Immunoediting of leukocyte functions within the tumor microenvironment promotes cancer metastasis development *

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Abstract. Attachment of tumor cells to the endothelium (EC) under flow conditions is critical for migration of tumor cells out of the vascular system to establish metastases. We found that neutrophils (PMN) increased melanoma cell extravasation. Endogenous IL-8 liberated from melanoma cells or from PMN induced by melanoma cells contributed to PMN-facilitated melanoma cell arrest on the EC in the microcirculation. Functional blocking of IL-8 receptors on PMN or neutralizing soluble IL-8 in the tumor circulation decreased the level of CD11b/CD18 up-regulation on PMN and subsequently reduced melanoma cell extravasation. We also found that targeting mutant V600EB-Raf interrupted melanoma cell extravasation in vitro and subsequent lung metastasis development in vivo. B-Raf encodes a RAS-regulated kinase that mediates cell growth and malignant transformation kinase pathway activation. Results showed that inhibition of V600EB-Raf reduced IL-8 secretion from melanoma cells and reduced the capacity of IL-8 production from the tumor microenvironment involving PMN. Furthermore, reduction in intercellular adhesion molecule-1 (ICAM-1) expression on melanoma cells was found after V600EB-Raf knockdown. These results provide new evidence for the complex role of secreted chemokine and PMN-melanoma adhesion in the recruitment of metastatic cancer cells to the EC, which are significant in fostering new approaches to cancer treatment through anti-inflammatory therapeutics.

Keywords: PMN, melanoma, endothelium, shear flow, cytokine signaling, extravasation

1. Introduction

Melanoma metastasis requires that tumor cells detach from a primary site and invade the surrounding stroma, survive immune defenses and turbulence of the blood circulation after invading into the circulatory system, extravasate through the endothelium (EC) lining of blood vessels and finally form a new colony in the surrounding tissue [21].
Inflammation facilitates tumor progression, including metastasis. Interleukin-8 (IL-8) is a chemokine that regulates polymorphonuclear neutrophil (PMN) mobilization and activity [2]. Several recent studies have shown that this cytokine also influences tumor cell behavior and indicated that melanoma cells, for example, by themselves cannot effectively adhere to and migrate through the EC under flow conditions [9,30]. Although melanoma cells lack necessary selectin or integrin adhesion molecules that we would find on PMN, Miele et al. [24] reported that a dose and time-dependent increase in surface expression of intercellular adhesion molecule-1 (ICAM-1) was found in human malignant melanoma cells. It has been hypothesized that IL-8 regulates ligand binding activity of the β2 integrins (e.g., CD11a/CD18 or LFA-1 and CD11b/CD18 or Mac-1) of PMN [5], which, through the binding of ICAM-1 of melanoma cells and of the ECs [18,19], are important for melanoma adhesion and subsequent transmigration under flow conditions (Fig. 1) [16]. IL-8 is of particular interest because of its ability to mediate PMN infiltration through CXC chemokine receptors 1 and 2 (CXCR1 and CXCR2) [33]. Although CXCR1 has not been shown to be expressed in melanoma cells [25], melanoma cells secrete high level of IL-8, which alters adhesion molecule expressions on PMN within a tumor microenvironment [31].

Malignant melanoma has a high propensity for metastatic spread, making it the most deadly form of skin cancer [14]. Several studies have implicated the mitogen-activated protein (MAP) kinase (Ras/Raf/Mek/Erk) pathway in melanoma metastasis by promoting processes such as cell proliferation, survival, invasion, and tumor angiogenesis [28,32]. Studies have also shown that mutation in B-Raf, a glutamic acid for valine substitution at codon 600 (V600E) in exon 15 happens in approximately 60% of melanomas [1]. B-Raf encodes a RAS-regulated kinase that mediates cell growth and malignant transformation [23]. The V600E B-Raf mutation is implicated in constitutive activation and hyper-activation of downstream proteins in the signaling cascade that promotes melanoma adhesion, migration, and proliferation [1,3]. Metastasis requires that melanoma cells pass into the vascular system (intravasation), survive the stresses encountered during blood flow, pass through the endothelial lining of vessels into the tissue (extravasation), and finally proliferate in the new tissue environment. The role played by mutant V600E B-Raf protein to facilitate these processes remains uncertain. Because B-Raf is the most mutated gene in melanomas, it is an attractive therapeutic target to inhibit metastatic spread [35]. However, little...
is known regarding regulation of IL-8 production and of interactions between PMN and melanoma by mutant V600E-B-Raf in melanoma cells, which may in turn, influence melanoma extravasation.

