Published in final edited form as:
Cancer Res. 2010 July 15; 70(14): 6071–6082. doi:10.1158/0008-5472.CAN-09-4442.

Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development

Sung Jin Huh[1], **Shile Liang**[7], **Arati Sharma**[1,5], **Cheng Dong**[5,7], and **Gavin P. Robertson**[1,2,3,4,5]

[1]Department of Pharmacology, The Pennsylvania State University College of Medicine, Hershey, PA 17033.

[2]Department of Pathology, The Pennsylvania State University College of Medicine, Hershey, PA 17033.

[3]Department of Dermatology and Surgery, The Pennsylvania State University College of Medicine, Hershey, PA 17033.

[4]The Foreman Foundation for Melanoma Research, The Pennsylvania State University College of Medicine, Hershey, PA 17033.

[5]Penn State Melanoma Therapeutic Program, The Pennsylvania State University College of Medicine, Hershey, PA 17033.

[7]Department of Bioengineering, The Pennsylvania State University, University Park, PA 16082.

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 intravenously injected into nude mice leading to 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 IL-8, attracting neutrophils and increasing tethering β_2 integrin expression by 75-100%. ICAM-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 20-50%, decreased β_2 integrin on neutrophils by ~50% and reduced neutrophil-mediated extravasation by 25-60%, 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.

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.

Corresponding Authors: Gavin P. Robertson. gprobertson@psu.edu, Telephone: (717) 531-8098, Fax: (717) 531-5013. Cheng Dong. cxd23@psu.edu, Telephone: (814) 865-8091, Fax: (814) 863-0490.

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 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). While 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 up-regulation of adhesion molecule β_2 integrin (e.g., CD11a/CD18 or LFA-1; and CD11b/CD18 or Mac-1) (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 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 PI3K and/or 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 AP-1 (21) to increase 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 MMP2 expression in bladder cancer and melanomas (19,23,24). Furthermore, melanoma cell secreted IL-8 can trigger neutrophils to secrete more protein to increase extracellular concentrations (25). While IL-8 has multiple roles in tumor development (16,18,19,26-29), 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, interferon-gamma (IFN-γ), or C5a (11). Melanoma cells secrete IL-8, which can attract and activate neutrophils by increasing β_2 integrins expression and induce further IL-8 secretion (30). Interaction between 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). While 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 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.

METHODS

Cell lines and neutrophils

Melanoma cell lines were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT). WM35 was maintained as described previously (35). To generate highly metastatic variants, 0.5×10^6 GFP-tagged A375M, C8161.Cl9 or UACC 903 cells were injected into the lateral tail vein of nude mice. Mice were sacrificed 2-4 weeks later and GFP-tagged tumors 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 grown in DMEM supplemented with 10% FBS containing penicillin/streptomycin. Procedure was repeated 2-3 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, St. Louis, MO) 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, Camarillo, CA). Fresh blood was collected from healthy donors according to an IRB approved protocol.

Small interfering RNA (siRNA) targeting IL-8

SiRNA (100 pmol) was introduced into 1.0×10^6 1205 Lu, C8161.Cl9 or UACC 903M via nucleofection using an Amaxa Nucleofector using Solution R/program K-17 (22,36-38). Transfection efficiency was >95% with 80-90% cell viability (35,37). Following siRNA introduction and 2-day recover in culture dishes, cells were replated in 96-well plates. 5-days later, cell viability was measured by MTS assay (Promega, Madison, WI) (39). Duplexed Stealth siRNA (Invitrogen, Carlsbad, CA) were used. siRNA sequences were: Scrambled: AAUUCUCCGAACGUGUCACGUGAGA; ^{V600E}*B-Raf:* GGUCUAGCUACAGAGAAAUCU CGAU; *IL-8#1*: GCAGCUCUGUGUGAAGGUGCAGUUU; *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., Camarillo, CA), α-enolase (Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (Sigma Chemical Co.).

IL-8 ELISA

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

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 bottom plate separated by a gasket (8,22,40). Before each experiment, EI cells expressing ICAM-1 comparable with IL-1β stimulated HUVEC were used as an endothelial substrate (provided by Dr. Scott Simon, UC Davis) and grown on sterile polyvinylpyrrolidone-free polycarbonate filters (8 μm pore size; NeuroProbe) precoated with fibronectin (30 μg/ml, for 3 hours; Sigma). 12 center wells of

the bottom plate were filled with soluble chemoattractant type IV collagen (100 μg/ml in RPMI 1640/0.1% 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 NIH ImageJ software (43). In IL-8 neutralization studies, neutrophils were treated with anti-human CXCR1/2 antibody (Sigma Chemical Co.) to block these receptors for 30 minutes at 37°C prior to 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, Carlsbad, CA) 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 (1μg/10^6 cells; Jackson ImmunoResearch, West Grove, PA) at 4°C, the cells were washed twice and fixed with 2% formaldehyde and analyzed by a GUAVA personal flow cytometer (GUAVA technologies Inc., Burlingame, CA).

