Cancer Research

Transiently Entrapped Circulating Tumor Cells Interact with Neutrophils to Facilitate Lung Metastasis Development

Sung Jin Huh[1], Shile Liang[6], Arati Sharma[1,5], Cheng Dong[5,6], and Gavin P. Robertson[1,2,3,4,5]

Abstract

It is unknown why only a minority of circulating tumor cells trapped in lung capillaries form metastases and involvement of immune cells remains uncertain. A novel model has been developed in this study showing that neutrophils regulate lung metastasis development through physical interaction and anchoring of circulating tumor cells to endothelium. Human melanoma cells were i.v. injected into nude mice leading to the entrapment of many cancer cells; however, 24 hours later, very few remained in the lungs. In contrast, injection of human neutrophils an hour after tumor cell injection increased cancer cell retention by ~3-fold. Entrapped melanoma cells produced and secreted high levels of a cytokine called interleukin-8 (IL-8), attracting neutrophils and increasing tethering β_2 integrin expression by 75% to 100%. Intercellular adhesion molecule-1 on melanoma cells and β_2 integrin on neutrophils interacted, promoting anchoring to vascular endothelium. Decreasing IL-8 secretion from melanoma cells lowered extracellular levels by 20% to 50%, decreased β_2 integrin on neutrophils by ~50%, and reduced neutrophil-mediated extravasation by 25% to 60%, resulting in ~50% fewer melanoma cells being tethered to endothelium and retained in lungs. Thus, transendothelial migration and lung metastasis development decreased by ~50%, showing that targeting IL-8 in melanoma cells has the potential to decrease metastasis development by disrupting interaction with neutrophils. *Cancer Res; 70(14); 6071–82. ©2010 AACR.*

Introduction

Melanoma is the most aggressive and metastatic form of skin cancer (1). Most deaths occur from disseminated, therapy-resistant tumors disrupting major organ function (2). Therefore, identification of proteins and molecular mechanisms regulating metastasis are important for effective long-term management of advanced disease.

Metastasis is a complex process requiring melanoma cell detachment from the primary tumor and migration to secondary sites in the body through the lymphatic or blood circulatory systems (3, 4). It is accompanied by secretion of factors including proteases and cytokines, performing autocrine or paracrine roles to promote metastasis (5). Tumor cells also secrete matrix metalloproteinases (MMP) to degrade extracellular matrix, disrupt cell-matrix interacting receptors, or alter expression of cell-cell adhesion molecules to escape the primary site and enter the circulatory system (3, 5, 6). Circulatory cancer cells must survive blood flow shear forces and immune system challenges (4) and, once entrapped in or adhering to capillaries, must extravasate into surrounding tissue and form tumors (5). Although extravasation is crucial for metastases, mechanisms regulating this complex process remain to be fully elucidated (5).

Interleukin-8 (IL-8) was originally identified as a neutrophil chemotactic factor involved in acute inflammatory responses (7), which activates neutrophils (8, 9) by upregulation of adhesion molecule β_2 integrin (e.g., CD11a/CD18 or LFA-1, and CD11b/CD18 or Mac-1; ref. 10) to recruit neutrophils to sites of inflammation by rolling along endothelium (11, 12). IL-8 is overexpressed in advanced stage carcinomas of the skin, breast, stomach, and prostate (7, 13), with neutrophils responding to this stimulus as it occurs during inflammation (13–15). IL-8 has high binding affinity for chemokine receptors 1 and 2 (CXCR1 and CXCR2) expressed on keratinocytes, fibroblasts, neutrophils, endothelial (15–19), and cancer cells (13, 15, 19, 20). Binding of IL-8 to CXCR1/CXCR2 activates phosphoinositide 3-kinase and/or mitogen-activated protein kinase (MAPK) pathways depending on cell type (15).

In melanomas, IL-8 lies downstream of constitutively active ^{V600E}B-Raf in the MAPK cascade, which can activate NF-κB and activator protein (21) to increase the transcription of *IL-8* (21, 22). IL-8 has both autocrine and paracrine roles regulating melanoma growth, angiogenesis, and metastasis (19). In animals, a humanized neutralizing antibody targeting IL-8 inhibits tumor growth and metastasis by reducing MMP 2 expression in bladder cancer and melanomas (19, 23, 24).

Authors' Affiliations: Departments of [1]Pharmacology, [2]Pathology, and [3]Dermatology and Surgery, [4]The Foreman Foundation for Melanoma Research, [5]Penn State Melanoma Therapeutic Program, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania and [6]Department of Bioengineering, The Pennsylvania State University, University Park, Pennsylvania

Corresponding Authors: Gavin P. Robertson, The Pennsylvania State University, R130, Hershey PA 17033. Phone: 717-531-8098; Fax: 717-531-5013; E-mail: gprobertson@psu.edu and Cheng Dong, Phone: 814-865-8091; Fax: 814-863-0490; E-mail: cxd23@psu.edu.

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Furthermore, melanoma cell–secreted IL-8 can trigger neutrophils to secrete more protein to increase extracellular concentrations (25). Although IL-8 has multiple roles in tumor development (16, 18, 19, 26–29), the mechanism leading to metastasis needs elucidation and neutrophil involvement remains uncertain.

Neutrophils are the most abundant white blood cells in humans and essential for immune system function (11). Neutrophils migrate toward sites of infection or inflammation by chemotaxis, responding to chemotactic gradients of IL-8, IFN-γ, or C5a (11). Melanoma cells secrete IL-8, which can attract and activate neutrophils by increasing β_2 integrin expression and induce further IL-8 secretion (30). Interaction between intercellular adhesion molecule-1 (ICAM-1) on melanoma cells and β_2 integrins (especially Mac-1) on neutrophils can enhance cell adhesion under flow conditions to an endothelial-like layer promoting extravasation across the layer. However, it is unknown whether this is a real process facilitating metastasis in animals (31–33). Although IL-8 can promote metastasis (34), the mechanism by which it occurs and the role played by neutrophils remains unclear.

This study provides data supporting a novel model showing that entrapped metastatic melanoma cells in the lungs produce and secrete IL-8 to attract neutrophils, which promotes tethering to the vascular endothelium. Prolonged melanoma cell retention in the lungs facilitated transendothelial migration and metastasis development. Reducing IL-8 expression using small interfering RNA (siRNA) decreased interaction between melanoma cells and neutrophils, resulting in fewer being tethered to the endothelium and retained in the lungs, thereby decreasing extravasation and metastasis development.

Materials and Methods

Cell lines and neutrophils

Melanoma cell lines were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone). WM35 was maintained as previously described (35). To generate highly metastatic variants, 0.5×10^6 green fluorescent protein (GFP)–tagged A375M, C8161.Cl9, or UACC 903 cells were injected into the lateral tail vein of nude mice. Mice were sacrificed 2 to 4 weeks later, and GFP-tagged tumors were isolated from the lung tissue. GFP-tagged melanoma cells present in the lung tumors were minced with scalpels into 0.5-mm^3 pieces and were grown in DMEM supplemented with 10% FBS containing penicillin/streptomycin. The procedure was repeated two to three times to generate highly metastatic variants of the parental cell lines.

Neutrophils were isolated from human blood using Histopaque 11191 and 10771 density gradients (Sigma) as described by the manufacturer and kept at 4°C in sterile PBS containing 0.1% human serum albumin until use. In some experiments, neutrophils were stimulated with recombinant human IL-8 (Biosource, Inc.). Fresh blood was collected from healthy donors according to an Institutional Review Board–approved protocol.