In the present study, we evaluated the role played by mutant V600E-B-Raf in melanoma extravasation and metastasis development. Short interfering RNA (siRNA) targeting mutant V600E-B-Raf was used to reduce expression and/or activity of mutant V600E-B-Raf in melanoma cells. Results suggest that inhibition of mutant V600E-B-Raf reduced melanoma metastasis in vivo by decreasing melanoma cell extravasation. The mechanism for reduced melanoma extravasation was due to decreased ICAM-1 expression, which occurred with decreased mutant V600E-B-Raf expression levels. In addition, endogenous secretion of the chemokine IL-8 from melanoma cells, the capacity of increased IL-8 production from a melanoma-PMN microenvironment, as well as PMN Mac-1 activation, were significantly decreased following inhibition of mutant V600E-B-Raf. Coupling these observations with previous studies showing that endogenously produced IL-8 and ICAM-1-β2 integrin binding mediate melanoma adhesion provides a rationale and mechanistic basis for targeting mutant V600E-B-Raf to inhibit melanoma extravasation and subsequent metastasis development.

2. Materials and methods

2.1. Cell preparations

Human melanoma cell lines WM35 and WM9 were provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA, USA) and maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Biosource, Inc., Camarillo, CA, USA) supplemented with 10% fetal bovine serum (FBS; Biosource, Inc.) and 100 units/ml penicillin-streptomycin (Biosource, Inc.) at 37°C under 5% CO2. C8161 cells (obtained from Dr. Danny Welch, University of Alabama, Birmingham, AL, USA) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM-F12) (Biosource, Inc.) supplemented with 10% FBS in a standard cell culture condition (5% CO2/37°C). Melanoma 1205Lu cells (provided by Dr. Gavin P. Robertson, Penn State Hershey Medical Center, Hershey, PA, USA) containing high levels of mutant V600E-B-Raf [29] were grown in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen). GFP-tagged versions of 1205Lu were used for metastasis assays. The presence of mutant V600E-B-Raf mutation in cell lines has been described recently [17].

EI cells are fibroblast L-cells transfected to express human E-selectin and ICAM-1 (provided by Dr. Scott Simon, UC Davis, CA, USA) and were maintained in culture as described elsewhere [10,18].

Fresh human blood was collected from healthy adults by venipuncture. PMN were isolated using a Histopaque® (Sigma) density gradient as described by the manufacturer and kept at 4°C in Dulbecco’s PBS (D-PBS) containing 0.1% human serum albumin until use. Cell preparations were 99.5% pure PMN, confirmed by a Diff-Quick stain (Dade Behring Inc., Newark, DE, USA). In the cases of PMN and melanoma cell co-culture, PMN resuspended in RPMI 1640 medium supplemented with 5% FBS were added onto the confluent melanoma cell lines (in a 1:1 ratio) directly and co-cultured for 4 hours. 95% of melanoma cells stay alive after a 4 hour contact culture with PMN (data not shown).

2.2. In vitro flow extravasation assay

A flow extravasation assay was performed in a modified 48-well chemotactic Boyden chamber consisting of a top and bottom plate separated by a gasket [7,8]. Prior to each experiment, a monolayer of EI cells was grown (typically 36 hours after cell seeding) on sterile polyvinylpyrrolidone (PVP)-free polycarbonate filters (8 µm pore size; Neuro Probe, Gaithersburg, MD, USA) pre-coated with fibronectin...
(30 µg/ml, 3 hours) (Sigma). The center 12 wells of the bottom plate were filled with soluble chemoattractant type IV collagen (CIV; 100 µg/ml in RPMI 1640/0.1% BSA) (BD Biosciences) and surrounding control wells were filled with medium (RPMI 1640/0.1% BSA). Studies have shown that melanoma cells express α2β1 integrin receptors for soluble collagen IV protein and migrate toward collagen IV stimulation [6,11,12]. The apparatus was assembled by placing a filter on the bottom plate followed by the addition of a sealing gasket and the top plate. The chamber was primed with 37°C medium to eliminate bubbles in the system. For the migration assay, 5 × 10^5 melanoma cells only or PMN together with melanoma cells (5 × 10^5 of each) were put in the chamber under shear flow conditions for 4 hours in a 37°C, 5% CO2 incubator. To quantify migration, migrated cells were stained with Protocol Brand Hema3 solution (Fisher Scientific, Pittsburgh, PA, USA) and counted using an inverted microscope (Diaphot 300, Nikon, Japan) with NIH Image software (v. β4.0.2).