Parallel-plate flow assays

C8161 cell adhesion to EI monolayer in the presence of neutrophils was performed in a parallel-plate flow chamber (Glycotech, Rockville, MD) mounted on the stage of a phase-contrast optical microscope (Diaphot 300, Nikon, Japan). A syringe pump (Harvard Apparatus, South Natick, MA) 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 2-3 min at a shear rate of 40 sec^{-1} for equilibration before the introduction of 1×10^6 cells/ml of neutrophils and C8161 cells. Neutrophils were either activated by IL-8 (5ng/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 0.1-0.3 dyn/cm^2 for 2 min, shear stresses were adjusted to the experimental range of 0.6 dyn/cm^2 and kept constant for 5 min. The aggregation of C8161 and neutrophils was quantified and normalized by the total number of collisions as:

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

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

Interaction of melanoma cells with human neutrophils in the lungs

100 pmol of siRNA 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 collected. Isolated neutrophils were stained with CellTracker Orange CMTMR (C2927, Invitrogen, Carlsbad, CA) according to manufactures 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, lungs 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 co-localized with neutrophils. Percentages were calculated by number of melanoma cells interacting with neutrophils/total number of melanoma cells in each field.

Mouse experimental metastasis assay

100 pmol of siRNA 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, injected into the lateral tail vein of nude mice. Mice were sacrificed 18 days later, necropsied, and lungs analyzed for presence of fluorescent metastatic lesions using a Nikon SMZ 1500 dissecting microscope with a Plan Apo 1.6x objective. Images were photographed at 48x magnification from lung ventral surface and number as well as area occupied by fluorescent metastatic lesions scored in pixels using IP lab imaging software (Scanalytics, Fairfax, VA). 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 and for comparison between two groups, t-test were used. Results were considered significant at a *P*-value of <0.05. Experiments were repeated at least twice.

RESULTS

Presence of neutrophils increased 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 injected. Significant numbers of entrapped cancer cells were observed in the lungs of animals after 30 minutes (Figure 1A); however, 24 hours later, few were present due to shear force related circulatory motion (Figure 1A). In contrast, injection of human neutrophils 1-hour after injection of GFP-tagged 1205 Lu melanoma cells led to a ~3-fold increase in retained cancer cells 24-hours later (Figures 1A and B).

Since 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 was placed a chemoattractant (8,22,40). Combining C8161 or 1205 Lu metastatic melanoma cells with neutrophils in this model led to a 4-5-fold increase in migrated cells across the monolayer compared to control melanoma cells alone (Figure 1C). This observations 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. While levels of IL-8 protein in normal human melanocytes or early stage melanoma WM35 cells were similar to those observed in poorly metastatic melanoma cells lines, highly metastatic variants generally contained significantly higher concentrations (Figure 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 that 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 (Figure 2B). Secreted IL-8 from C8161.Cl9 cells were not as high as those observed for A375M and UACC 903M cells compared to the parental versions. Thus, in comparison to 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.

Since 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 co-cultures. Neutrophils were stimulated with recombinant human IL-8 causing an increase of 30-50% in endogenous levels compared to untreated neutrophils (Figure 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 (Figure 2D). Thus, IL-8 secreted from melanoma cells induced IL-8 levels secreted into neutrophil-melanoma co-culture 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 effect on levels of secreted protein measured, using an established published approach (22). SiRNA targeting two different regions of the IL-8 mRNA were introduced into 1205 Lu, UACC 903M and C8161.Cl9 cell lines via nucleofection and Western blotting used to show that protein levels were reduced by 80-100% compared to buffer or scrambled siRNA controls (Figure 3A). Since siRNA siIL-8 #1 was ineffective in C8161.Cl9 cells only siIL-8 #2 was used in this cell line (Figure 3A). Similar to other reports using siRNA to target genes (38), 30-70% lower levels of IL-8 protein compared to controls were observed in UACC 903M, 1205Lu and C8161Cl.9 cells for 6-8 days after nucleofection demonstrating efficacy of this model for decreasing IL-8 levels in cultured cells (Figure 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 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 (Figure 3B). Thus, siRNA can reduce levels of IL-8 produced in and secreted by melanoma cells, making this a suitable model to dissect 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

Since metastatic melanoma cells tended to express and secrete IL-8, siRNA was used to reduce protein levels and 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 to untransfected cells or those controls nucleofected with buffer or scrambled siRNA (Figure 3C). To determine whether decreasing IL-8 protein levels in metastatic melanoma cells would alter the cells' tumorigenic potential, siRNA was introduced into 1205 Lu cells, which were then injected subcutaneously into nude mice. 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 to control cells nucleofected with buffer or scrambled siRNA (Figure 3D). Thus, decreasing IL-8 did not alter growth of cultured cells or the tumorigenic potential of metastatic melanoma cells.