SiRNA targeting IL-8

SiRNA (100 pmol) was introduced into 1.0×10^6 1205 Lu, C8161.Cl9 or UACC 903M through nucleofection using an Amaxa Nucleofector using Solution R/program K-17 (22, 36–38). Transfection efficiency was >95% with 80% to 90% cell viability (35, 37). Following siRNA introduction and 2-day recovery in culture dishes, cells were replated in 96-well plates. Five days later, cell viability was measured by MTS assay (Promega; ref. 39). Duplexed Stealth siRNA (Invitrogen) were used. siRNA sequences were as follows: scrambled, AAUUCUCCGAACGUGUCACGUGAGA; ^{V600E}*B-Raf*, GGUCUAGCUACAGAGAAAUCU CGAU; *IL-8#1*, GCAGCUCUGUGUGAAGGUGCAGUUU; and *IL-8#2*, CCAAGGAGUGCUAAAGAACUUAGAU.

Western blot analysis

Procedure for Western blotting was as previously reported (35). Blots were probed with antibodies to IL-8 (Biosource, Inc.), α-enolase (Santa Cruz Biotechnology), and β-actin (Sigma Co.).

IL-8 ELISA

After introducing siRNA by nucleofection, cells were replated in media, replaced 24 hours later with fresh DMEM (Invitrogen) supplemented with 10% FBS (Hyclone). The next day, media were collected to quantify IL-8 secretion. For melanoma-neutrophil cocultures, cell-free supernatants were collected by centrifugation at $430 \times g$ for 5 minutes, and IL-8 was quantified using ELISA (primary and secondary antibody pairs, recombinant IL-8; R&D Systems). Intra-assay variation was 10% to 15%.

Flow chamber extravasation assay

Cell migration under cell culture flow conditions was measured using a modified 48-well chemotactic Boyden chamber consisting of a top and a bottom plate separated by a gasket (8, 22, 40). Before each experiment, EI cells expressing ICAM-1 comparable with IL-1β–stimulated human umbilical vascular endothelial cells were used as an endothelial substrate (provided by Dr. Scott Simon, UC Davis, Davis CA) and grown on sterile polyvinylpyrrolidone-free polycarbonate filters (8-μm pore size; NeuroProbe) precoated with fibronectin (30 μg/mL, for 3 h; Sigma). Twelve center wells of the bottom plate were filled with soluble chemoattractant type IV collagen [100 μg/mL in RPMI 1640/0.1% bovine serum albumin (BSA); BD Biosciences], and surrounding control wells filled with RPMI 1640/0.1% BSA. Melanoma cells express $\alpha_2\beta_1$ integrin receptors for soluble collagen IV protein and migrate toward collagen IV following stimulation (41, 42). For the migration assay, melanoma cells only or neutrophils together with melanoma cells (5.0×10^5 of each) were placed in the chamber under shear flow conditions (4 dyn/cm^2) for 4 hours in a 37°C, 5% CO_2 incubator. Migrated cells were stained with Protocol Brand Hema3 solution (Fisher Scientific) and counted using an inverted microscope (Diaphot 330, Nikon) with the NIH ImageJ software (43). In IL-8 neutralization studies, neutrophils were treated with anti-human CXCR1/2 antibody (Sigma)

to block these receptors for 30 minutes at 37°C before the assay. For blocking secreted IL-8 function, 1 μg/mL anti-human IL-8 (R&D systems, Inc.) was added into the flow loop during the flow-extravasation assay.

Flow cytometry analysis for Mac-1 expression on neutrophils

Mac-1 expression on neutrophils was measured by incubating cells with anti-CD11b antibody (Invitrogen) in 1% BSA for 20 minutes at 4°C followed by two washes. After an additional 20-minute incubation with TRITC-conjugated goat anti-mouse Fab_2 fragment (1 μg/10^6 cells; Jackson ImmunoResearch) at 4°C, the cells were washed twice and fixed with 2% formaldehyde and analyzed by a GUAVA personal flow cytometer (GUAVA Technologies, Inc.).

Parallel-plate flow assays

C8161 cell adhesion to EI monolayer in the presence of neutrophils was performed in a parallel-plate flow chamber (Glycotech) mounted on the stage of a phase-contrast optical microscope (Diaphot 300). A syringe pump (Harvard Apparatus) was used to generate a steady flow field in the flow chamber. A Petri dish (35 mm) with a confluent EI cell monolayer (acting as a ligand-binding substrate) was attached to the flow chamber by vacuum. The flow chamber was perfused with appropriate media at 37°C over the EI monolayer for 2 to 3 minutes at a shear rate of 40 s^{-1} for equilibration before the introduction of 1 × 10^6 cells/mL of neutrophils and C8161 cells. Neutrophils were either activated by IL-8 (5 ng/mL for 30 minutes) or treated with anti-Mac-1 (R&D systems) blocking antibodies. After allowing neutrophils and C8161 cells to contact the EI monolayer at a shear stress of 0.1 to 0.3 dyn/cm^2 for 2 minutes, shear stresses were adjusted to the experimental range of 0.6 dyn/cm^2 and kept constant for 5 minutes. The aggregation of C8161 and neutrophils was quantified and normalized by the total number of collisions as:

$$\text{C8161} - \frac{\text{Neutrophil}}{\text{Aggregation}} = \frac{\text{Number of C8161} - \text{PMN Aggregation}}{\text{Number of C8161} - \text{PMN Collisions}}$$

The numerator is the number of C8161-neutrophil aggregation on the EI monolayer at the end of the entire flow assay as a result of collision between entering C8161 cells and tethered neutrophils. The denominator is the total number of C8161-neutrophil collisions near the EI monolayer surface and is counted as a transient accumulative parameter throughout the entire flow assay.

Animal studies

Tumor formation was measured in 4- to 6-week-old female athymic-Foxn1^{nu} nude mice purchased from Herlan. SiRNA (500 pmol) was nucleofected into 5.0 × 10^6 cells, and after 48 hours of recovery, 1.0 × 10^6 cells/0.2 mL in 10% FBS-DMEM were injected s.c. above rib cages. Dimensions of developing tumors were measured on alternate days using calipers.

Interaction of melanoma cells with human neutrophils in the lungs

siRNA (100 pmol) was nucleofected into 1.0 × 10^6 GFP-tagged 1205 Lu cells, and 36 hours later, 0.5 × 10^6 cells/0.2 mL HBSS were collected. Isolated neutrophils were stained with CellTracker Orange CMTMR (C2927, Invitrogen) according to the manufacturer's protocol. Melanoma cells were injected i.v. into the left lateral tail vein of athymic-Foxn1^{nu} nude mice, and human neutrophils were injected i.v. into the right lateral tail vein. After 24 hours, mice were sacrificed, and lungs were removed and analyzed for melanoma cells interacting with neutrophils using a Nikon SMZ 1500 dissecting microscope with fluorescence detection capabilities (for GFP, ex470/em500; for CellTracker Orange CMTMR, ex550/em600). Melanoma-neutrophil interaction was quantified by percentages of melanoma cells colocalized with neutrophils. Percentages were calculated by the number of melanoma cells interacting with neutrophils/total number of melanoma cells in each field.

Mouse experimental metastasis assay

siRNA (100 pmol) was nucleofected into 1.0 × 10^6 GFP-tagged 1205 Lu, C8161.Cl9, or UACC 903M cells, and 36 hours later, 0.5 × 10^6 cells/0.2 mL HBSS were injected into the lateral tail vein of nude mice. Mice were sacrificed 18 days later, necropsied, and lungs were analyzed for presence of fluorescent metastatic lesions using a Nikon SMZ 1500 dissecting microscope with a Plan Apo 1.6× objective. Images were photographed at ×48 magnification from lung ventral surface and number as well as area occupied by fluorescent metastatic lesions scored in pixels using the IP Laboratory imaging software (Scanalytics). Assays were replicated at least twice.

