an intrasplenic model of liver metastasis. This effect was reversed when inflamed neutrophils were co-inoculated with cancer cells. In addition, early adhesion within liver sinusoids was inhibited in the absence of neutrophils and partially restored with a short perfusion of isolated activated neutrophils. Intravital microscopy showed that cancer cells adhered directly on top of arrested neutrophils, indicating that neutrophils may act as a bridge to facilitate interactions between cancer cells and the liver parenchyma. The adhesion of lipopolysaccharide-activated neutrophils to cancer cells was mediated by neutrophil Mac-1/ICAM-1. Our findings, therefore, show a novel role for neutrophils in the early adhesive steps of liver metastasis. *Cancer Res; 72(16); 3919–27. ©2012 AACR.*

Introduction

A growing body of literature links metastasis and inflammation (1, 2). Cancer is an inflammatory condition and the available treatments are associated with an acute inflammatory state, be it through tissue trauma, infectious complications, or tumor-mediated inflammation (3). A portion of this literature has focused on the clinical observation that elevated neutrophil counts or elevated neutrophil-to-lymphocyte ratios are predictive of poor outcomes and distant metastasis in patients with a wide array of epithelial malignancies including lung (4), gastric (5), ovarian (6), hepatic (6), pancreatic (7), and colon carcinomas (8). Neutrophils become activated in response to inflammatory stimuli and have widespread effects on organs that commonly harbor metastases such as the lung and the liver (9). The growing literature on neutrophilia in the perioperative period and, more specifically, in the pretreatment setting indicates that it is associated with poor oncologic outcomes. Inflammation arising from the primary tumor or from treatment-associated complications affect neutrophil function and numbers and may be related to cancer progression (3). Thus, an improved understanding of how neutrophils may participate in the metastatic cascade could provide new insight for patients who are eligible to receive therapies with curative intent.

Patients with developing carcinomas shed circulating tumor cells (CTC) that ultimately are the seeds of distant metastasis (1, 10). Malignancies with poor outcomes are generally related to early implantation of CTCs within metastatic sites. CTCs can adhere directly to the endothelium of a target organ such as the liver (11). Inflammatory stimuli including tumor-derived cytokines or complications of treatments such as infections can cause an upregulation of inflammation-inducible cell adhesion molecules (CAM) that promote the adhesion and recruitment of CTCs to a host organ (12). Neutrophils promote endothelial dysfunction and can directly cause changes in CAM expression of endothelial cells (13). Thus, there is reason to believe that patients with developing carcinomas causing a systemic inflammatory state may experience neutrophil recruitment to potential host organs that facilitates the implantation of CTCs by both direct and indirect action. Indeed, there is now increasing *in vitro* evidence that supports the possibility of such CTC–neutrophil interactions (14–21). Very limited data are available for the presence of a functional role of neutrophils in the process of CTC recruitment to metastatic sites *in vivo* and the mechanistic basis of such interactions have yet to be characterized in animal models (22). This was the primary goal of our study.

Neutrophils experience changes in their surface CAMs that affect their adherence and migratory abilities within putative host metastatic tissues as a result of inflammatory cues (23).

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One such molecule is Mac-1 (CD11b, $\alpha_M\beta_2$). It is the α-subunit of the predominant β_2 (CD18) integrin present on granulocytes (24). Mac-1 becomes highly expressed on the surface of neutrophils in response to inflammatory cues such as lipopolysaccharide (LPS), an endotoxin produced by Gram-negative bacteria (25). We have previously shown that systemic inflammation via LPS promotes cancer cell adhesion within the liver using intravital microscopy (11). One of its main binding partners is ICAM-1, which is expressed on endothelial cells as well as a number of human and mouse lung cancer cells. It is, therefore, plausible that ICAM-1–expressing CTCs may interact with neutrophil Mac-1 within the microcirculation of a host metastatic organ.

As an example, patients requiring operative resection for non–small cell carcinoma of the lung are subject to important tissue trauma and are at high risk for infectious complications during the postoperative period (26). The tissue manipulation required during surgery is associated with an important release of CTCs (27–29). Together, the combination of acute systemic inflammatory stimuli with high numbers of CTCs presents a fertile ground for the initiation of metastasis (3). The clinical evidence linking postoperative complications and high CTC numbers to poor oncologic outcomes is abundant (30–34). This study contends that neutrophils, as key effectors of the acute systemic inflammatory reaction, play a functional role to promote the implantation of CTCs and, thus, facilitate metastatic spread to distant organs such as the liver.

Materials and Methods

Animals and cell lines

Six-week-old C57BL6 mice (Charles River, St. Constant, Quebec) weighing approximately 25 g were maintained in the Royal Victoria Hospital Animal Facility and used for all the experiments, with the exception of the experiments conducted on the spinning-disc confocal microscope, for which mice were housed at the Institute for Infection, Immunity and Inflammation in Calgary, Alberta. Lysm-enhanced GFP mice, in which the enhanced *GFP* gene was knocked into the murine lysozyme M locus, were used to identify PMN/cancer cell interactions *in vivo* (35, 36). Mac-1–null *Itgam*$^{-/-}$ mice were used to study the effect of Mac-1 on PMN/cancer cell interactions *in vivo* using intravital microscopy (37). The *Itgam*$^{-/-}$ mice have a targeted disruption of the CD11b locus that prevents formation of the β_2-integrin Mac-1 by preventing the heterodimerization of CD11b and CD18. All experiments were conducted in accordance with guidelines prescribed by the institutional animal care committee.