2.3. Enzyme-linked immunosorbent assay (ELISA) determination

At the end of in vitro cell-culture assays, for both the melanoma cells alone or the melanoma cells co-cultured with PMN, cell-free supernatants were collected by centrifugation at 430g for 5 minutes and IL-8 was quantified by a sandwich ELISA following standard protocols. Primary and secondary antibody pairs were obtained from R&D Systems (catalog number MAB208 and BAF208). Standard human recombinant IL-8 was also obtained from R&D Systems (catalog number 208-IL) and a standard curve was included in each ELISA plate. The plates were read on a Packard Spectracount at 405 nm and the data was analyzed using I-Smart Software. Intra assay variation was typically 10–15%.

2.4. Western blots

Cells were collected and washed with cold PBS, then whole cell extracts were prepared by resuspending cells in lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA [pH 8.0], 2 mM Na3VO3, 10 mM NaF, 10 mM Na4P2O7, 1% NP-40, 1 mM PMSF, 2 ng/ml peptatin A). Lysates were incubated on ice for 30 minutes followed by centrifugation at 16,000g for 1 minute at 4°C. The pellet was discarded and the supernatant was mixed with 2 × SDS running buffer (0.2% bromophenol blue, 4% SDS, 100 mM Tris [pH 6.8], 200 mM DTT, 20% glycerol) in a 1:1 ratio. Samples were boiled for three minutes and 15 µl were loaded onto a 12% or 15% SDS-PAGE gel and proteins were transferred to a 0.2 µm PVDF membrane (Millipore Co., Billerica, MA, USA) by electroblotting. Primary antibodies included rabbit anti-human IL-8 (Biosource, Inc.), and anti-β-actin IgG1 (Sigma Chemical Co.) was used for protein control loading. Secondary antibodies were peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG. Proteins were detected using the Enhanced Chemiluminescence Detection System (Amersham Pharmacia Biotech, Arlington Heights, IL, USA).

2.5. In vitro and in vivo siRNA studies

For in vitro studies, 1205Lu melanoma cells containing high levels of mutant V600E B-Raf were grown in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen). SiRNA (100 pmol) was introduced into 1 × 10^6 1205Lu cells via nucleofection with an Amaxa Nucleofector (Koeln, Germany) using Solution R/program K-17 as described previously [29]. The resultant transfection efficiency following nucleofection was >90% and knockdown of mutant V600E B-Raf protein in 1205Lu
melanoma cells persisted beyond 8 days in culture compared to control cells nucleofected with scrambled siRNA. Specificity of using this siRNA approach to knockdown mutant V600E-B-Raf has been described previously [28,29]. Duplexed stealth siRNA (Invitrogen) was used for these studies. SiRNA-nucleofected 1205Lu cells were used for in vitro flow extravasation and ELISA studies, compared with studies using non-nucleofected 1205Lu melanoma cells, as well as 1205Lu cells nucleofected with buffer only or scrambled siRNA.

For in vivo studies, siRNA (100 pmoles) was nucleofected into GFP-tagged 1205Lu cells, which were then replated in culture dishes. 36 hours later, $1 \times 10^6$ of the 1205Lu cells in 0.2 ml of Hank’s balanced salt solution were injected intravenously into the lateral tail vein of nude mice. The effect of siRNA knockdown in melanoma xenograft modules can be maintained up to day 17.5 [28]. The mice were sacrificed 17 days later and their lungs were analyzed for the presence of fluorescent tumors using a Nikon SMZ 1500 dissecting microscope with a Plan Apo 1.6× objective and fluorescence detection capabilities. Images of five random fields were photographed at a magnification of 4.8× from the ventral surface of each lung and the number of fluorescent tumors as well as the area scored in pixels occupied by each tumor was quantified using IP lab imaging software (Scanalytics, Fairfax, VA, USA). Duplicate experiments consisting of 8 animals were used per group.