Statistical analysis

Statistical significance for multiple comparisons was determined using one-way ANOVA or nonparametric one-way ANOVA Kruskal-Wallis test followed by Dunnett's multiple comparison tests. For comparison between two groups, *t* test was used. Results were considered significant at a *P* value of <0.05. Experiments were repeated at least twice.

Results

Presence of neutrophils increased the number of melanoma cells retained in lungs

To test the hypothesis that entrapped metastatic melanoma cells secrete inflammatory cytokines attracting neutrophils, resulting in cellular interactions promoting melanoma cell retention in the lungs, GFP-tagged 1205 Lu human melanoma cells were injected into the lateral tail vein of nude mice, and 1 hour later, unlabeled human neutrophils were injected. Significant numbers of entrapped cancer cells were observed in the lungs of animals after 30 minutes (Fig. 1A); however, 24 hours later, few were present due to shear force–related circulatory motion (Fig. 1A). In contrast, injection of human neutrophils 1 hour after injection of GFP-tagged 1205 Lu melanoma cells led

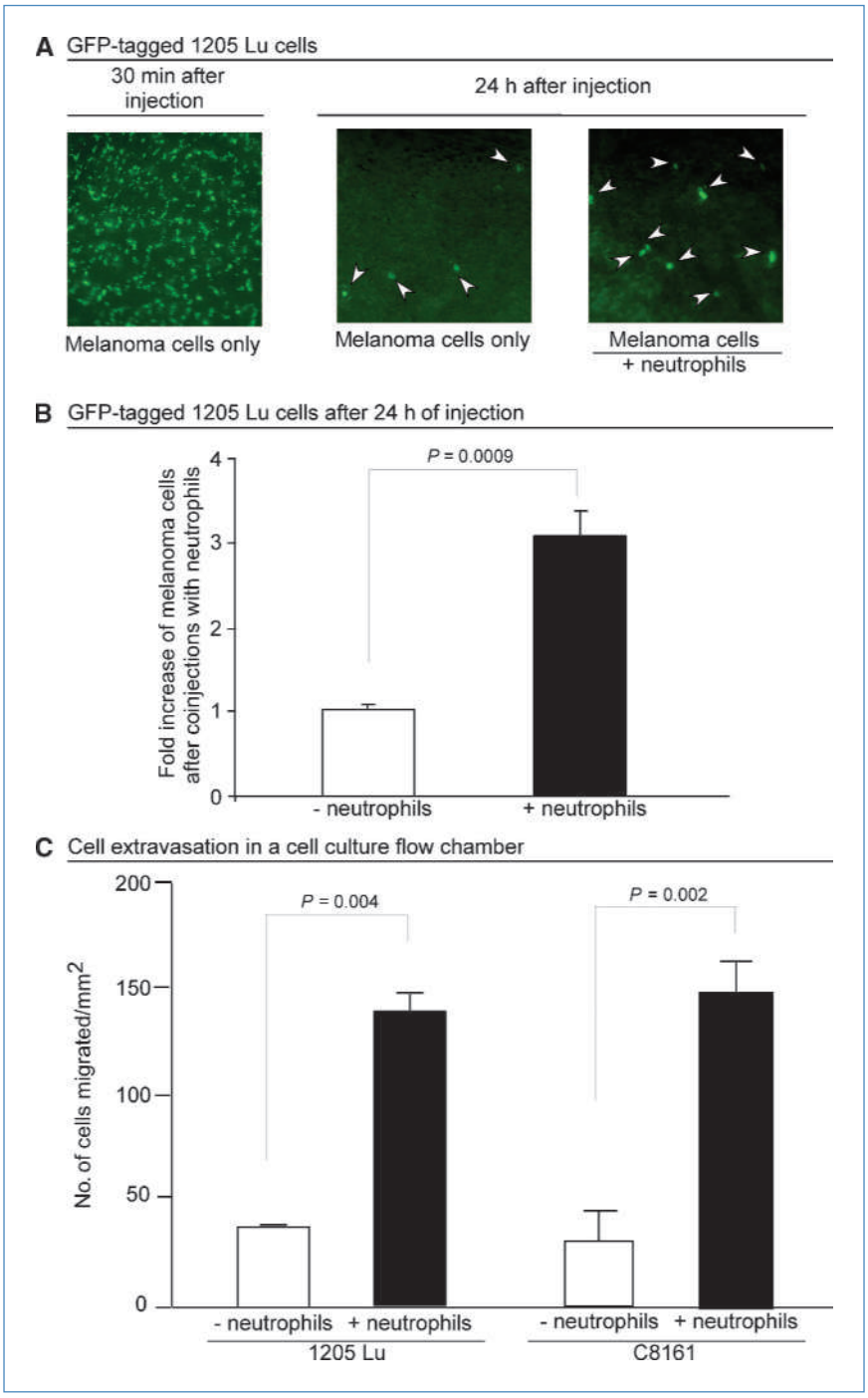

Figure 1. Neutrophils increase number of melanoma cells retained in lungs. A, i.v. injected melanoma cells become transiently entrapped in lungs 30 min after injection (left). Twenty-four hours later, very few remained (middle). I.v. injection of neutrophils 1 h later increased melanoma cell retention in the lungs (right). Arrowheads, GFP-tagged melanoma cells (×48). B, melanoma cells that were retained in the lungs were scored in the presence or absence of injected neutrophils, showing a ~3-fold increase following neutrophil injection. C, melanoma cells migrated more frequently across an endothelial-like cell layer in a flow chamber when cocultured with neutrophils. Columns, mean representing at least two independent experiments; bars, SEM.

to a ~3-fold increase in retained cancer cells 24 hours later (Fig. 1A and B).

Because neutrophils were hypothesized to be regulating tumor cell retention, interaction was studied using an *in vitro* flow migration chamber mimicking blood flow over an endothelial-like cell layer under which a chemoattractant was placed (8, 22, 40). Combining C8161 or 1205 Lu metastatic melanoma cells with neutrophils in this model led to a 4- to 5-fold increase in migrated cells across the monolayer compared with control melanoma cells alone (Fig. 1C). This observation led to the hypothesis that neutrophils might assist entrapped melanoma cells in being anchored in place and retained in the lung circulation by resisting circulatory shear forces to promote metastasis development. However, identity of potential cytokines regulating this process was unknown.

High levels of IL-8 are produced in and secreted from metastatic melanoma cells

IL-8 is a neutrophil-activating chemokine known to promote melanoma metastasis, but the mechanism remains to be identified in animals (34, 44). To determine whether IL-8 secreted from melanoma cells plays a role together with neutrophils in retaining entrapped melanoma cells in lungs, protein expression and secretion were initially compared between highly metastatic variants and related original parental melanoma cell lines. Although levels of IL-8 protein in normal human melanocytes or early-stage melanoma WM35 cells were similar to those observed in poorly metastatic melanoma cell lines, highly metastatic variants generally contained significantly higher concentrations (Fig. 2A). The exception was UACC 903 that expressed similar levels to that observed in WM35 cells; however, derivative UACC 903M cells contained 7-fold higher levels than the parental cell line. Derivative melanoma cell lines A375M, UACC 903M, and C8161.Cl9 with higher metastatic potential also secreted more IL-8 into the culture media than each respective parental cell line (Fig. 2B). Secreted IL-8 from C8161.Cl9 cells was not as high as that observed for A375M and UACC 903M cells compared with the parental versions. Thus, compared with less metastatic melanoma cells, derivative metastatic variants tended to secrete more IL-8, suggesting it may play a role in neutrophil-mediated retention of melanoma cells in lungs.