Lewis lung carcinoma H-59 cells that stably express GFP after plasmid transfection (a kind gift from Dr. Pnina Brodt) were obtained and cultured as previously described (38). They have been tested and authenticated in accordance with our institutional policies for contaminants using PCR within the past year. Near-confluent monolayers of H-59 cells were detached using a PBS-EDTA solution and resuspended in PBS for injection. In some cases, untransfected H-59 cells were used and could be labeled using cell tracker orange (Invitrogen, Molecular Probes). A549 human lung carcinoma cells that were obtained within the past 6 months from the American Type Culture Collection were handled as previously described to conduct adhesion assays with isolated human neutrophils. ICAM-1 expression has previously been established in this cell line (39). Circulating neutrophils were isolated on a Ficoll-hypaque gradient (VWR) from anesthetized C57BL6 mice after cardiac puncture using a heparinized syringe as previously described. Neutrophil isolates were 98% viable when assessed by trypan blue exclusion. In some experiments, neutrophils were treated with LPS (Sigma) 100 ng/mL for 45 minutes *in vitro*.

Liver metastasis model

Intrasplenic injections for the study of gross metastasis formation were conducted as previously described (40). Briefly, C57BL6 mice were anesthetized and their spleens were injected with 10^5 H-59-GFP cells in PBS and euthanized at 72 hours and 2 weeks later. Surface metastases were counted under a stereomicroscope at 2 weeks, whereas microscopic metastases were counted using a $\times10$ objective on a Nikon Eclipse TE300 microscope over 20 liver sections per mouse with 5 mice per group. For mice with innumerable metastases, the total number was capped at 200. Some mice were depleted of PMNs by intraperitoneal injection of PMN-depleting antibodies (RB6-8C5 150 μg i.v.; Cedarlane; ref. 41) and some PMN-depleted animals received H-59-GFP cells that were coincubated with LPS-treated (LPS 100 ng/mL for 45 minutes) mouse PMNs for 1 hour.

Intravital microscopy of the liver

Mice were prepared for hepatic intravital microscopy and received intra-arterial injections of H-59 cells. Briefly, quantification of H-59 cell recruitment to the liver was done with direct *in vivo* visualization of the hepatic microvasculature under epifluorescence on a Nikon Eclipse TE300 microscope (Nikon) equipped with a $\times20$ objective and a video camera (Panasonic Digital KR222, Panasonic). Images were recorded on an iMac G5 (Apple, Cupertino, CA) equipped with video-acquisition software. Some experiments were conducted on a spinning-disc confocal Olympus IX81 inverted microscope using an $\times10$/0.40 Uplan FL N objective to visualize PMN/H-59-GFP interactions *in vivo*. The microscope was equipped with a confocal light path (WaveFX, Quorum) on the basis of a modified Yokogawa CSU-10 head (Yokogawa Electric Corporation, Tokyo, Japan). Some animals received the anti-GR-1 mAb conjugated to Alexa-568 (1.6 μg intra-arterially; eBiosciences) to label PMNs *in vivo*. For some experiments, the GR-1 label was conjugated to Alexa Fluor 647 and, for others, Alexa Fluor 555. Some mice received bacterial LPS i.v. (0.5 mg/kg) as a model of acute inflammation and others received RB6-8C5 150 μg i.p. for PMN depletion. Isolated PMNs were stimulated with LPS 100 ng/mL for 45 minutes and 2×10^5 PMNs were injected per mouse 10 minutes before H-59 cell infusion.

In each experiment, 1.5×10^6 H-59-GFP cells were injected intra-arterially via a carotid artery cannula. The livers were visualized at 30, 45, and 60 minutes postinjection. Cells that arrested within un-occluded sinusoids of the liver for greater than 30 seconds were considered adherent, and were counted per microscopic field over 6 fields of view at $\times20$ magnification

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and ×10 for spinning-disc confocal microscopy experiments. The number of adherent cells for this time period were pooled and averaged to represent early cancer cell adhesion within the first hour after injection.

Immunohistochemistry

To investigate the colocalization of GFP-expressing H-59 cells and neutrophils, liver specimens of mice previously used for intravital microscopy were fixed in 4% paraformaldehyde and immersed in 30% sucrose before freezing at −80°C. Specimens were embedded in OCT compound, and frozen sections were cut at 10 μm and immunostained with a rat anti-mouse neutrophil specific antibody (L8993AP; Cedarlane) followed by Alexa 568-conjugated goat anti-rat (Invitrogen). Sections were observed under a laser-scanning confocal microscope (Zeiss). The degree of colocalization was measured in control, LPS-treated, and PMN-depleted mice by counting the percentage of cancer cells with an associated neutrophil per field of view in 5 random fields for 3 separate animals.