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

To determine whether reducing IL-8 protein levels would decrease extracellular concentrations when melanoma cells were co-cultured with neutrophils, IL-8 secretion from the neutrophil-melanoma co-cultures was measured following 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 measured by ELISA. Melanoma cells cultured alone were set as a value of 1 and fold change in IL-8 after co-culture reported (Figure 4A). Melanoma cells co-cultured with neutrophils had 1.3 to 2-fold higher levels of extracellular IL-8 compared to melanoma cells cultured alone. In contrast, decreasing IL-8 protein levels in melanoma cells reduced concentrations present in the extracellular neutrophil-melanoma cell co-culture environment compared to untransfected cells or controls nucleofected with buffer only or scrambled siRNA (Figure 4A). Thus, decreasing IL-8 present in melanoma cells reduced concentrations in the extracellular neutrophilmelanoma 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 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 co-cultured with neutrophils. FACS analysis measured β_2 integrin levels, specifically Mac-1, on neutrophils present in co-cultures. Mac-1 levels present on neutrophils cultured alone were set at 1 and fold change on surface of neutrophils cultured with melanoma cells reported (Figure 4B). Neutrophils cultured with control melanoma cells nucleofected with buffer or scrambled siRNA had 80% higher levels of Mac-1 compared to those nucleofected with siRNA targeting IL-8 (Figure 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.

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 30-60% for UACC 903M cells and by 25% for C8161.Cl9 cells compared to untransfected or control cells nucleofected with buffer only or scrambled siRNA (Figure 4C).

To validate the role of IL-8 in neutrophil-mediated melanoma extravasation under flow conditions, melanoma cells were co-cultured 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 60-75% for 1205 Lu cells compared to untreated melanoma-neutrophil co-cultures or use of an anti-IgG antibody control (Figure 4C). Therefore, secreted IL-8 levels induced melanoma-neutrophil interaction and subsequent extravasation under flow conditions. To confirm 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 approximately 50% compared to IL-8 stimulated neutrophil-melanoma cells. 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 demonstrate 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 (Figure 5A). The existence of neutrophil significantly increased C8161Cl.9 migration compared to C8161Cl.9 alone under flow conditions by ~8-fold. Stimulation of neutrophils with IL-8 further increased C8161Cl.9 migration under flow conditions compared to 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 to IL-8 stimulated neutrophils-melanoma co-cultures. 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 demonstrate 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, co-localized green melanoma cells and red neutrophils were photographed and

quantified (Figures 5B & 5C). Decreasing IL-8 expression in melanoma cells reduced co-localization with neutrophils by ~60% compared to buffer and scrambled siRNA controls (Figure 5C). CellTracker Orange CMTMR-labeled neutrophils only were also injected to the tail vein without melanoma cells. However, twenty-four 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

While 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 50-60% for 1205 Lu (Figure 6A) and 30-40% for UACC 903M (Figure 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 to that observed with just endogenous mouse neutrophils and siRNA-mediated targeting of IL-8 reduced interaction to that observed in control cells (Figure 6C; scale set to that in Figure 6A, Figure 6D; scale set to that in Figure 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 regarding the multiple functions played by IL-8 necessitates studies such as this one dissecting its different roles, which appear 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 controling 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 in order 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 co-cultures (25), which occurred in this study. IL-8 secretion from neutrophils further can 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 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.

While the importance of neutrophil-mediated melanoma extravasation in lung tissue is demonstrated 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 employ 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 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 advance-stage melanomas, possibly resulting in a cooperative synergistically acting drug regime.

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.

Acknowledgments

The authors acknowledge Pu Zhang, Jennifer Fritz, Raghavendra Gowda and Chinying Chung for providing technical support.

Grant Support: NIH CA-127892-01A1, ACS RSG-04-053-01-GMC and The Foreman Foundation for Melanoma Research (G.P.R.); NIH CA-97306, CA-125707 and NSF CBET-0729091 (C.D.); and Johnson & Johnson Seed Grant (C.D. and G.P.R.).

REFERENCES

1. Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. Nature. 2007; 445:851–7. [PubMed: 17314971]
2. Shevde LA, Welch DR. Metastasis suppressor pathways--an evolving paradigm. Cancer Lett. 2003; 198:1–20. [PubMed: 12893425]
3. Chiang AC, Massague J. Molecular basis of metastasis. N Engl J Med. 2008; 359:2814–23. [PubMed: 19109576]