Because IL-8 was secreted from metastatic melanoma cells, its effect was next examined on IL-8 expression in and secretion from neutrophils or neutrophil-melanoma cocultures. Neutrophils were stimulated with recombinant human IL-8 causing an increase of 30% to 50% in endogenous

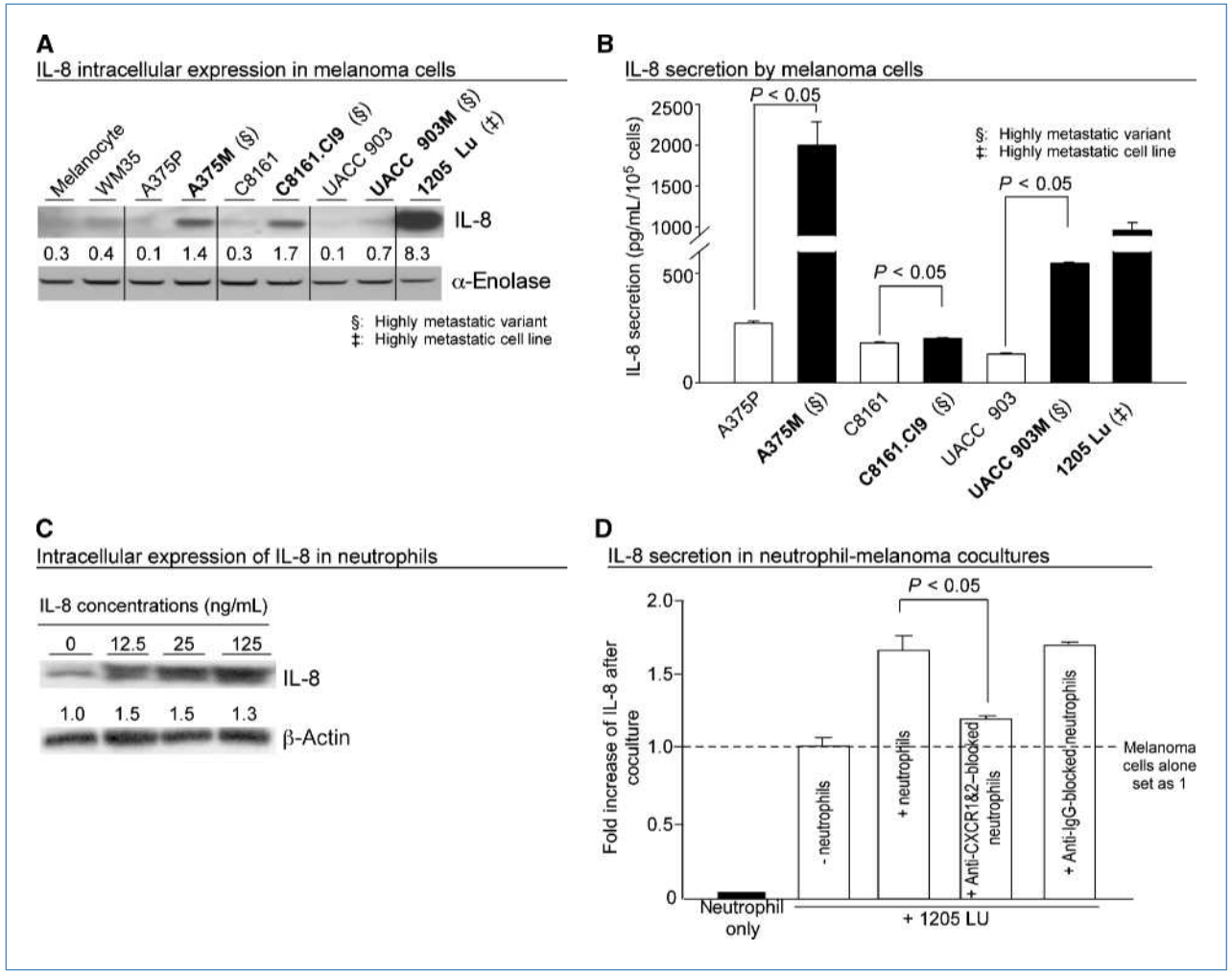

Figure 2. Increased IL-8 was present in metastatic variants compared with less aggressive parental melanoma cell lines. Endogenous (A) and secreted IL-8 (B) tended to be higher in melanoma cell lines with greater metastatic potential (labeled bold; § and ‡) compared with matched less metastatic parental cells. C, human recombinant IL-8 (12.5–125 ng/mL) led to a corresponding increase in endogenous IL-8 expression in neutrophils. D, cocultured melanoma and neutrophils led to increased IL-8 secretion. Anti-CXCR1/2 antibodies neutralized secreted IL-8 compared with negative control mouse anti-IgG. Columns, mean representing at least two independent experiments; bars, SEM.

levels compared with nontreated neutrophils (Fig. 2C). Adding 1205 Lu melanoma cells to neutrophils increased IL-8 secretion from neutrophils by ~40%, which could be blocked by pretreating neutrophils with anti-CXCR1/2 antibodies to saturate the IL-8 receptors on the neutrophils (Fig. 2D). Thus, IL-8 secreted from melanoma cells induced IL-8 levels secreted into neutrophil-melanoma coculture media.

SiRNA-mediated targeting of IL-8 reduced protein expression in and secretion from melanoma cells

To determine whether IL-8 played a role regulating melanoma tumor cell retention in lungs, a siRNA-based model was developed to decrease protein expression in cells and the effect on levels of secreted protein was measured, using an established published approach (22). SiRNA targeting two different regions of the IL-8 mRNA was introduced into 1205 Lu, UACC 903M, and C8161.Cl9 cell lines through nucleofection, and Western blotting was used to show that protein levels were reduced by 80% to 100% compared with buffer or scrambled siRNA controls (Fig. 3A). Because siRNA siIL-8 #1 was ineffective in C8161.Cl9 cells, only siIL-8 #2 was used in this cell line (Fig. 3A). Similar to other reports using siRNA to target genes (38), 30% to 70% lower levels of IL-8 protein compared with controls were observed in UACC 903M, 1205Lu, and C8161Cl.9 cells for 6 to 8 days after nucleofection, showing efficacy of this model for decreasing IL-8 levels in cultured cells (Fig. 3A).

To show that decreasing protein levels in melanoma cells reduced secreted IL-8 concentrations, conditioned media were collected from melanoma cells nucleofected with siRNA targeting IL-8 or controls, and protein levels were quantified using ELISA. SiRNA targeting IL-8 reduced secretion for 1205 Lu cells by ~3.5-fold from 300 to 80 pg/mL/10^5 cells, for UACC 903M cells by ~3-fold from 50 to 15 pg/mL/10^5 cells, and for C8161.Cl9 cells by ~2-fold from 28 to ~12 pg/mL/10^5 cells (Fig. 3B). Thus, siRNA can reduce the levels of IL-8 produced in and secreted by melanoma cells, making this a suitable model to dissect the involvement of this chemokine in melanoma cell retention in the lungs and its involvement with neutrophils.