Cell adhesion assays

To determine the effect of neutrophils on the ability of H-59 cells to adhere to endothelial cells, human umbilical vein endothelial cells (HUVEC; Cell Systems Corp.) were grown to confluence in 48-well plates and pretreated with isolated mouse PMN (RPMI, control PMN, or LPS-activated PMN) for 3 hours before H-59-GFP co-culture with HUVECs. In other experiments, H-59-GFP cells were coincubated with isolated mouse PMN (RPMI, control PMN, or LPS-activated PMN) for 1 hour in a shaking water bath at 37°C before co-culture with HUVECs. Preincubated H-59-GFP cells or coincubated PMN/H-59-GFP cells were plated onto HUVECs for 1 hour and counted at ×20 magnification over 10 fields. Each experiment was repeated with similar results at least 3 times.

The role of Mac-1 in PMN–H-59 cell interactions was studied by plating H-59 cells to confluence on 48-well plates. CFSE (Molecular Probes, Invitrogen)-stained isolated mouse PMNs, LPS-treated (100 ng/mL for 45 minutes) or otherwise, were co-cultured for 1 hour in the presence of antibodies to Mac-1 (Cedarlane), ICAM-1 (Cedarlane), and CD44 (BD Pharmingen). After 2 washes in PBS, the remaining PMNs were counted at ×20 magnification over 10 fields of view.

Statistics

A Mann–Whitney *U* test was used to determine the significance between populations for the gross metastasis experiment. A chi-square test was used to analyze differences between groups in the 72-hour microscopic metastasis experiment. Otherwise, ANOVA was used with a *post hoc* Bonferroni correction for multiple comparisons and data are presented as mean ± SEM. Statistical significance was set at $P < 0.05$. GraphPad Prism 5 software was used for all statistical analysis and graphing.

Results

Neutrophil depletion reduces gross metastasis

Neutrophils routinely patrol liver sinusoids, interacting directly with endothelial cells. Adhesion or arrest within liver sinusoids is a necessary precursor to further growth and the development of metastasis. We have previously shown that metastatic ability of Lewis lung cancer clones closely correlates with adhesion to hepatic sinusoids (11). We sought to determine whether the presence of neutrophils in the liver microcirculation could affect the eventual development of gross metastasis. Intrasplenic injection of H-59 cells followed by a 2-week incubation period produces significant numbers of metastatic nodules. Mice depleted of circulating neutrophils, using the anti-GR-1 antibody clone RB6-8C5, 24 hours before intrasplenic injection reduced gross metastatic foci from a median of 45 ($n = 14$) in control mice to 4 ($P = 0.007$; $n = 13$; Fig. 1). To confirm that neutrophils were capable of promoting metastatic outgrowth as a result of early interactions, neutrophils were isolated from the blood of untreated syngeneic mice, activated with LPS and incubated for 1 hour with H-59 cells before intrasplenic injection in neutrophil-depleted mice. This treatment reversed the effects of neutrophil depletion resulting in a median number of metastases of 71.5 ($P = 0.047$; $n = 6$).

To address the possibility that the microscopic tumor burden differed from what was seen in Fig. 1 in terms of gross metastatic burden, we repeated the experiment and counted microscopic tumor foci at 72 hours postinoculation. In this experiment, we observed 9 tumor foci in the control group compared with 3 in the neutrophil-depleted group over 100 observations ($P = 0.037$; $n = 5$). The neutrophil depleted with LPS-activated neutrophil reinfusion group had 8 tumor foci over 100 observations in 5 mice ($P = 0.061$ compared with the PMN-depleted group and $P = 0.399$ compared with control).

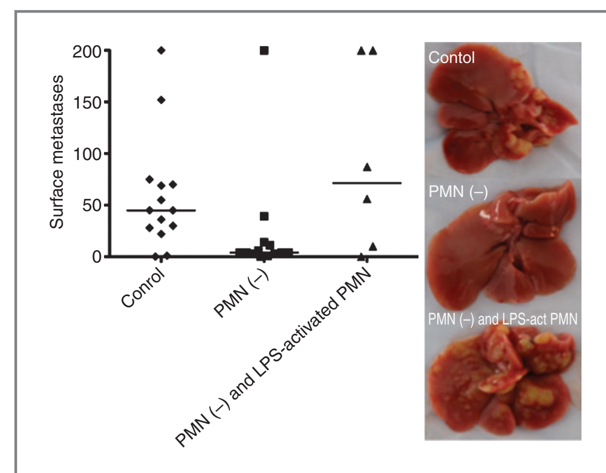

Figure 1. PMN depletion [PMN (−)] significantly reduced the development of surface metastases in a liver metastasis model using intrasplenic injection of H-59 cells from a median of 45 to 4 ($P = 0.007$). When LPS-activated PMNs were coincubated with H-59 cells before injection in a PMN-depleted mouse median surface, metastases increased from 4 to 71.5 ($P = 0.047$). Representative photographs of livers from each condition harvested at 2 weeks post-intrasplenic injection are shown. PMN (+) $n = 14$, PMN (−) $n = 13$ and PMN (−) with LPS-activated PMN $n = 6$.

