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Platelets and P-Selectin Control Tumor Cell Metastasis in an Organ-Specific Manner and Independently of NK Cells

Lucy A. Coupland¹, Beng H. Chong², and Christopher R. Parish¹

Abstract

The prometastatic role of platelets has long been recognized with proposed mechanisms of action including shielding tumor cells from natural killer (NK) cell destruction and aiding endothelial attachment and extravasation of tumor cells with platelet P-selectin being implicated in these processes. However, many aspects of the prometastatic function of platelets remain unclear. In this study, we used mouse models of metastatic breast cancer and melanoma to investigate the platelet effect, focusing on organ specificity, the relationship with NK cells and the relative importance of platelet-derived versus endothelial-derived P-selectin. We found that platelets promote lung metastasis in the absence of NK cells in both acute and spontaneous metastasis models. In addition, the prometastatic action of platelets was found to be organ specific, clearly enhancing lung metastasis but not affecting B16F1 liver metastasis, in fact, liver metastasis was enhanced in the absence of platelets. Furthermore, the profound antimetastatic activity of NK cells was equally effective in the presence or absence of platelets and chronologically distinct from the prometastatic role of platelets. Finally, it was shown that endothelial-derived P-selectin is just as important as platelet-derived P-selectin in promoting lung metastasis and also plays an important role in liver metastasis. Taken together, our findings help clarify the roles of platelets, NK cells and P-selectin in metastasis, and they identify P-selectin as an attractive therapeutic target for preventing metastasis in multiple organs. *Cancer Res*; 72(18); 4662–71. ©2012 AACR.

Introduction

Platelets have long been implicated in hematogenous metastasis with the first shown association published in 1968 (1). The proposed mechanisms by which platelets promote metastasis include: (i) formation of a thrombus around tumor cells within the bloodstream thus protecting tumor cells from shear stresses and aiding tumor cell entrapment in capillary beds; (ii) promotion of adhesion to the blood vessel wall via platelet-specific proteins including P-selectin and $\alpha\text{IIb}\beta_3$; (iii) release from platelets of permeability factors and degradative enzymes to assist tumor cell migration across the blood vessel wall; and (iv) release from platelets of angiogenic and other growth factors to assist establishment of secondary tumors (2, 3). Other research suggests that platelets shield tumor cells from natural killer (NK) cell attack (4, 5), impair NK cell effector function via platelet-derived TGF- β (6) and transfer MHC-I to tumor cells thereby preventing NK cell recognition (7). How-

ever, because platelets promote the extravasation of metastasizing tumor cells, it is unlikely that the prometastatic role of platelets is limited to the inhibition of NK cell effector function. In this article we examined whether the prometastatic role of platelets is global or organ specific, the relationship between the prometastatic activity of platelets and the antimetastatic action of NK cells, and the importance of both endothelial- and platelet-derived P-selectin in tumor metastasis.

Materials and Methods

Mice

C57BL/6 (CD45.1 and CD45.2), BALB/c and NOD.SCID mice (Animal Resources Centre, Perth, Australia); thrombopoietin receptor deficient (*c-mpl*^{-/-}) C57BL/6 mice (Warren Alexander, Walter and Eliza Hall Institute of Medical Research [WEHI], Melbourne, Australia); Bcl-X_L mutant Plt20 and *c-mpl* mutant BC219 BALB/c mice (Benjamin Kile, WEHI); P-selectin deficient (*P-selectin*^{-/-}) C57BL/6 mice (Michael Hickey, Monash University, Melbourne, Australia) and NOD.SCID/ γC ^{-/-} mice (Jackson Laboratories) were housed under specific pathogen-free conditions. Experimental procedures were approved by the ANU Animal Experimentation Ethics Committee.

Antibody depletion of platelets and NK cells *in vivo*

Mice were depleted of platelets by i.v. injection of 20 μg (unless otherwise indicated) 1B5 mAb to murine $\alpha\text{IIb}\beta_3$ (Barry Coller, Rockefeller University, New York, NY) and NK cells were depleted using 50 μL /mouse of rabbit polyclonal anti-asialo GM1 antiserum (WAKO Pure Chemical Industries, Osaka, Japan) at the times indicated.

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Tumor cell lines and metastasis assays

Murine B16F1 melanoma, and 4T1.2 breast cancer (Robin Anderson, Peter MacCallum Cancer Institute, Melbourne, Australia) cell lines were cultured in F15 medium containing 10% fetal calf serum, 1% glutamine, and 0.1% penicillin, streptomycin and neomycin. For acute metastasis assays mice were injected i.v. with 1 to 2×10^5 tumor cells and organs removed 14 days later. For spontaneous metastasis assay mice were injected s.c. with 1×10^5 tumor cells and organs removed 24 days later. Organs were placed in 10% formalin (B16F1) or Bouin's solution (4T1.2) and metastases counted under a dissecting microscope.

Generation of P-selectin bone marrow chimeras

Two transplant formats were used as described in the supplementary section.

Tumor cell interactions with CFSE-labeled platelets *in vitro*

C57BL/6, BALB/c and *P-selectin*^{-/-} mice were injected i.v. with CFSE as described previously (8) to label platelets *in vivo*. Approximately 18 hours later, 200 μ L blood was collected into acid-citrate dextrose and washed fluorescent platelets added to tumor cell suspensions (ratio $\sim$ 1000:1) for 15 minutes at room temperature before analysis by flow cytometry for tumor cell-bound fluorescent platelets.