2.6. In vivo co-localization of mouse PMN and human melanoma cells

Co-localization of mouse PMN and human melanoma cells in the lungs were used to verify interactions between cells and provide evidence of tethering of melanoma cells to the endothelial lining mediated by PMN. Briefly, $1 \times 10^6$ GFP-tagged human metastatic melanoma cells (1205Lu) in 0.2 ml of Hank’s balanced salt solution were injected intravenously into the lateral tail vein of nude mice. 24 hours later, PE-conjugated rat anti-mouse Gr-1 monoclonal antibody (BD Pharmingen) at a concentration of 1 mg/kg body weight in 100 µl of PBS was injected intravenously into the lateral tail vein of mice. The mice were sacrificed 2 hours later and their lungs were analyzed using a Nikon SMZ 1500 dissecting microscope with fluorescence detection capabilities.

2.7. Statistics

All results were shown using mean ± SEM unless otherwise stated. The Student’s $t$-test was used for pairwise comparisons and the One-way Analysis of Variance (ANOVA) was used for groupwise comparisons, followed by the appropriate post hoc tests (Dunnett’s, Tukey’s or Dunn’s). Results were considered significant at $p < 0.05$.

3. Results

3.1. Inhibiting mutant V600E-B-Raf reduced melanoma cell extravasation

Melanoma cell lung metastasis in vivo requires adhesion to and subsequent extravasation through the endothelial lining of lung vessels. To evaluate the role of mutant V600E-B-Raf in facilitating melanoma extravasation, 1205Lu cell extravasation was tested using an in vitro flow extravasation model (Fig. 2). Under static condition, melanoma only (without PMN) migration was reduced by 25% following mutant V600E-B-Raf inhibition (data not shown). Under flow conditions of 4 dyn/cm² in the absence of PMN, very few migrations of melanoma cells were observed and there were no migratory differences between
control cells and those in which mutant \( V_{600E}B-Raf \) had been inhibited (Fig. 2, “− Neutrophil”). Results showed that PMN promoted 1205Lu melanoma cell migration under flow conditions of 4 dyn/cm\(^2\) (Fig. 2, “+ Neutrophil”) compared to melanoma cells alone (Fig. 2, “− Neutrophil”), confirming that PMN affected melanoma adhesion and subsequent extravasation through an EC monolayer. Inhibition of mutant \( V_{600E}B-Raf \) significantly reduced PMN-mediated melanoma cell extravasation by around 2 fold under flow conditions (Fig. 2, “+ Neutrophil”), indicating that inhibition of mutant \( V_{600E}B-Raf \) may affect interactions between PMN and melanoma cells. These data showed that inhibition of mutant \( V_{600E}B-Raf \) significantly decreases melanoma extravasation across the EC monolayer, suggesting one potential mechanism for regulating metastasis development.

3.2. Co-culturing PMN with melanoma cells induces IL-8

In order to determine the relationship between tumor metastatic potential and cytokine expression in melanoma-PMN co-cultures, four human melanoma cell lines, 1205Lu, WM9, C8161 and WM35, were tested for their abilities to induce cytokine and chemokines in melanoma-PMN co-cultures. These cell lines have a range of metastatic potentials as reported in the literature as well as determined by migration assays [27], with 1205Lu being the most metastatic, WM9 and C8161 more intermediate in their ability to extravasate and WM35 having the lowest metastatic potential of all four lines. A correlation was found between metastatic potentials of melanoma cells and IL-8 secretion profiles (Fig. 3A). PMN co-cultured either in a Transwell or in direct-contact with 1205Lu, WM9 and C8161 increased IL-8 expression above the summed background level, and co-culturing PMN with WM35 did not induce IL-8 production. The induction of IL-8 was comparable whether cells were cultured in direct-contact or separated in a Transwell culture system, indicating that melanoma cells modified PMN activity through a soluble factor.