Growth and tumorigenic potential of metastatic melanoma cells is not affected by decreasing IL-8 levels

Because metastatic melanoma cells tended to express and secrete IL-8, siRNA was used to reduce protein levels and

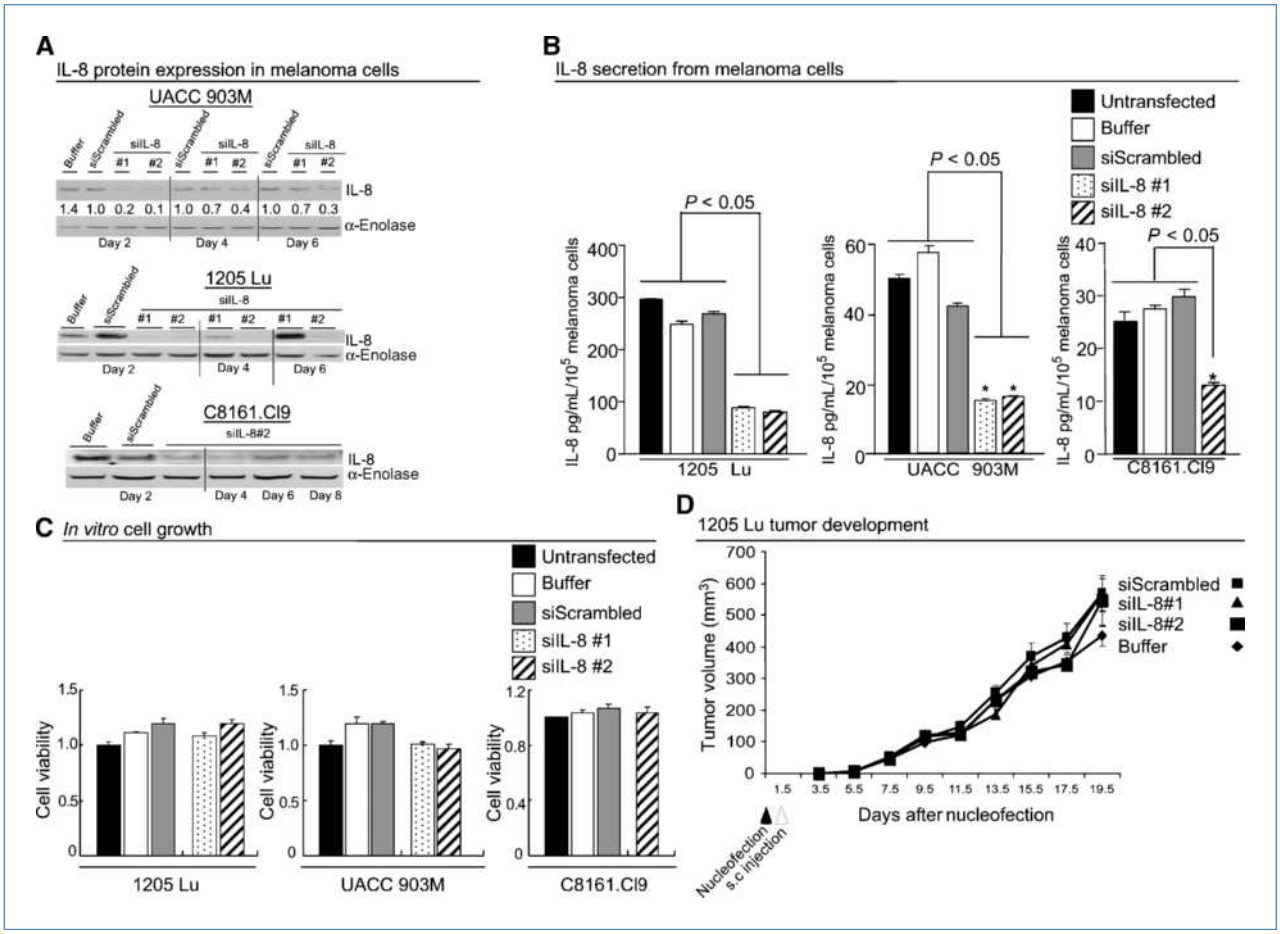

Figure 3. IL-8 does not regulate cellular growth or tumorigenesis. SiRNA-mediated knockdown of IL-8 protein expression (A) and secretion (B) from melanoma cells. Decreasing IL-8 levels did not affect growth of cultured metastatic melanoma cells (C) or the tumorigenic potential of cells (D). Columns, mean representing at least two independent experiments; bars, SEM.