4. Gupta GP, Massague J. Cancer metastasis: building a framework. Cell. 2006; 127:679–95. [PubMed: 17110329]
5. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer. 2002; 2:563–72. [PubMed: 12154349]
6. Soengas MS, Lowe SW. Apoptosis and melanoma chemoresistance. Oncogene. 2003; 22:3138–51. [PubMed: 12789290]
7. Payne AS, Cornelius LA. The role of chemokines in melanoma tumor growth and metastasis. J Invest Dermatol. 2002; 118:915–22. [PubMed: 12060384]
8. Slattery MJ, Dong C. Neutrophils influence melanoma adhesion and migration under flow conditions. Int J Cancer. 2003; 106:713–22. [PubMed: 12866031]
9. Drost EM, MacNee W. Potential role of IL-8, platelet-activating factor and TNF-alpha in the sequestration of neutrophils in the lung: effects on neutrophil deformability, adhesion receptor expression, and chemotaxis. Eur J Immunol. 2002; 32:393–403. [PubMed: 11813158]
10. Diamond MS, Springer TA. The dynamic regulation of integrin adhesiveness. Curr Biol. 1994; 4:506–17. [PubMed: 7922371]
11. Nathan C. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol. 2006; 6:173–82. [PubMed: 16498448]
12. Rajarathnam K, Sykes BD, Kay CM, et al. Neutrophil activation by monomeric interleukin-8. Science. 1994; 264:90–2. [PubMed: 8140420]
13. Xie K. Interleukin-8 and human cancer biology. Cytokine Growth Factor Rev. 2001; 12:375–91. [PubMed: 11544106]
14. Schaider H, Oka M, Bogenrieder T, et al. Differential response of primary and metastatic melanomas to neutrophils attracted by IL-8. Int J Cancer. 2003; 103:335–43. [PubMed: 12471616]
15. Waugh DJ, Wilson C. The interleukin-8 pathway in cancer. Clin Cancer Res. 2008; 14:6735–41. [PubMed: 18980965]
16. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. J Immunol. 2003; 170:3369–76. [PubMed: 12626597]
17. Murdoch C, Monk PN, Finn A. Cxc chemokine receptor expression on human endothelial cells. Cytokine. 1999; 11:704–12. [PubMed: 10479407]
18. Bauskin AR, Brown DA, Junankar S, et al. The propeptide mediates formation of stromal stores of PROMIC-1: role in determining prostate cancer outcome. Cancer Res. 2005; 65:2330–6. [PubMed: 15781647]
19. Melnikova VO, Bar-Eli M. Bioimmunotherapy for melanoma using fully human antibodies targeting MCAM/MUC18 and IL-8. Pigment Cell Res. 2006; 19:395–405. [PubMed: 16965268]
20. Dhawan P, Richmond A. Role of CXCL1 in tumorigenesis of melanoma. J Leukoc Biol. 2002; 72:9–18. [PubMed: 12101257]
21. Zivkovic M, Poljak-Blazi M, Zarkovic K, Mihaljevic D, Schaur RJ, Zarkovic N. Oxidative burst of neutrophils against melanoma B16-F10. Cancer Lett. 2007; 246:100–8. [PubMed: 16564616]
22. Liang S, Sharma A, Peng HH, Robertson G, Dong C. Targeting mutant (V600E) B-Raf in melanoma interrupts immunoediting of leukocyte functions and melanoma extravasation. Cancer Res. 2007; 67:5814–20. [PubMed: 17575149]
23. Luca M, Huang S, Gershenwald JE, Singh RK, Reich R, Bar-Eli M. Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. Am J Pathol. 1997; 151:1105–13. [PubMed: 9327744]
24. Mian BM, Dinney CP, Bermejo CE, et al. Fully human anti-interleukin 8 antibody inhibits tumor growth in orthotopic bladder cancer xenografts via down-regulation of matrix metalloproteases and nuclear factor-kappaB. Clin Cancer Res. 2003; 9:3167–75. [PubMed: 12912969]
25. Peng HH, Liang S, Henderson AJ, Dong C. Regulation of interleukin-8 expression in melanoma-stimulated neutrophil inflammatory response. Exp Cell Res. 2007; 313:551–9. [PubMed: 17141217]