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Intravital microscopy reveals that neutrophil depletion reduces lung cancer cell adhesion at early time points in the metastatic cascade

Acute systemic inflammation via LPS increases H-59 cell adhesion to hepatic sinusoids (Control 14.1 ± 0.4 vs. LPS 22 ± 0.84 cells/HPF; $P < 0.0001$) and this phenomenon is reversed by neutrophil depletion (Fig. 2). Similar to the intrasplenic model, neutrophil depletion resulted in a significant decrease in H-59 cell adhesion within 1 hour of injection (Control 14.1 ± 0.4 vs. PMN-depleted 11.7 ± 0.52 cells/HPF; $P < 0.01$). This effect was most prominent in the acutely inflamed host (LPS 22 ± 0.84 vs. LPS and PMN depletion 8.9 ± 0.4 cells/HPF; $P < 0.0001$). To confirm that activated neutrophils alone were capable of promoting metastasis, we isolated peripheral blood neutrophils from syngeneic sibling mice, treated them with LPS and, after washing, injected them into mice prepared for intravital microscopy. When neutrophil-depleted mice received LPS-activated peripheral blood neutrophils 10 minutes before H-59 injection, hepatic adhesion of H-59 cells was significantly increased from a mean of 11.6 ± 0.40 to 16.5 ± 0.53 cells/HPF (Fig. 2). This treatment partially restored the metastatic recruitment observed from the systemically inflamed mice that received intravenous LPS 4 hours before H-59 injection (22.0 ± 0.84 cells/HPF).

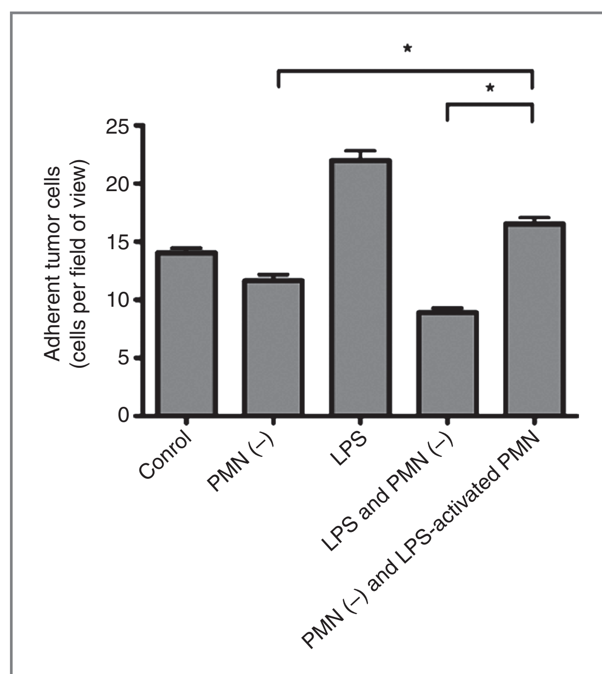

Figure 2. Systemic LPS increases H-59 recruitment from control conditions and is reversed by PMN depletion ($P < 0.0001$). PMN depletion reduces H-59 adhesion marginally under control conditions and more prominently in the acutely LPS inflamed host ($P < 0.0001$). Reinfusion of LPS-activated PMNs from a syngeneic sibling for 10 minutes significantly increases H-59 adhesion to liver sinusoids. *, $P < 0.0001$. Between 3 and 8 mice were used for each experimental group.

Intravital microscopy shows lung cancer cells directly interacting with arrested neutrophils *in vivo*

The results of the previous experiments indicated that neutrophils prime the microenvironment for the arrival of CTCs in such a way that their adhesion and outgrowth is facilitated. We, therefore, used the higher resolution of spinning-disc confocal microscopy to image the *in vivo* interactions between CTCs and native neutrophils within the liver. Mice were prepared for intravital microscopy and injected with anti-GR-1 fluorescently conjugated antibody to highlight circulating neutrophils. Following this preparation, GFP-tagged H-59 cells were injected via a carotid cannula, as previously described. Within minutes, we identified direct physical interactions between arrested neutrophils and infused GFP-tagged H-59 cells in hepatic sinusoids. The time series presented shows the arrival and arrest of an H-59 cell directly over a GR-1–labeled PMN (Arrow, Fig. 3A and 3B and Supplementary Movie). This cancer cell remained adherent for the entire imaging period, while another H-59 cell that was not associated with a neutrophil did not remain stably adhered (arrowhead, Fig. 3C and D). In addition, a circulating neutrophil was noted to be patrolling through the sinusoid containing the H-59–PMN complex even at 45-minute postadhesion, indicating that this sinusoid remained patent (arrow, Fig. 3E and F).

Neutrophils frequently colocalize with arrested lung cancer cells *in vivo* in both inflamed and baseline conditions

Given the finding that neutrophils can be physically linked to the arrest of CTCs and that their absence leads to reduced metastatic burden, we investigated how frequently such colocalization occurred during the early phases of metastasis within liver sinusoids. The livers of mice having undergone intravital microscopy to assess cancer cell adhesion under control, LPS-inflamed, and neutrophil-depleted conditions were frozen and sectioned for immunofluorescence using a mouse neutrophil-specific antibody. The percentage of H-59 cells that were colocalized was high in both control (82.5%) and LPS-inflamed (77.6%) conditions and, as expected, was nil in the neutrophil-depleted mouse (Fig. 4A).

To further confirm this finding, the Lysm-enhanced GFP transgenic mouse model was used to identify neutrophil–CTC interactions. Cell tracker orange–stained H-59 cells were injected via the carotid artery and visualized within liver sinusoids during the first hour postinjection by spinning-disc confocal microscopy. Circulating neutrophils express GFP in the Lysm-GFP mouse and these were also found to frequently colocalize with arrested H-59 cells (Fig. 4B).