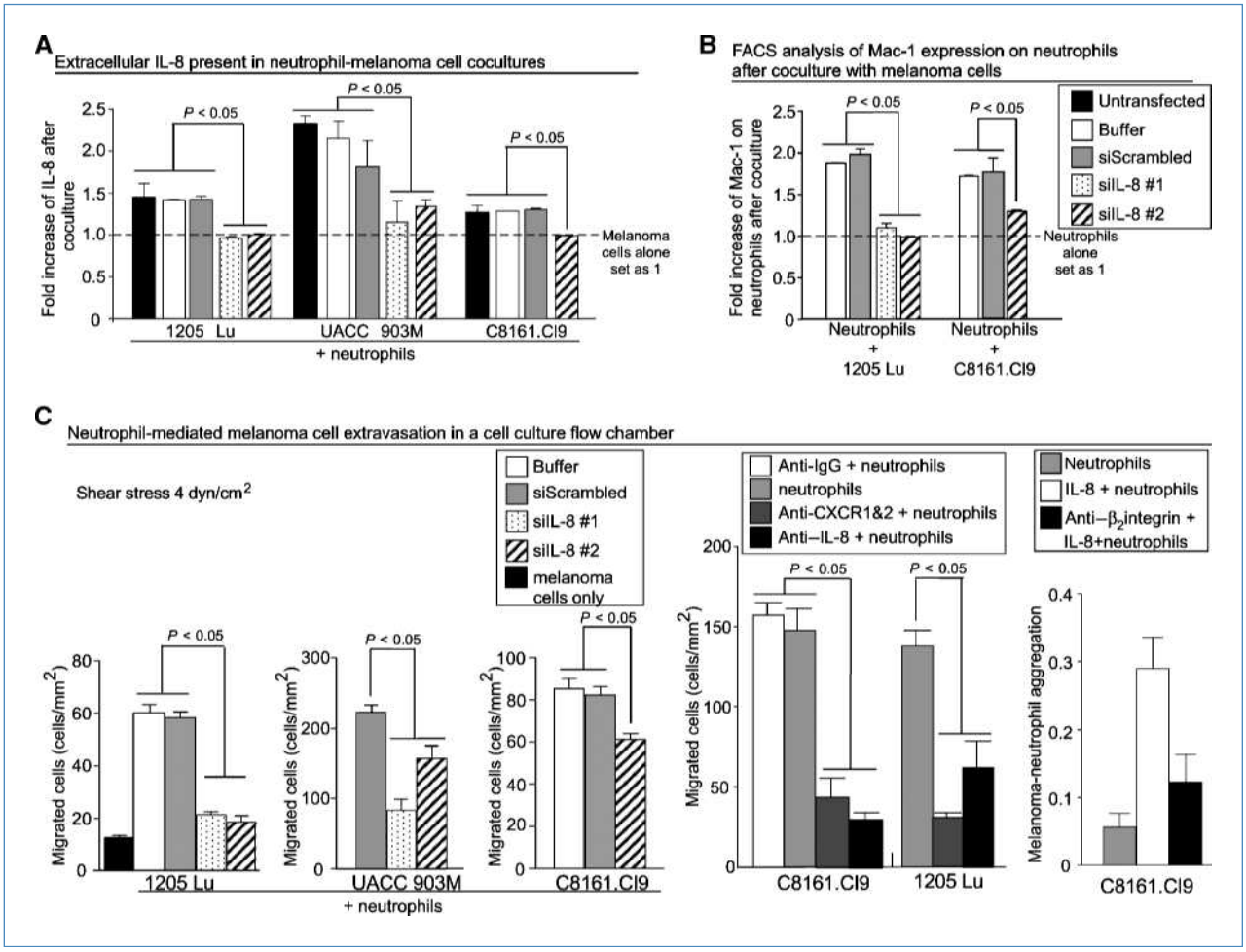

Figure 4. Decreasing IL-8 protein levels in metastatic melanoma cells reduced neutrophil-mediated extravasation across an endothelial-like cell layer under flow conditions by disrupting ICAM-1/Mac-1 binding. SiRNA-mediated targeting of IL-8 in melanoma cells decreased extracellular protein present in neutrophil cocultures (A) and reduced Mac-1 expression on neutrophils (B). Values were normalized to background Mac-1 expression on neutrophils alone. C, decreasing secreted IL-8 using siRNA or neutralizing the chemokine using antibodies directed against CXCR-1/2 or IL-8 decreased melanoma cell migration across an endothelial-like layer under flow conditions. Columns, mean representing at least two independent experiments; bars, SEM.

effects on cultured cell growth and tumorigenic potential measured. Decreasing IL-8 protein levels had negligible effect on the proliferative potential of 1205 Lu, UACC 903M, or C8161.Cl9 cells compared with untransfected cells or those controls nucleofected with buffer or scrambled siRNA (Fig. 3C). To determine whether decreasing IL-8 protein levels in metastatic melanoma cells would alter the tumorigenic potential of the cells, siRNA was introduced into 1205 Lu cells, which were then injected s.c. into nude mice. The size of developing tumors was measured on alternate days up to 19.5 days. Consistent with a negligible effect on growth of cultured cells, decreasing IL-8 protein levels did not alter the rate at which 1205 Lu tumors developed compared with control cells nucleofected with buffer or scrambled siRNA (Fig. 3D). Thus, decreasing IL-8 did not alter the growth of cultured cells or the tumorigenic potential of metastatic melanoma cells.

SiRNA-mediated targeting of IL-8 decreased extracellular levels in neutrophil-melanoma cocultures

To determine whether reducing IL-8 protein levels would decrease extracellular concentrations when melanoma cells were cocultured with neutrophils, IL-8 secretion from the neutrophil-melanoma cocultures was measured following the introduction of siRNA targeting IL-8 into melanoma cells. Media, in which melanoma cells were cultured alone or with neutrophils, were collected, and IL-8 concentration was measured by ELISA. Melanoma cells cultured alone were set as a value of 1, and fold change in IL-8 after coculture was reported (Fig. 4A). Melanoma cells cocultured with neutrophils had 1.3- to 2-fold higher levels of extracellular IL-8 compared with melanoma cells cultured alone. In contrast, decreasing IL-8 protein levels in melanoma cells reduced concentrations present in the extracellular neutrophil-melanoma cell coculture environment compared with

untransfected cells or controls nucleofected with buffer only or scrambled siRNA (Fig. 4A). Thus, decreasing IL-8 present in melanoma cells reduced concentrations in the extracellular neutrophil-melanoma cell environment.

Decreasing IL-8 levels in melanoma cells disrupted neutrophil-melanoma interactions

β_2 Integrin on neutrophils and ICAM-1 on melanoma cells promote neutrophil-melanoma cell interactions (32, 45), which can enhance the attachment of melanoma cells to the endothelium under flow conditions aiding transit across this layer (8, 40). Melanoma cell lines 1205 Lu, C8161.Cl9, and UACC 903M all express ICAM-1 (8, 22, 45); therefore, to show that melanoma secreted IL-8–regulated β_2 integrin expression on neutrophils and thereby neutrophil-melanoma cell interaction, siRNA was used to decrease IL-8 secretion in 1205 Lu or C8161.Cl9 melanoma cells, which were then cocultured with neutrophils. Fluorescence-activated cell sorting analysis measured β_2 integrin levels, specifically Mac-1, on neutrophils present in cocultures. Mac-1 levels present on neutrophils cultured alone were set at 1, and fold change on the surface of neutrophils cultured with melanoma cells was reported (Fig. 4B). Neutrophils cultured with control melanoma cells nucleofected with buffer or scrambled siRNA had 80% higher levels of Mac-1 compared with those nucleofected with siRNA targeting IL-8 (Fig. 4B). Thus, IL-8 secreted from melanoma cells regulates Mac-1 levels on neutrophils, which can enhance neutrophil-melanoma cell interaction, potentially aiding tethering to the vascular endothelium.