26. Inoue K, Slaton JW, Eve BY, et al. Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. Clin Cancer Res. 2000; 6:2104–19. [PubMed: 10815938]
27. Koch AE, Polverini PJ, Kunkel SL, et al. Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science. 1992; 258:1798–801. [PubMed: 1281554]
28. Singh RK, Varney ML. IL-8 expression in malignant melanoma: implications in growth and metastasis. Histol Histopathol. 2000; 15:843–9. [PubMed: 10963128]
29. Strieter RM, Kunkel SL, Elner VM, et al. Interleukin-8. A corneal factor that induces neovascularization. Am J Pathol. 1992; 141:1279–84. [PubMed: 1281615]
30. Hoskins MH, Dong C. Kinetics analysis of binding between melanoma cells and neutrophils. Mol Cell Biomech. 2006; 3:79–87. [PubMed: 16903259]
31. Liang S, Slattery MJ, Dong C. Shear stress and shear rate differentially affect the multi-step process of leukocyte-facilitated melanoma adhesion. Exp Cell Res. 2005; 310:282–92. [PubMed: 16154563]
32. Liang S, Slattery MJ, Wagner D, Simon SI, Dong C. Hydrodynamic shear rate regulates melanoma-leukocyte aggregation, melanoma adhesion to the endothelium, and subsequent extravasation. Ann Biomed Eng. 2008; 36:661–71. [PubMed: 18253835]
33. Lynam E, Sklar LA, Taylor AD, et al. Beta2-integrins mediate stable adhesion in collisional interactions between neutrophils and ICAM-1-expressing cells. J Leukoc Biol. 1998; 64:622–30. [PubMed: 9823767]
34. Bar-Eli M. Role of interleukin-8 in tumor growth and metastasis of human melanoma. Pathobiology. 1999; 67:12–8. [PubMed: 9873223]
35. Stahl JM, Sharma A, Cheung M, et al. Deregulated Akt3 activity promotes development of malignant melanoma. Cancer Res. 2004; 64:7002–10. [PubMed: 15466193]
36. Madhunapantula SV, Sharma A, Robertson GP. PRAS40 deregulates apoptosis in malignant melanoma. Cancer Res. 2007; 67:3626–36. [PubMed: 17440074]
37. Sharma A, Tran MA, Liang S, et al. Targeting mitogen-activated protein kinase/extracellular signal-regulated kinase kinase in the mutant (V600E) B-Raf signaling cascade effectively inhibits melanoma lung metastases. Cancer Res. 2006; 66:8200–9. [PubMed: 16912199]
38. Sharma A, Trivedi NR, Zimmerman MA, Tuveson DA, Smith CD, Robertson GP. Mutant V599EBRaf regulates growth and vascular development of malignant melanoma tumors. Cancer Res. 2005; 65:2412–21. [PubMed: 15781657]
39. Tran MA, Gowda R, Sharma A, et al. Targeting V600EB-Raf and Akt3 using nanoliposomal-small interfering RNA inhibits cutaneous melanocytic lesion development. Cancer Res. 2008; 68:7638–49. [PubMed: 18794153]
40. Dong C, Slattery MJ, Liang S, Peng HH. Melanoma cell extravasation under flow conditions is modulated by leukocytes and endogenously produced interleukin 8. Mol Cell Biomech. 2005; 2:145–59. [PubMed: 16708476]
41. Hodgson L, Dong C. [Ca2+]i as a potential downregulator of alpha2beta1-integrin-mediated A2058 tumor cell migration to type IV collagen. Am J Physiol Cell Physiol. 2001; 281:C106–13. [PubMed: 11401832]
42. You J, Mastro AM, Dong C. Application of the dual-micropipet technique to the measurement of tumor cell locomotion. Exp Cell Res. 1999; 248:160–71. [PubMed: 10094823]
43. Rasband, W. ImageJ. U.S. National Institutes of Health; Bethesda (MD):
44. Singh RK, Gutman M, Radinsky R, Bucana CD, Fidler IJ. Expression of interleukin 8 correlates with the metastatic potential of human melanoma cells in nude mice. Cancer Res. 1994; 54:3242–7. [PubMed: 8205546]
45. Liang S, Fu C, Wagner D, et al. Two-dimensional kinetics of beta 2-integrin and ICAM-1 bindings between neutrophils and melanoma cells in a shear flow. Am J Physiol Cell Physiol. 2008; 294:C743–53. [PubMed: 18199704]
46. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008; 454:436–44. [PubMed: 18650914]
47. Dissemond J, Weimann TK, Schneider LA, et al. Activated neutrophils exert antitumor activity against human melanoma cells: reactive oxygen species-induced mechanisms and their modulation

by granulocyte-macrophage-colony-stimulating factor. J Invest Dermatol. 2003; 121:936–8. [PubMed: 14632216]

48. Criscuoli ML, Nguyen M, Eliceiri BP. Tumor metastasis but not tumor growth is dependent on Src-mediated vascular permeability. Blood. 2005; 105:1508–14. [PubMed: 15486073]
49. Galaup A, Cazes A, Le Jan S, et al. Angiopoietin-like 4 prevents metastasis through inhibition of vascular permeability and tumor cell motility and invasiveness. Proc Natl Acad Sci U S A. 2006; 103:18721–6. [PubMed: 17130448]
50. Glinskii OV, Huxley VH, Glinsky GV, Pienta KJ, Raz A, Glinsky VV. Mechanical entrapment is insufficient and intercellular adhesion is essential for metastatic cell arrest in distant organs. Neoplasia. 2005; 7:522–7. [PubMed: 15967104]