In order to identify which cell type contributed to the increased amount of IL-8 in PMN-melanoma co-cultures, cell lysates were prepared from PMN and melanoma cells following Transwell co-culture, and levels of IL-8 were detected by Western blots. As shown in Fig. 3B, melanoma cells with higher metastatic potentials (1205Lu, WM9 and C8161) increased IL-8 production in PMN, while WM35 did
not induce IL-8 protein in PMN. In contrast, IL-8 expression in melanoma cells did not dramatically change when co-cultured in the presence or absence of PMN (data not shown). However, high levels of endogenous IL-8 were found from highly metastatic melanoma cells.

3.3. Inhibiting mutant V600E B-Raf reduced IL-8 production from a melanoma microenvironment

The endogenously produced IL-8 could potentially mediate PMN interactions with tumor cells. In order to evaluate the role played by mutant V600E B-Raf in chemokine production from melanoma cells, which could affect melanoma cell extravasation, cells nucleofected with siRNA against mutant V600E B-Raf were examined for IL-8 production. Results showed that inhibition of mutant V600E B-Raf significantly reduced IL-8 production from melanoma cells alone. Specifically, IL-8 produced by control melanoma cells (untransfected melanoma and melanoma nucleofected with buffer only or scrambled siRNA) was around 500 pg/ml/10⁵ cells (Fig. 4A). However, inhibition of mutant V600E B-Raf resulted in roughly 2 fold less IL-8 production (Fig. 4A). In addition, IL-8 production from the melanoma-PMN microenvironment was also inhibited after knockdown of mutant V600E B-Raf. Results showed that, following co-culture of PMN and control melanoma cells, IL-8 production increased about 1.5 fold compared with the summed background levels of melanoma and PMN cultured alone (Fig. 4B). In contrast, equivalent or slightly-reduced levels of IL-8 were detected in the co-cultures between PMN and 1205Lu melanoma cells nucleofected with siRNA targeting mutant V600E B-Raf (Fig. 4B). These data suggest that knockdown of mutant V600E B-Raf significantly reduced IL-8 production from melanoma cells alone, and reduced capacity of IL-8 production from the tumor microenvironment involving immune cells, such as PMN.

3.4. Disruption of ICAM-1-β₂ integrins binding between melanoma and PMN following inhibition of mutant V600E B-Raf retarded melanoma extravasation

Functional binding of ICAM-1 expressed on melanoma to β₂ integrin (LFA-1 and Mac-1) ligands expressed on PMN could result in cooperative tethering of PMN-melanoma aggregate to the EC,
which subsequently promotes melanoma extravasation. Flow cytometry confirmed that siRNA-mediated ICAM-1 knockdown resulted in reduced expressions of ICAM-1 (Fig. 5A) on melanoma cells. Interestingly, inhibition of mutant $V_{600E}$B-Raf reduced expression of ICAM-1 on melanoma cells to a similar level of that observed following siRNA ICAM-1 knockdown (Fig. 5A).

In vitro flow extravasation results indicated siRNA-mediated down-regulation of ICAM-1 via an inhibition of $V_{600E}$B-Raf also reduced melanoma cell migration significantly compared with the controls (Fig. 5B). These results suggest an important role for ICAM-1 in melanoma extravasation under flow conditions. In addition, extravasation of melanoma cells was reduced even more significantly following inhibition of mutant $V_{600E}$B-Raf (Fig. 5B) compared with siRNA ICAM-1 knockdown cases, suggesting that inhibition of mutant $V_{600E}$B-Raf has multiple effects other than reducing ICAM-1 expression.

Our results above have shown that inhibition of mutant $V_{600E}$B-Raf retards IL-8 production in melanoma-PMN microenvironment. Melanoma-PMN co-culture has been shown to increase IL-8 levels and activate Mac-1 expression on PMN [27,31]. We then investigated the effects of inhibiting mutant $V_{600E}$B-Raf in melanomas on the expression of Mac-1 on PMN. Results showed that Mac-1 expression on PMN increased approximately 2 fold following co-culture with control melanoma cells (Fig. 5C). In contrast, in the cases where PMN was co-cultured with 1205Lu cells following inhibition of mutant $V_{600E}$B-Raf, Mac-1 expression on the PMN was unchanged and significantly less than on the controls (Fig. 5C).