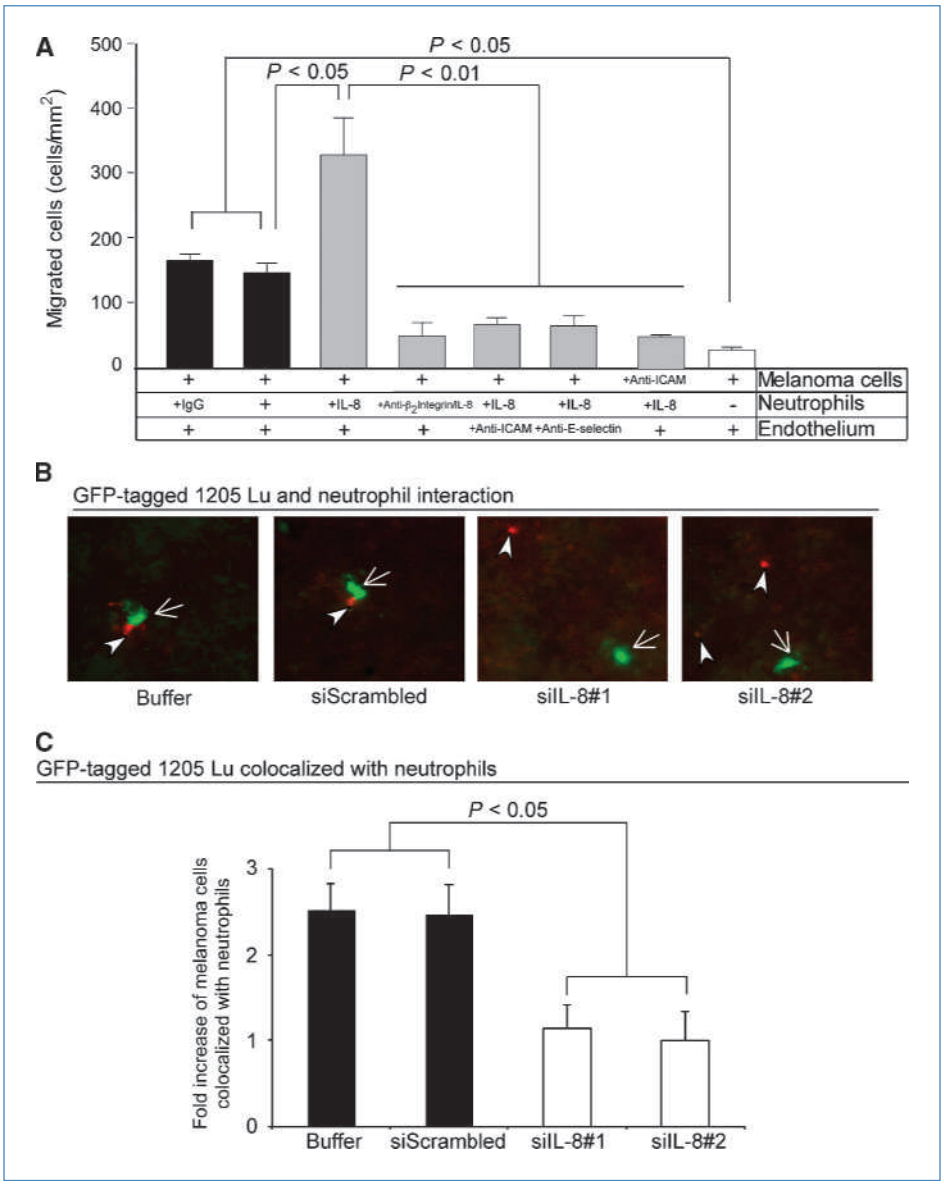

Figure 5. Decreasing IL-8 secretion from melanoma cells reduced interaction with exogenously added human neutrophils in lungs. A, neutrophils facilitate melanoma cell migration through an adhesion-mediated mechanism. Decreased IL-8 secretion from melanoma cells reduced human neutrophil colocalization with melanoma cells in the lungs of nude mice (B and C). B, colocalized neutrophils (red; arrowheads) and melanoma cells (green; arrows) were more abundant in controls compared with melanoma cells having reduced IL-8 secretion (×100). Columns, mean representing at least two independent experiments; bars, SEM.

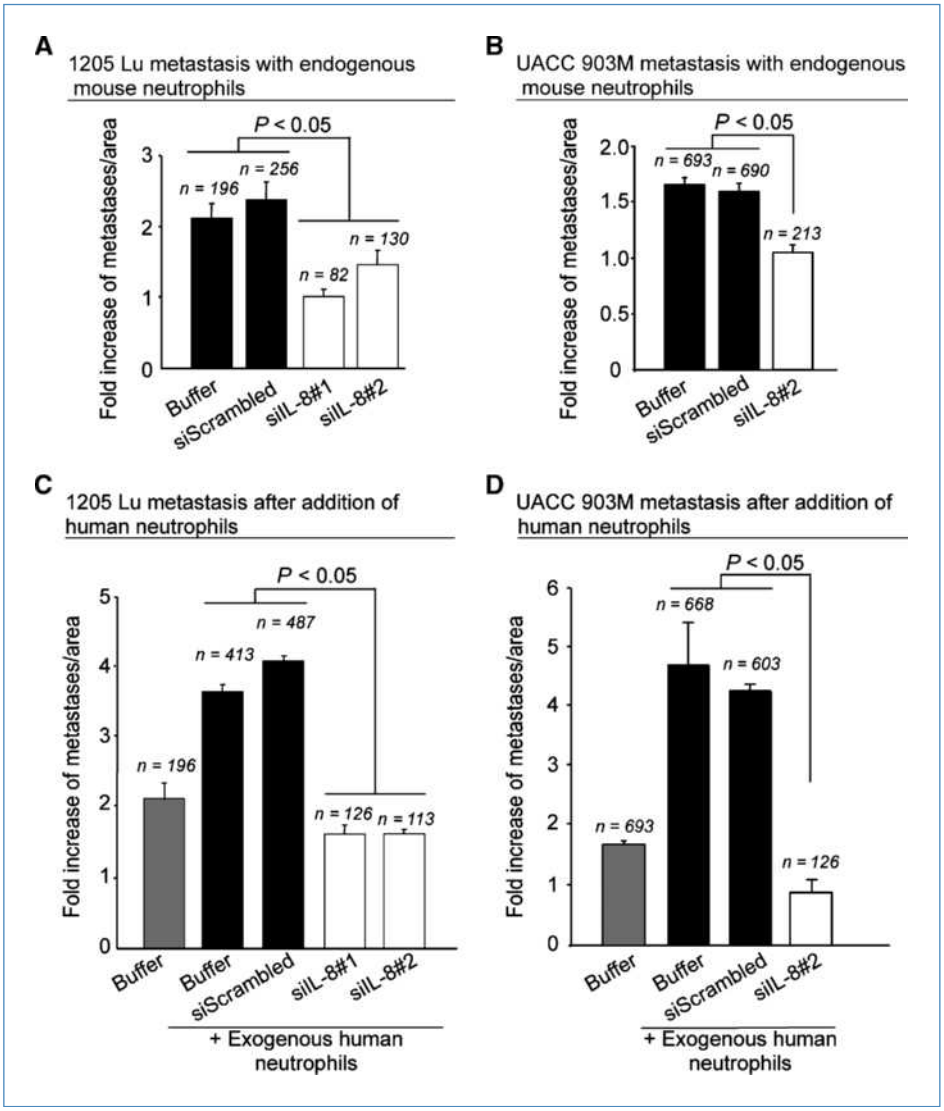

Figure 6. Decreasing IL-8 expression in melanoma cells reduced lung metastases formation. SiRNA-mediated targeting of IL-8 in melanoma cells, decreased development of GFP-tagged 1205 Lu (A), or UACC 903M (B) lung metastasis development in the presence of endogenous mouse neutrophils. C and D, injection of exogenous human neutrophils doubled the rate at which melanoma lung metastases developed. Decreasing IL-8 secretion from melanoma cells reduced lung metastasis formation to control levels. Columns, mean representing at least two independent experiments; bars, SEM.

IL-8 secreted from melanoma cells induced neutrophil-mediated melanoma extravasation under flow conditions

To determine whether IL-8 secreted by melanoma cells would regulate neutrophil-tethering to the endothelial-like cell layer and subsequently regulate melanoma cell extravasation across it under flow conditions, IL-8 protein levels in 1205 Lu, UACC 903M, and C8161.Cl9 cells were decreased using siRNA. Migration of melanoma cells across the endothelial-like cell layer was quantified in the presence or absence of neutrophils within the flow chamber under shear stress of 4 dyn/cm^2 for 4 hours. The existence of neutrophils increased 1205 Lu melanoma cell migration under flow condition by ~6-fold. Decreasing IL-8 secretion from melanoma cells reduced neutrophil-mediated melanoma cell transit across the endothelial cell layer by 70% for 1205 Lu cells, by 30% to 60% for UACC 903M cells, and by 25% for C8161.Cl9 cells compared with untransfected or control cells nucleofected with buffer only or scrambled siRNA (Fig. 4C, left).

To validate the role of IL-8 in neutrophil-mediated melanoma extravasation under flow conditions, melanoma cells were cocultured with neutrophils in the absence and presence of neutralizing anti-CXCR1/2 or anti–IL-8 antibodies. Targeting IL-8 receptors on neutrophils or blocking IL-8 in media reduced neutrophil-mediated melanoma extravasation by 75% for C8161.Cl9 cells and by 60% to 75% for 1205 Lu cells compared with nontreated melanoma-neutrophil cocultures or use of an anti-IgG antibody control (Fig. 4C, middle). Therefore, secreted IL-8 levels induced melanoma-neutrophil interaction and subsequent extravasation under flow conditions. To confirm that neutrophil-mediated melanoma extravasation under flow conditions is through the β_2 integrins of neutrophils, we have quantified heterotypic aggregation of neutrophil and melanoma in a parallel-plate flow assay. Blocking β_2 integrins of neutrophils reduced IL-8–stimulated neutrophil-melanoma aggregation by ~50% compared with IL-8–stimulated neutrophil-melanoma cells (Fig. 4C, right). Thus, β_2 integrin on neutrophils promotes neutrophil-melanoma cell interactions to mediate extravasation.