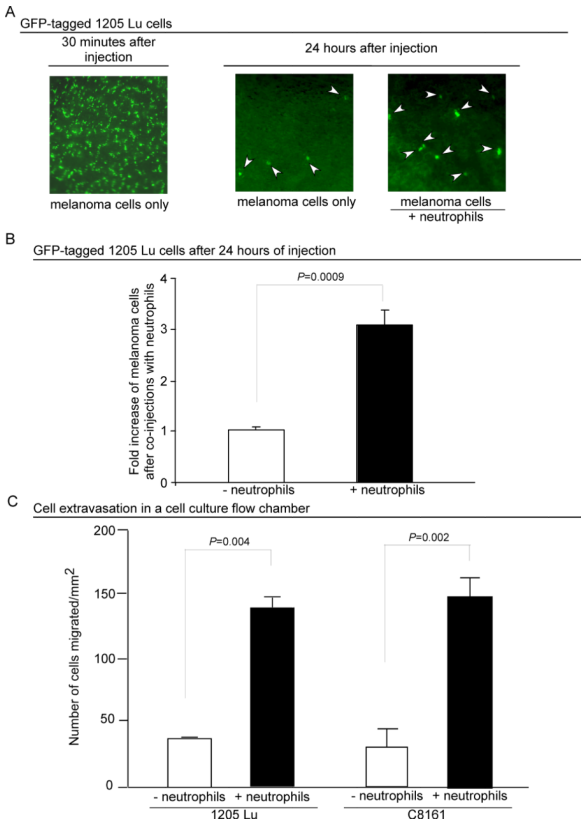

Figure 1. Neutrophils increase number of melanoma cells retained in lungs

(A) Intravenously injected melanoma cells become transiently entrapped in lungs 30 minutes after injection (left panel). Twenty-four hours later very few remained (middle panel). Intravenous injection of neutrophils 1-hour later, increased melanoma cell retention in the lungs (right panel). Arrowheads show GFP-tagged melanoma cells (48x). **(B)** Melanoma cells retained in lungs was 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 co-cultured with neutrophils. All data are mean ± SEM, representing at least 2 independent experiments.

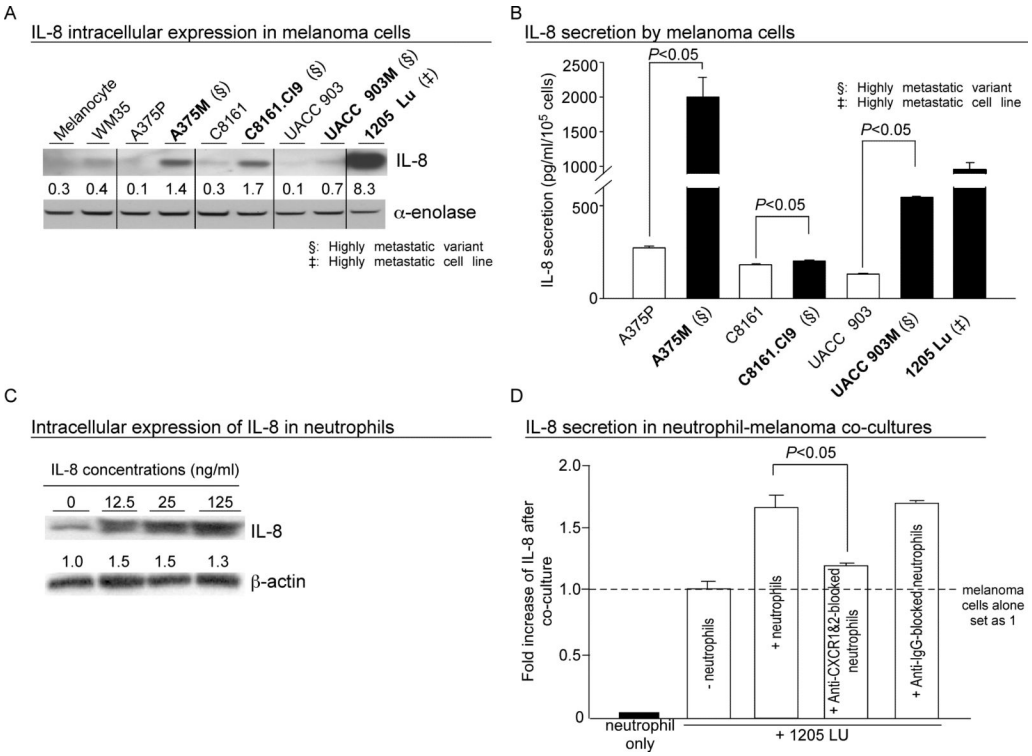

Figure 2. Increased IL-8 was present in metastatic variants compared to less aggressive parental melanoma cell lines

Endogenous **(A)** and secreted IL-8 **(B)** tended to be higher in melanoma cell lines with greater metastatic potential (labeled bold; § and ‡) compared to matched less metastatic parental cells. **(C)** Human recombinant IL-8 (12.5-125 ng/ml) led to a corresponding rise in endogenous IL-8 expression in neutrophils. **(D)** Co-cultured melanoma and neutrophils led to increased IL-8 secretion. Anti-CXCR1/2 antibodies neutralized secreted IL-8 compared to negative control mouse anti-IgG. All data are mean ± SEM, representing at least 2 independent experiments.