Collectively, these data indicated that knockdown of mutant $V_{600E}$B-Raf inhibited ICAM-1 expressions on the surface of melanoma cells as well as Mac-1 up-regulation on PMN after melanoma-PMN co-culture, which in turn retard PMN-facilitated melanoma extravasation through disruption of ICAM-1/β₂ integrin binding (Fig. 6).
Fig. 5. Disruption of ICAM-1-β2 integrin binding in PMN-mediated melanoma extravasation. (A) ICAM-1 expression on 1205Lu melanoma cells was reduced after knockdown of mutant \(V_{600E}\)B-Raf and ICAM-1 using siRNA. (B) Knockdown of mutant \(V_{600E}\)B-Raf and ICAM-1 inhibited PMN-mediated melanoma extravasation, values are mean ± SEM for \(n \geq 3\). (C) Mac-1 expression on PMN after co-culture with melanoma cells. Mac-1 expression on PMN increased significantly after PMN co-cultured with control melanoma cells (untransfected melanoma and melanoma nucleofected with buffer only or scrambled siRNA). However, the co-culture between PMN and melanoma cells treated with siRNA against \(V_{600E}\)B-Raf did not significantly increase Mac-1 expression on PMN. Values were normalized to background Mac-1 expression on PMN alone and shown as means ± SEM for \(n \geq 3\).

3.5. SiRNA mediated inhibition of mutant \(V_{600E}\)B-Raf reduced melanoma metastases

Since B-Raf is the most mutated gene in melanomas, it is an attractive therapeutic target to inhibit melanoma metastasis. To develop a more thorough understanding of its function in metastasis, siRNA was used to knock down expression of mutant \(V_{600E}\)B-Raf in the metastatic human melanoma cell line 1205Lu. Experimental metastasis development was studied by nucleofecting siRNA into melanoma cells, allowing recovery in culture for 1.5 days followed by intra venous injection of cells into the tail vein of nude mice. Seventeen days later, the mice were euthanized and the number and size of lung metastases was scored. Figure 7A shows that reducing expression/activity of mutant \(V_{600E}\)B-Raf significantly decreased the metastases of 1205Lu cells. A significant 5–7 fold decrease in tumors >1500 pixels
Fig. 6. Links among different molecular and cellular players in melanoma-PMN signaling. Melanoma cells constitutively secrete IL-8 and induce IL-8 production in PMN, indicating potential paracrine stimulation of IL-8 within a tumor microenvironment. NF-κB activities are required in melanoma-PMN signaling [17,27]. Endogenous secretion of IL-8 from melanoma cells, as well as the capacity of increased IL-8 production from a melanoma-PMN microenvironment, is significantly decreased following inhibition of mutant V600E-B-Raf. Inhibition of mutant V600E-B-Raf also decreases PMN Mac-1 activation when co-cultured with melanoma, reduces melanoma ICAM-1 expression, and affects ICAM-1-β2 integrin binding that supports melanoma adhesion to the EC.

Fig. 7. In vivo lung metastasis study. (A) SiRNA-mediate inhibition of mutant V600E-B-Raf in 1205Lu cells reduced formation of melanoma lung metastases. Number of tumors within particular size ranges (<1500 or >1500 pixels) were quantified in a minimum of 6 fields per lung from 5 to 10 animals. Values are means ± SEM. (B) Co-localization of melanoma cells and mouse PMN in vivo. Left panel, green GFP-tagged melanoma cells; middle panel, red PE-labeled PMN; right panel, co-localization of green GFP-tagged melanoma cells and red PE-labeled PMN.

was found following inhibition of V600E-B-Raf versus control animals injected with cells nucleofected with scrambled siRNA (Fig. 7A).

Previous studies have shown that human PMN facilitates melanoma extravasation in vitro [30]. To
investigate the interaction of melanoma cells with mouse PMN, co-localizations of green GFP-tagged melanoma cells and red PE-tagged mouse PMN in the lungs were studied. Results showed green GFP-tagged 1205Lu cells and red PE labeled PMN can be detected microscopically in the lung stroma (Fig. 7B). More importantly, approximately 13% melanoma cell and mouse PMN were co-localized together, indicating that mouse PMN are also facilitating melanoma cell adhesion to the endothelium, which could promote extravasation and thus metastases.