Neutrophil-melanoma extravasation is through a cell adhesion–mediated mechanism under flow conditions

To show that neutrophil-melanoma migration is mediated through β_2 integrin/ICAM-1 adhesion, β_2 integrin and ICAM-1 were blocked using antibodies on neutrophils and melanoma cells, respectively (Fig. 5A). The existence of neutrophil significantly increased C8161Cl.9 migration compared with C8161Cl.9 alone under flow conditions by ~8-fold. Stimulation of neutrophils with IL-8 further increased C8161Cl.9 migration under flow conditions compared with IL-8–unstimulated neutrophils. ICAM-1 plays an important role in neutrophil-facilitated melanoma cell migration. Targeting β_2 integrin on neutrophils or ICAM-1 on melanoma cells and the endothelium decreases neutrophil-mediated melanoma cell migration by 7-fold compared with IL-8–stimulated neutrophil-melanoma cocultures. In addition, blocking E-selectin on the monolayer endothelium inhibited IL-8–stimulated neutrophil interaction and subsequent C8161Cl.9 cell migration by ~7-fold. Thus, ICAM-1 on melanoma cells and β_2 integrin on neutrophils interacted, promoting anchoring to vascular endothelium.

Entrapped melanoma cells in lungs secrete IL-8 to attract neutrophils, thereby promoting retention

To show that metastatic melanoma cells entrapped in lungs secrete IL-8, which recruited neutrophils resulting in cellular interactions promoting melanoma cell retention, GFP-tagged 1205 Lu human melanoma cells nucleofected with siRNA targeting IL-8 were injected into the lateral tail vein of nude mice. One hour later, human CellTracker Orange CMTMR–stained human neutrophils were injected in the opposite tail vein. Twenty-four hours later, colocalized green melanoma cells and red neutrophils were photographed and quantified (Fig. 5B and C). Decreasing IL-8 expression in melanoma cells reduced colocalization with neutrophils by ~60% compared with buffer and scrambled siRNA controls (Fig. 5C). CellTracker Orange CMTMR–labeled neutrophils only were also injected to the tail vein without melanoma cells. However, 24 hours after tail vein injection, the number of neutrophils entrapped in the lungs was negligible (data not shown).

Thus, entrapped melanoma cells secrete IL-8 to attract neutrophils, which then interact with the melanoma cells promoting shear-resistant retention within the lung circulation to enhance extravasation under flow conditions and subsequent metastasis development.

Decreasing secreted IL-8 from metastatic melanoma cells reduced lung metastasis development

Although decreasing IL-8 secreted from melanoma cells led to less interaction with neutrophils and retention of fewer cells in lungs, it was uncertain whether retained cells would develop into lung metastases. Therefore, siRNA was used to decrease IL-8 protein levels in GFP-tagged 1205 Lu or UACC 903M cells that were injected into the tail vein of nude mice with only the endogenous mouse neutrophils present. Total number of metastatic nodules in the lungs was quantified by fluorescence microscopy 18 days later. Reducing IL-8 expression in and secretion from melanoma cells decreased number of metastases by 50% to 60% for 1205 Lu (Fig. 6A) and 30% to 40% for UACC 903M (Fig. 6B) cells. Similar results were observed for C8161.Cl9 cells (data not shown).

To show that addition of human neutrophils into the mouse bloodstream could further promote metastasis, GFP-tagged 1205 Lu cells or UACC 903M cells having endogenous or reduced IL-8 expression were injected into the lateral tail vein of a nude mouse. One hour later, human CellTracker Orange CMTMR–stained human neutrophils were injected in the opposite tail vein. Total number of metastatic nodules in the lungs was quantified by fluorescence microscopy 18 days later. Addition of human neutrophils doubled the number of metastases developing in the lungs of mice compared with that observed with just endogenous mouse neutrophils and siRNA-mediated targeting of IL-8 reduced interaction to that observed in control cells (Fig. 6C; scale set to that in Fig. 6A and D; scale set to that in Fig. 6B). Thus, decreasing IL-8 levels secreted by melanoma cells reduced interaction with neutrophils, which led to the development of fewer lung metastases.

Discussion

Controversy about the multiple functions played by IL-8 necessitates studies such as this one, dissecting its different roles, which seem to be dependent on tumor stage, microenvironment, and intercellular interactions. This report shows that entrapped circulating melanoma cells in lungs secrete IL-8 to recruit neutrophils, which aids anchoring the cancer cells in the lungs to promote metastasis (32). Although IL-8 lies downstream of ^{V600E}B-Raf in the MAPK pathway, which is known for promoting proliferation (22), this study suggests it does not modulate growth but rather regulates metastasis by controlling interaction with neutrophils (16, 18, 27).

Tumor infiltration by immune cells occurs in most tumors (46). Neutrophils infiltrate early melanomas to inhibit growth (14, 21, 47) and are also present in advanced tumors secreting IL-8 (14). Early melanomas might not secrete IL-8 to reduce neutrophil infiltration and immune cell–mediated tumor cell death. However, as shown in this study, more aggressive metastatic melanomas have increased intracellular and secreted IL-8 levels to recruit neutrophils, serving to aid shear-resistant adhesion to the vascular endothelium to promote extravasation and metastasis.

Mechanistically, decreasing secreted IL-8 from melanomas disrupted interactions between ICAM-1 expressed on melanoma cells and β_2 integrins (especially Mac-1) on neutrophils, which reduced anchoring of entrapped melanoma cells to the lung endothelium. Previously, IL-8 secretion from melanoma cells has been shown to induce secretion from neutrophils in cocultures (25), which occurred in this study. IL-8 secretion from neutrophils can further enhance the strength, stability, or affinity of interaction between neutrophils and melanoma cells to promote metastasis. Thus, melanoma cell–recruited neutrophils can play an important role in modulating metastasis by holding transiently entrapped

melanoma cells in place within the circulation in the lungs for a sufficient period of time to facilitate extravasation across the endothelial lining to promote development of metastases.

Although the importance of neutrophil-mediated melanoma extravasation in lung tissue is shown in this report, it is speculated that this process might also occur in other organs. It is also possible that neutrophils hold melanoma cells in place in the capillaries until the cells grow into a secondary tumor, which is a possibility that has not been explored in this study. However, neutrophil-mediated melanoma extravastion may only be a sufficient and not a necessary mechanism to promote metastasis. It is possible that metastasis involves more than one mechanism, possibly also involving cancer cell entrapment only, passage through a leaky vasculature, or metastasis through the lymphatic system (48–50).

Technologies with potential to therapeutically target and reduce IL-8 levels in entrapped or circulating melanoma cells might use a liposome carrying siRNA (39) or an antibody targeting secreted IL-8 (15, 19, 24). The obstacle for liposomal and antibody-mediated targeting of IL-8 would be the removal of all protein in the animal, which could cause adverse side effects. Targeting the liposomes or antibody might overcome these obstacles. If IL-8–targeting agents were combined with other therapeutics inhibiting the activity of major signaling pathways deregulated in melanomas, such as the ^{V600E}B-Raf and Akt3 cascades (35, 38), it might be possible to more effectively treat advanced-stage melanomas, possibly resulting in a cooperative synergistically acting drug regimen.

In summary, secreted IL-8 from entrapped melanoma cells did not affect cellular growth or tumor development but attracted neutrophils and upregulated their β_2 integrin expression. This promoted shear-resistant binding between ICAM-1–expressing melanoma cells and neutrophils to the endothelium, thereby aiding melanoma extravasation and subsequent lung metastases development. Thus, IL-8 plays an important role in neutrophil-mediated melanoma cell retention in the lungs and, if targeted, could have significant therapeutic potential to reduce metastasis development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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