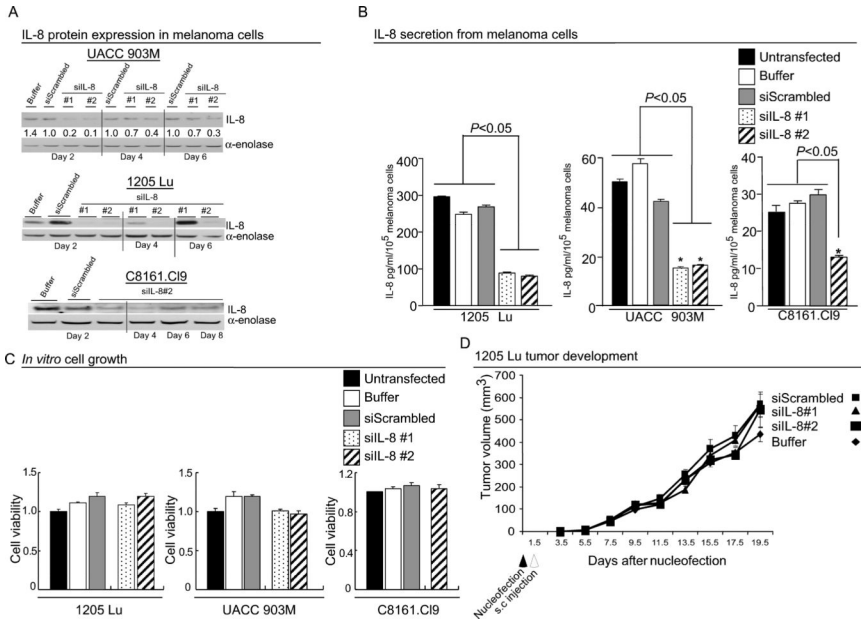

Figure 3. IL-8 does not regulate cellular growth or tumorigenesis

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 cells' tumorigenic potential **(D)**. All data are mean ± SEM, representing at least 2 independent experiments.

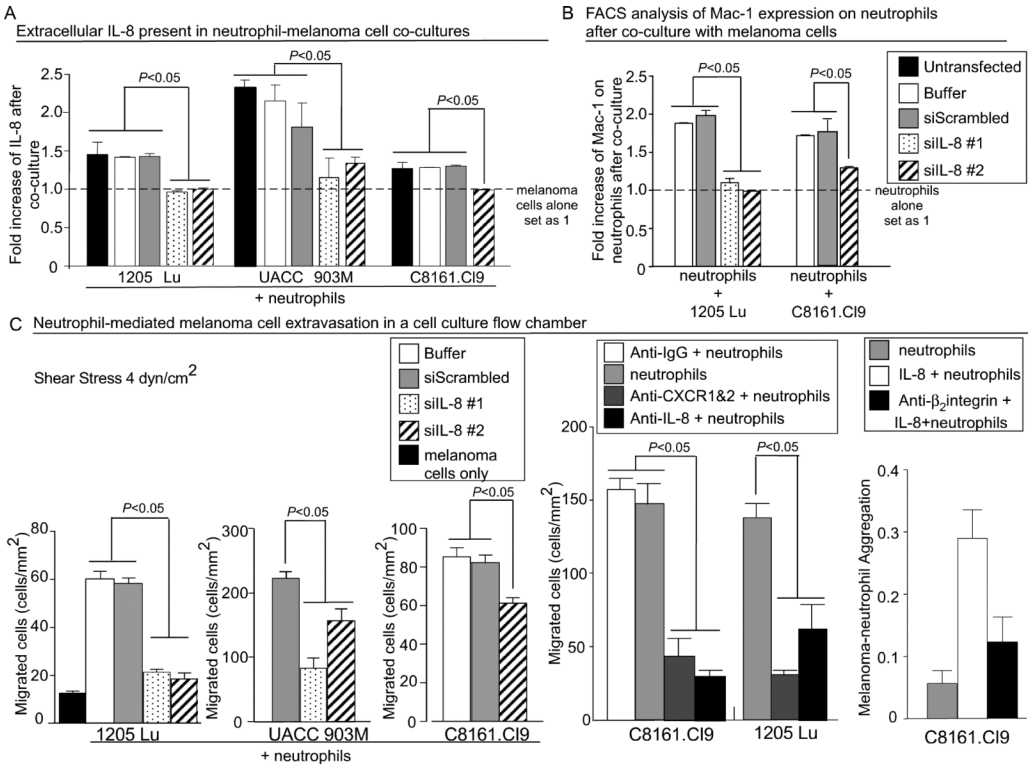

Figure 4. Decreasing IL-8 protein levels in metastatic melanoma cells reduced neutrophil-mediated extravasation across an endothelial like cell layer under flow conditions by disrupting ICAM-1/Mac-1 binding

siRNA-mediated targeting of IL-8 in melanoma cells decreased extracellular protein present in neutrophil co-cultures **(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. All data are mean ± SEM, representing at least 2 independent experiments.