Reducing expression (activity) of V600E-B-Raf significantly retarded the metastases of 1205Lu cells (Fig. 8A) containing mutant V600E-B-Raf. Fewer green fluorescent tumors were observed in the lungs of animals injected with melanoma cells in which V600E-B-Raf had been inhibited, suggesting that fewer cells were extravasated through the endothelial lining or had become entrapped in lung vessels and proliferated in the lung tissue to form metastases (Fig. 8B). In contrast, control animals injected with cells nucleofected with scrambled siRNA or buffer had significantly more metastases, occupying a larger overall area of lung than cells in which V600E-B-Raf had been inhibited. Metastases were placed into two pixel-based categories based on size, i.e. those <1500 and those >1500 pixels. A significant 5–7 fold decrease in tumors >1500 pixels was observed following V600E-B-Raf inhibition compared to control 1205Lu cells nucleofected with buffer or scrambled siRNA (Fig. 8C). Collectively, these data show that inhibition of B-Raf in melanoma cells containing B-Raf mutation reduces development of metastatic lung tumors.

4. Discussion

Previous studies have shown that melanoma cells express α2β1 integrin receptors for soluble collagen IV protein, which is one of the proteins found in the extracellular matrix in vivo [11]. Knockdown of mutant V600E-B-Raf not only reduced melanoma cell extravasation under dynamic flow conditions (Fig. 2) but also migration toward chemoattractant (collagen IV) under static conditions in vitro [17], compared with control cases (untransfected melanoma and melanoma nucleofected with buffer only or scrambled siRNA). These results indicate that mutant V600E-B-Raf might have significant influence not only in receptor-mediated tumor cell adhesion but also in tumor cell motility. Such effects should not be collagen IV specific, since melanoma cells do migrate to soluble laminin, collagen IV and fibronectin [6], although we have not directly tested whether inhibiting V600E-B-Raf would affect collagen IV receptor α2β1 on melanoma cells.

In melanoma cells, the interactions of α4β1 (VLA-4) on sialyl-Lewisxa-negative melanoma cells and vascular adhesion molecule (VCAM-1) on inflamed EC supported melanoma adhesion to and subsequent extravasation through the EC in low shear flow [20]. Other studies showed that colon carcinoma cells express carbohydrate antigens such as sialyl-Lea and sialyl-Lea that can bind to the selectins on the EC [13]. Colon carcinoma cells also express CD44 variant isoforms (CD44v), which may mediate their adhesion to the EC via binding to selectins expressed on the EC [26]. We have found that melanoma cells express high levels of ICAM-1, but do not express sialyl-Lea or sialyl-Lea. In addition, they only express CD44 standard isoforms (CD44s) from our recent experiments (data not shown), which was suggested to bind E- and P-selectin with low affinity [26]. Interactions between ICAM-1 on melanoma cells and β2 integrins on PMN promote shear-resist tethering of melanoma cells to the vascular EC under flow conditions, which is mediated by endogenous IL-8 [27,31]. Consistent with this observation, we have recently found that IL-8 neutralization leads to a reduction in melanoma transmigration [8,16].

IL-8 plays a crucial role in regulating cell function for host defense and natural immunity [34]. IL-8 is released by various cell types, including PMN, monocytes, T lymphocytes and endothelial cells, upon
exposure to inflammatory stimuli, such as TNF-α, IL-1 and LPS [2,22]. IL-8, in particular, is a major mediator for β2 integrin activation in PMN-regulated adhesion to the EC, as well as a chemoattractant for PMN migration [5].
A unique strategy using transient siRNA-mediated knockdown of mutant V600E-B-Raf has been used to demonstrate that the development of metastatic lung tumors was significantly reduced due to the disruption of extravasation through endothelium by targeted inhibition of mutant V600E-B-Raf. As a result of the inhibition of mutant V600E-B-Raf, endogenous IL-8 production as well as interactions between ICAM-1 on melanoma and β2 integrins on PMN were disrupted, which in turn reduced melanoma extravasation. This is a novel finding to identify the role of mutant V600E-B-Raf played in PMN-facilitated melanoma extravasation, suggesting a potential mechanism for preventing the metastatic process.