Neutrophils promote adhesion of lung cancer cells to cultured endothelial cells

Neutrophils have multiple functions during the process of inflammation and can act via multiple mechanisms. Our goal was to determine whether neutrophils could facilitate the adhesion of cancer cells to cultured endothelial cells. Neutrophils were isolated from the peripheral blood of C57Bl6 mice under control or LPS-activated conditions. Both uninflamed (40.5 ± 2.64 cells/HPF) and LPS-activated (46.9 ±

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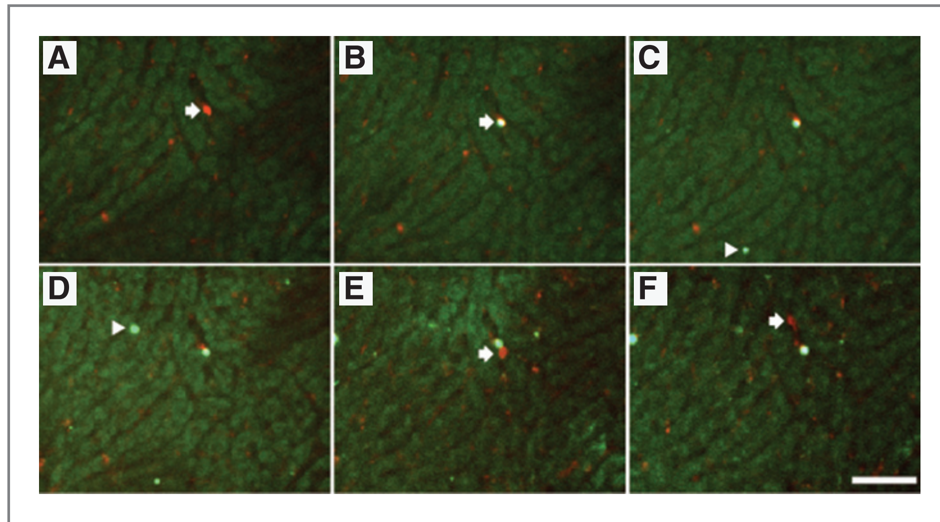

Figure 3. Time lapse imaging of the liver under spinning-disc confocal microscopy following intra-arterial injection of H-59-GFP cells. Circulating neutrophils are shown in red as a result of anti-GR-1-Alexa-568 mAb labeling. A, images taken at time 0 before intra-arterial infusion of H-59-GFP. (B, 50 seconds; C, 90 seconds; D, 10 minutes; E, 22 minutes; and F, 30 minutes postinfusion of H-59 cells). Arrows in A and B show a colocalized H-59-GFP cell and a GR–1-labeled PMN. Arrowheads in C, D, and E show recruited cancer cells that adhere independently from neutrophils. Arrows in E and F track a patrolling neutrophil within the sinusoid that retains the neutrophil/H-59 complex, suggesting patency of this sinusoid. Scale bar, 25 μm.

3.78 cells/HPF) neutrophils contributed to a significant increase ($P < 0.0001$) in adhesion of H-59 cells when added to confluent monolayers of endothelial cells for 3 hours before the addition of cancer cells as compared with neutrophil-free buffer (27.8 ± 2.67 cells/HPF) In a similar fashion, co-incubation of cancer cells with PMNs for 1 hour before co-culture with HUVECs resulted in a significant increase in H-59 adhesion whether PMNs were pretreated with LPS (13.3 ± 1.41 cells/HPF) or not (17.8 ± 1.24 cells/HPF) compared with control buffer (6.05 ± 0.78; $P < 0.0001$; Supplementary Fig. S1).

Neutrophil activation with LPS promotes adhesion to human and murine lung cancer cells *in vitro* and this effect is partially reversed by Mac-1 or ICAM-1 blockade

Initially, we determined that neutrophils were capable of adhering to confluent H-59 monolayers (17.8 ± 2.64 PMN/HPF) and this adhesion was potentiated when neutrophils were LPS-activated (75.9 ± 6.14 PMN/HPF; $P < 0.0001$; Fig. 5A). H-59 cells were tested for their expression of ICAM-1 using flow cytometry to determine whether Mac-1 based binding would be possible and, indeed, they expressed ICAM-1 constitutively at their surface (Supplementary Fig. S2). When PMNs were added to H-59 monolayers in the presence of Mac-1 or ICAM-1 function-blocking antibodies, there was a significant reduction when PMNs were LPS activated, but not under control conditions (Fig. 5A). CD44 is a known PMN-CAM expressed at their surface. A function-blocking CD44 antibody of the same isotype as the Mac-1 and ICAM-1 antibodies used did not lead to a significant decrease in PMN adhesion both under control and LPS-activated conditions.