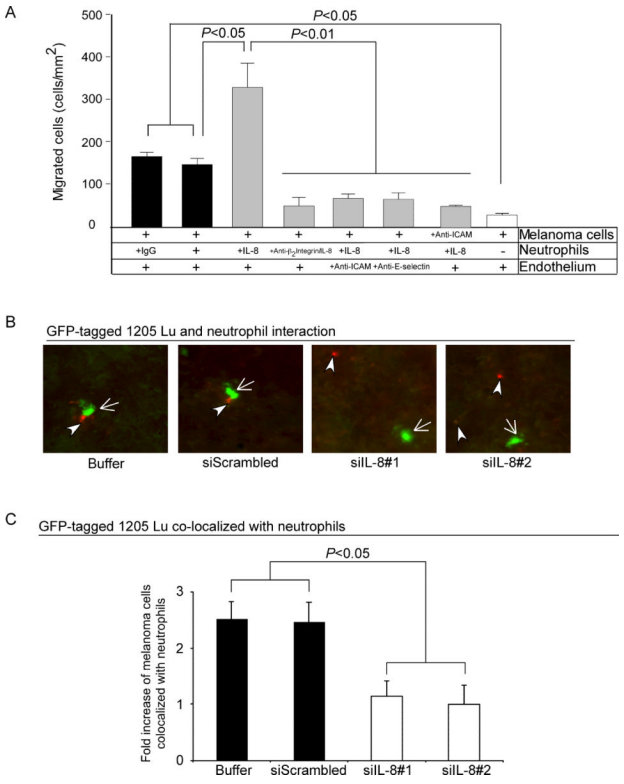

Figure 5. Decreasing IL-8 secretion from melanoma cells reduced interaction with exogenously added human neutrophils in lungs

(A) Neutrophils facilitate melanoma cell migration through an adhesion-mediated mechanism. Decreased IL-8 secretion from melanoma cells, reduced human neutrophil co-localization with melanoma cells in the lungs of nude mice **(B and C)**. **(B)** Co-localized neutrophils (red; arrow heads) and melanoma cells (green; arrows) were more abundant in controls compared to melanoma cells having reduced IL-8 secretion (100x). All data are mean ± SEM, representing at least 2 independent experiments.

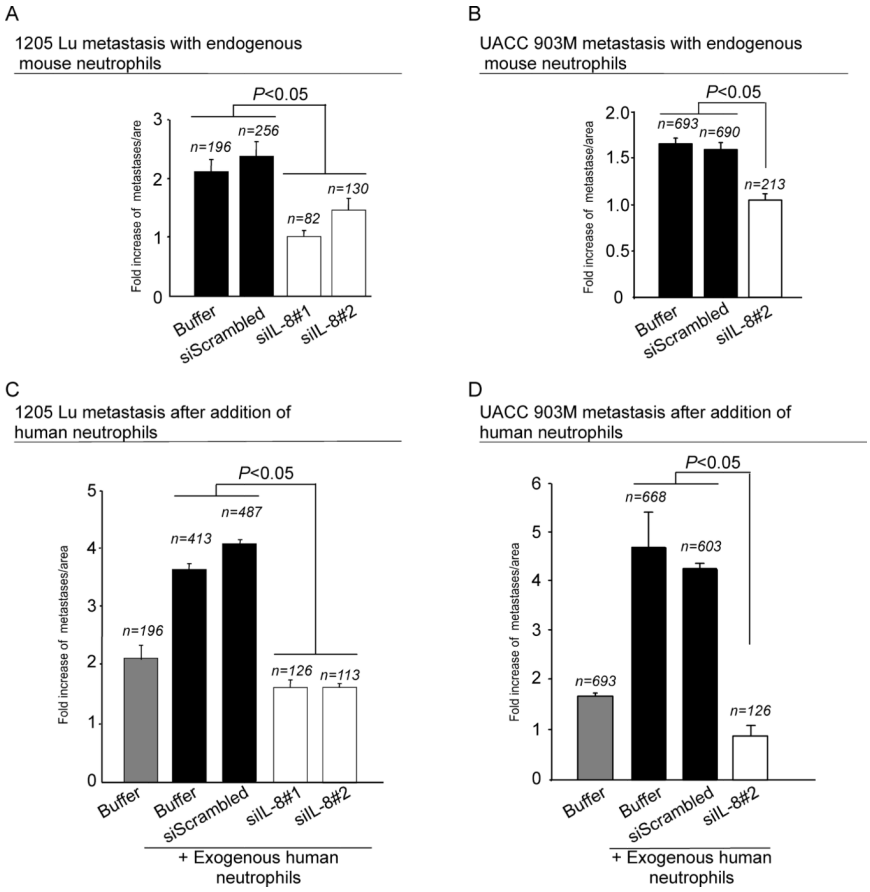

Figure 6. Decreasing IL-8 expression in melanoma cells reduced lung metastases formation

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 rate at which melanoma lung metastases developed. Decreasing IL-8 secretion from melanoma cells reduced lung metastasis formation to control levels. All data are mean ± SEM, representing at least 2 independent experiments.