Therefore, disruption of the signals between these cell types could be used for therapeutic advantages [17,18]. IL-8 expression in human melanoma cells correlates with the level of anoxia and the aggressiveness of the melanoma [15]. We have found that endogenously secreted IL-8 from melanoma cells induced an increase in IL-8 production from PMN after co-culture of PMN and melanoma cells. Although co-culture of PMN and control melanoma cells increased IL-8 production, our results also indicate that IL-8 production after co-culture of PMN and melanoma following inhibition of mutant V600E-B-Raf was unchanged or even reduced. Therefore, reduced IL-8 secretion from a melanoma-PMN microenvironment after inhibition of mutant V600E-B-Raf could attribute to both reduced IL-8 production from melanoma cells and reduced IL-8 from PMN due to the reduced amount of IL-8 from melanoma cells.

In this study, results showed that inhibition of mutant V600E-B-Raf in melanoma reduced PMN-facilitated melanoma migration significantly. We found that ICAM-1 expression on melanoma cells was reduced after treatment with siRNA against mutant V600E-B-Raf. In addition, we have shown that PMN-melanoma co-culture can increase Mac-1 expression on PMN, which was inhibited after knockdown of mutant V600E-B-Raf. It has also been reported that IL-8 can trigger functional up-regulation of ligand binding activity of β2 integrins, Mac-1 in particular, on PMN [4,5]. Our results show reduced IL-8 from PMN-melanoma co-cultures following inhibition of mutant V600E-B-Raf, possibly explaining the mechanism for reduced Mac-1 expression on PMN after co-culture with melanoma following inhibition of mutant V600E-B-Raf. Collectively, these data indicate one mechanism of reduced melanoma extravasation under flow conditions after mutant V600E-B-Raf inhibition, which is through interrupting the ICAM-1-β2 integrin binding mechanism necessary for PMN-facilitated melanoma extravasation. While results presented in this paper mainly focused on the roles of IL-8, B-Raf mutation, and different adhesion molecules played in melanoma cell extravasation, effects of IL-8 on melanoma adhesion to the EC via tethered PMN were recently reported in separated papers from our laboratory [16,31] using an in vitro parallel-plate flow assay, in which we have shown that IL-8 increase the number of melanoma cells bound to PMN and EC.

Through the use of a unique strategy involving transient siRNA-mediated knockdown of V600E-B-Raf protein expression (activity), experimental lung metastases could be inhibited 4–5 fold in metastatic melanoma cells containing mutant B-Raf but not those lacking the mutant protein. The model focused on lung metastasis since lungs are a major organ to which melanoma cells metastasize. A lack of clinical responsiveness targeting B-Raf using the pharmacological agent BAY43-9006 has raised serious doubt regarding the utility of therapeutically targeting this signaling cascade to inhibit melanoma development [15]. We have shown that siRNA-mediated inhibition of mutant V600E-B-Raf signaling reduced lung metastases, indicating that this pathway is important for melanoma metastasis. Metastasis can be inhibited by disrupting extravasation through the endothelial lining of lung vessels or by reducing proliferation in the lung microenvironment. The present studies have demonstrated that V600E-B-Raf plays a critical role in regulating both of these processes, thereby regulating metastasis. Interaction of melanoma
cells with PMN facilitates attachment to the endothelium, thereby promoting extravasation into the lung stroma.

Extravasation is a prerequisite for circulation-mediated dissemination of metastatic cancer cells to various tissues, although development of metastatic tumor in the lung may or may not depend on extravasation. The co-localization of melanoma cells and PMN may also result from blocking the local microcirculation by mechanical trapped tumor cells rather than binding of tumor cells to PMN, which could be an alternative, but not exclusive, mechanism in tumor development in the lung. Unpublished \textit{in vivo} results from our laboratory have recently revealed a significant reduction in melanoma colonies in the lung when PMN were depleted, suggesting that the number of extravasated tumor cells observed \textit{in vitro} are well correlated with the number of metastasis in the lung \textit{in vivo}. Endogenously produced IL-8 and ICAM-1/β2 integrin binding mediate melanoma extravasation provides a rationale and mechanistic basis for targeting mutant \textit{V600E}B-Raf to inhibit melanoma adhesion, extravasation and subsequent metastasis development.

Collectively, we have provided novel insight into these processes, showing that in melanoma, \textit{V600E}B-Raf promotes metastasis by facilitating interaction with PMN and extravasation across the endothelial layer under flow conditions and by conferring increased proliferative potential in the lung microenvironment.

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