The same experiment was repeated, this time using the human lung carcinoma cell line A549 and human neutrophils. Almost identical trends were found. LPS caused a significant increase in neutrophil adhesion to A549 monolayers compared with control (2.67 ± 0.34 vs. 4.8 ± 0.56 PMN/HPF; $P < 0.05$). Both Mac-1 and ICAM-1 blockade cause significant reductions

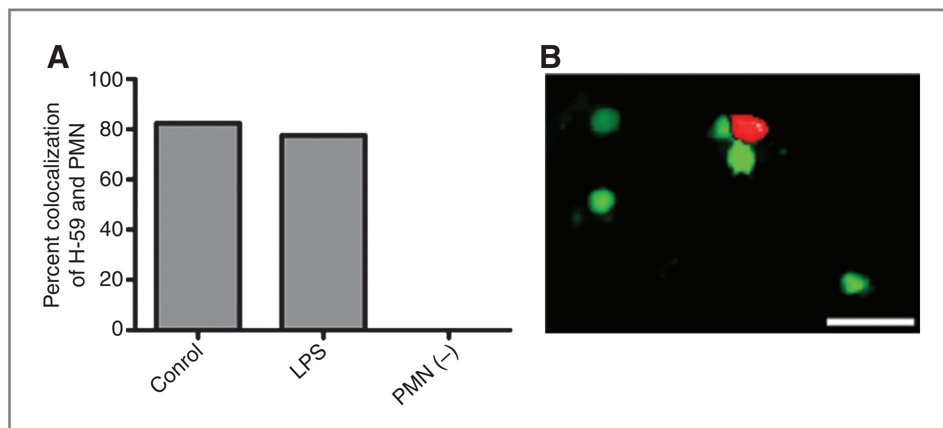

Figure 4. A, H-59 cells colocalize to a high degree in both control and LPS-inflamed animals, whereas there is no colocalization in the PMN (–) anti–GR-1–treated mice. Three mice per group were used to quantify colocalization. B, intravital spinning-disc confocal microscopy reveals colocalization within liver sinusoids of GFP-tagged neutrophils in the LysM-GFP mouse with cell tracker orange–stained H-59 cells injected intra-arterially.

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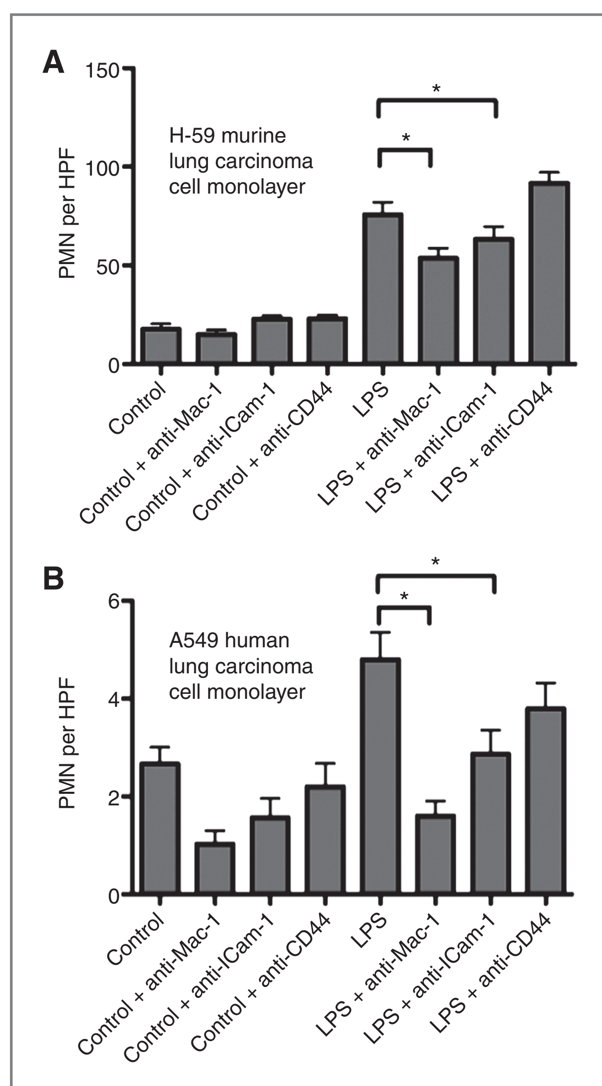

Figure 5. LPS promotes the adhesion of neutrophils to H-59 cells and this effect is partially mediated by Mac-1/ICAM-1. PMNs, whether LPS-activated or not, were incubated over confluent monolayers of H-59 cells in the presence of function-blocking antibodies. LPS activation of PMNs led to a significant increase in PMN/H-59 adhesion ($P < 0.0001$). A, addition of Mac-1 or ICAM-1 mAb significantly reduced the effect of LPS. Identical experiments were carried out using the human lung cancer cell line A549 and human neutrophils. B, similarly, LPS promoted human neutrophil/A549 adhesion and this effect was mediated by Mac-1/ICAM-1 interactions. *, $P < 0.05$. Three independent experiments were carried out.

in PMN adhesion in the LPS-stimulated group (1.6 ± 0.31; $P < 0.0001$ and 2.87 ± 0.49; $P < 0.05$). Similar to the mouse cells, there was no difference in the unstimulated group, and CD44 had no effect on PMN–A549 interactions.

Mac-1 KO mice display reduced cancer cell adhesion in response to LPS stimulation despite unchanged neutrophil recruitment

Given the results of the antibody-blocking experiments, we aimed to study the early metastatic behavior of H-59 cells within liver sinusoids of Mac-1 KO mice as compared with wild type (WT). As expected, neutrophil recruitment to liver sinusoids after LPS stimulation in the Mac-1 KO was normal as compared with WT controls (Fig. 6B). In terms of H-59 recruitment within the first hour after intracarotid injection, there was a trend toward reduced adhesion in untreated Mac-1 KO mice (3.53 ± 0.87 cells/HPF) as compared with WT (6.22 ± 1.01 cells/HPF; $P = 0.078$). However, after LPS-stimulation, WT mice displayed a 2-fold increase in cancer cell adhesion (as previously shown) while H-59 adhesion was unchanged in the Mac-1 KO after LPS as compared with the Mac-1 KO-untreated control (Fig. 6A). Furthermore, while PMN depletion in the WT returned H-59 recruitment to control levels, it had no effect in the Mac-1 KO, indicating that the effect seen in the Mac-1 KO is neutrophil mediated (Fig. 6A).

Discussion

The link between cancer progression and inflammation is now well established (1). Numerous lines of investigation have shown that inflammatory cells are key participants in cancer progression and metastasis (42). This study is, to our knowledge, the first to identify a functional role for neutrophils in the adhesion of CTCs in the liver. Neutrophil depletion resulted in a significant decrease in both gross metastases using an intrasplenic model of liver metastasis. Activated neutrophil infusion 10 minutes before the administration of cancer cells resulted in increased adhesion of H-59 cells in another model of metastasis using intravital microscopy. Again at 72 hours

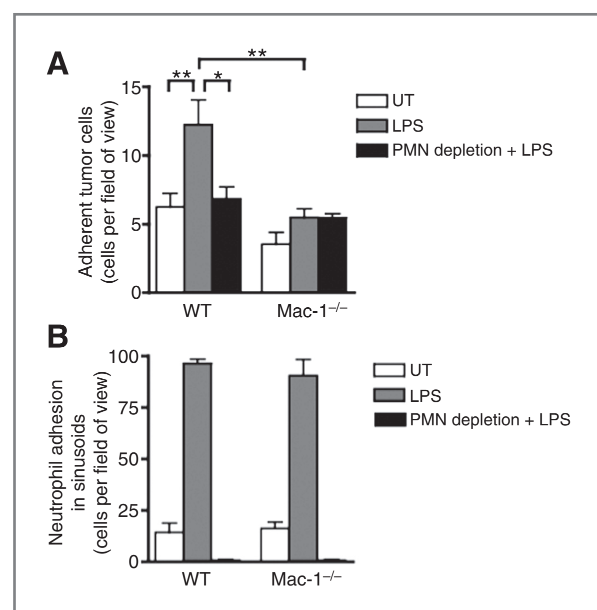

Figure 6. Mac-1$^{-/-}$ mice are insensitive to the effects of LPS on H-59 cell adhesion in liver sinusoids. A, using intravital microscopy, H-59 cell adhesion was unchanged in the Mac-1$^{-/-}$ mice after LPS administration as compared with the 2-fold increase seen in the wild-type mouse after LPS-induced acute systemic inflammation. There was a trend toward reduced tumor cell adhesion in the Mac-1$^{-/-}$ as compared with wild-type at baseline conditions. This effect did not result from changes in neutrophil recruitment following LPS administration (B). *, $P < 0.05$; **, $P < 0.01$. Five to 8 mice were used per experimental condition.

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postinjection, neutrophil depletion caused a profound reduction in microscopic liver metastases, which was rescued when activated peripheral blood neutrophils were admixed to the cancer cell inoculum. These results indicate that early interactions between neutrophils and cancer cells within sinusoids of the liver promote cancer progression at a key juncture in the metastatic process.

Such interactions were documented *in vivo* using spinning-disc confocal intravital microscopy. Cancer cells could be seen to arrest from flow directly over an adhered neutrophil inside liver sinusoids within minutes of cancer cell injection. Moreover, such interactions were witnessed in non-inflamed and LPS-inflamed hosts. In fact, when quantified, the percentage colocalization of H-59 cells and neutrophils was similar in both control and LPS-inflamed mice. This finding correlates well with the profound reduction in liver metastasis after neutrophil depletion in uninflamed mice. To further show that such interactions were taking place without the potential interference of antibodies to label circulating neutrophils, the LysM-GFP mouse that expresses GFP within neutrophils was used for intravital microscopy. Once again, direct physical interactions between GFP-tagged neutrophils and H-59 cancer cells were identified within the first hour after dissemination.

Nonetheless, the reduction in adhesion seen at 60 minutes by intravital microscopy in non–LPS-stimulated conditions was not nearly as significant as compared with both the reduction in micro-metastasis at 72 hours and gross metastasis at 2 weeks after neutrophil depletion. This finding argues against a purely adhesion-based mechanism to explain the functional role of neutrophils in producing the dramatic effects seen at 2 weeks. However, under LPS-stimulated conditions, the reduction of H-59 adhesion was far more significant after neutrophil depletion. Furthermore, this effect appears to be almost entirely mediated by Mac-1 as shown by intravital microscopy using the Mac-1 KO. Together, these findings indicate that cancer cell–neutrophil Mac-1–mediated adhesion represents a key mechanism for metastatic recruitment in the livers of inflamed mice. Interestingly, the Mac-1 KO also displayed approximately 40% less H-59 adhesion as compared with uninflamed controls and this trend approached statistical significance. It is possible that, in the uninflamed setting, Mac-1 continues to play a role to facilitate adhesion as a result of mild surface expression. Nonetheless, adhesive mechanisms, although still important, do not seem to explain the full extent of the phenotype seen at 72 hours and 2 weeks in the uninflamed setting. Indeed, neutrophils may help stabilize arrested cancer cells and may, in fact, assist their proliferation.

CTCs may use adhered neutrophils as an anchor to tether themselves to the endothelium. Such adhered tumor cells may benefit from the matrix-degrading enzymes present within neutrophils to extravasate into the parenchyma of the host organ. Emerging data with regard to the angiogenic effects of neutrophils in the tumor microenvironment are consistent with a supportive role from the very inception of metastasis (43, 44). Multiple cancers express chemotactic factors such as interleukin-8 (IL-8) that recruit neutrophils to take residence within the tumor microenvironment (45). A recent study by Huh and colleagues showed that IL-8–expressing melanoma cells physically interact with neutrophils within hours of inoculation (22). These interactions were β_2-integrin/ICAM-1 mediated and resulted in increased tumor cell retention and transendothelial migration. IL-8 expression was directly linked to this effect, thus indicating that the ability of cancer cells to attract neutrophils offers a survival advantage in the metastatic environment. This study is only the second, to our knowledge, to show physical interactions that are functionally related to the process of early metastasis *in vivo*. Our results differ from those of Huh and colleagues in that the interactions they noted occurred after the arrest of the CTC within the lung parenchyma. Neutrophils (endogenous and exogenously supplied) were found to have a stabilizing effect on melanoma cell retention and proliferation. In our study, it was the presence of neutrophils before the implantation of cancer cells that promoted the eventual implantation and outgrowth of CTCs. It is quite likely that both mechanisms occur during these early phases of metastasis and, in this regard, both studies are complementary and highlight the important role of neutrophils in the metastatic cascade.

Neutrophils routinely extravasate into resident tissues to patrol the environment where they may induce conformational changes within the endothelium that may also facilitate the passage of a recruited cancer cell (46). CTCs must gain access to the host organ's parenchyma to proliferate and there is data from *in vitro* studies to support the concept that neutrophils may facilitate this process (15, 16, 22). As such, CTCs may use adhered neutrophils to "piggy-back" their way into the parenchyma of a host organ. Additional studies examining the nature of these interactions further into the metastatic cascade are required to characterize the exact nature of how neutrophils might promote metastasis and, more specifically, in the uninflamed setting to understand how neutrophils promote metastasis given the limited impact of adhesion-based CTC recruitment.

Our model aimed at eliminating neutrophils in tumor-naïve mice before the implantation of CTCs. The RB6-8C5 clone has been shown to eliminate 97% of circulating neutrophils within 24 hours of administration without significantly affecting other populations (47). In addition, we documented this effect in Fig. 6B, where neutrophil levels were quantified by intravital microscopy. It should be noted that, while neutrophils constitute the vast majority of circulating leukocytes targeted by GR-1 depletion, other monocyte subsets numbers are mildly affected by this mAb (48). Given the budding literature on myeloid-derived suppressor cells and their role in the metastatic cascade, it is important to note the possibility that a portion of our observed effects from GR-1–mediated depletion are explained by cell populations other than neutrophils. Nonetheless, the results from the LysM-GFP mouse do corroborate neutrophil-specific interactions with CTCs. In addition, neutrophils return to normal circulating levels within 72 hours of depletion (48). Thus, our results strongly support a role for neutrophils during the early steps of metastasis before the implantation of CTCs in the liver.

Although our data indicate that direct interactions between neutrophils and cancer cells during the early phases of hematogenous metastasis are important, this study does not address

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some of the other mechanisms in which neutrophils may affect this process. It is quite likely that neutrophils alter the host metastatic microenvironment in an indirect fashion. Neutrophils secrete large amounts of proinflammatory cytokines that can activate endothelial cells, making them more likely to capture CTCs. These cytokines could directly influence putative CTCs within the primary tumor microenvironment and/or CTCs in circulation leading to altered CAM expression that may affect their ability to arrest and become mature metastases. Further studies are required to understand how neutrophil products contribute to CTC implantation.

Surgery is considered definitive treatment for numerous epithelial malignancies including lung cancer. However, the tissue trauma of surgery itself, together with its infectious complications such as Gram-negative sepsis, is well known to induce neutrophilia and Mac-1 upregulation on circulating neutrophils. This, in combination with a high CTC burden, is likely to result in poor oncologic outcomes by promoting the adhesion of CTCs and their eventual proliferation into gross tumors. Our results indicate that neutrophil–lung cancer cell interactions are likely to be an important mechanism by which the progression of early malignancy is facilitated. More specifically, neutrophil Mac-1 interactions with CTC ICAM-1 are an attractive target requiring further investigation. Mac-1 inhibition may prove to be a useful adjunct to limit the dissemination of CTCs in patients undergoing potentially curative therapies for hematogenously spreading malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.D. Spicer, L.E. Ferri
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.D. Spicer, B. McDonald, J.J. Cools-Lartigue, S.C. Chow, L.E. Ferri
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.D. Spicer, B. McDonald, J.J. Cools-Lartigue, L.E. Ferri
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Giannias
Imaging experiments: P. Kubes

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