Measurement of cell surface protein expression by tumor cells

Tumor cells suspended in PBS/1% bovine serum albumin (5×10^6 /mL) were incubated with rat mAbs specific for murine α v-integrins (biotinylated, BioLegend; clone RMV-7) and tissue factor (Genentech; clone 1H1; ref. 9) or isotype controls for 30 minutes at 4°C, washed and, respectively, APC Streptavidin (BD Pharmingen) or a PE-conjugated mAb specific for rat IgG2a (BD Pharmingen) added before analysis by flow cytometry.

Thrombin pretreatment of tumor cells

B16F1 melanoma cells resuspended in Hanks buffered saline solution (HBSS; 5×10^6 /mL) were incubated with or without thrombin (2.5 U/mL) at 37°C for 15 minutes, gently agitated 5 minutes and washed 4 times in HBSS before i.v. injection.

Statistics

Comparisons of metastases between experimental groups were used using the Student *t* test when variance was equal between groups, as determined by the *F*-test, and the Welch *t* test when the variance between groups was unequal. Significance is given as a 2-tailed *P* value.

Results

Platelets are required for lung metastasis of B16F1 melanoma and 4T1.2 breast cancer cells

To determine the role of platelets in B16F1 melanoma and 4T1.2 breast cancer lung metastasis, C57BL/6 and BALB/c mice were injected 1 to 3 hours before i.v. tumor cell injection with the α IIB β 3-specific mAb, 1B5, resulting in >95% reduction

in platelet numbers within 10 minutes and lasting 24 to 48 hours after mAb injection (Fig. 1A–D). It was found that B16F1 lung metastases were undetectable in the platelet depleted mice and 4T1.2 lung metastases were only 6% the level of control mice ($P < 0.05$, Fig. 1).

To confirm the prometastatic role of platelets, metastasis experiments were undertaken in *c-mpl*^{-/-} C57BL/6 (lack the thrombopoietin receptor, $\leq$ 15% normal platelet numbers) and Plt20 BALB/c mice (nonfunctional anti-apoptotic protein, Bcl-X_L, $\sim$ 30% normal platelet numbers). In the *c-mpl*^{-/-} mice B16F1 lung metastasis numbers were, respectively, 11% ($P < 0.0005$) and 24% ($P < 0.05$) of those observed in control C57BL/6 mice and heterozygous (*c-mpl*^{+/-}) littermates (Fig. 1E). In contrast, no significant change in 4T1.2 lung metastases was observed between the Plt20 mutant and wild-type (WT) BALB/c mice (Fig. 1E). When C57BL/6 mice were administered 2 μ g of the 1B5 mAb to achieve a similar platelet number reduction as Plt20 mutant mice (i.e., $\sim$ 30% normal platelet numbers), no significant reduction in lung metastases was seen compared with control mice (Fig. 1E). Collectively these data indicate that the level of platelet reduction required to negatively impact upon metastasis is substantial ($\geq$ 85%) as $\sim$ 70% platelet depletion was shown to be insufficient to affect lung metastasis with both tumor cell lines (Fig. 1E).

NK cells strongly inhibit metastasis and mask the influence of platelets on liver and lung metastasis

To determine whether B16F1 melanoma metastasis is susceptible to NK cell control C57BL/6 mice depleted of NK cells and NOD.SCID/ γ c^{-/-} mice, genetically NK cell deficient (also B and T cell deficient), were used. Initial experiments showed that splenic NK cells were depleted by $\sim$ 80% 1 hour following injection of an anti-asialo GM1 pAb, whereas NKT, T and B cell splenic numbers were not affected (Supplementary Fig. S1), a similar depletion of NK cells (77%) being observed 48 hours later (data not shown).

NK-depleted C57BL/6 mice were found to have a 33-fold increase in lung ($P < 0.0005$) and a 54-fold increase in liver metastases ($P < 0.05$) compared with untreated control mice (Fig. 2A). Similarly, NOD.SCID/ γ c^{-/-} mice were found to have a 3-fold higher tumor burden in the lungs ($P < 0.005$) and 58-fold higher in the liver ($P < 0.0005$) than the NK cell sufficient NOD.SCID controls (Fig. 2A). Analysis of whether 4T1.2 tumor cell metastasis is also susceptible to NK cells yielded similar results, NK-cell depleted mice having a 47-fold higher incidence of lung metastases ($P < 0.000005$), although liver metastases were not found in either control or NK cell-depleted mice (Fig. 2B).

The respective roles of platelets and NK cells in the metastatic process was then assessed by depleting mice of NK cells and/or platelets before i.v. injection of B16F1 melanoma or 4T1.2 breast cancer cells. As a second model, *c-mpl*^{-/-} and control C57BL/6 mice were depleted of NK cells before B16F1 melanoma cell injection. In the B16F1 model, mice depleted of platelets had no, or very few, detectable lung or liver metastases (Fig. 3A). In contrast, mice depleted of NK cells had a dramatic increase in B16F1 lung (46-fold $P < 0.005$) and liver (66 ± 11 tumors versus 0 in controls) metastases (Fig. 3A). Mice

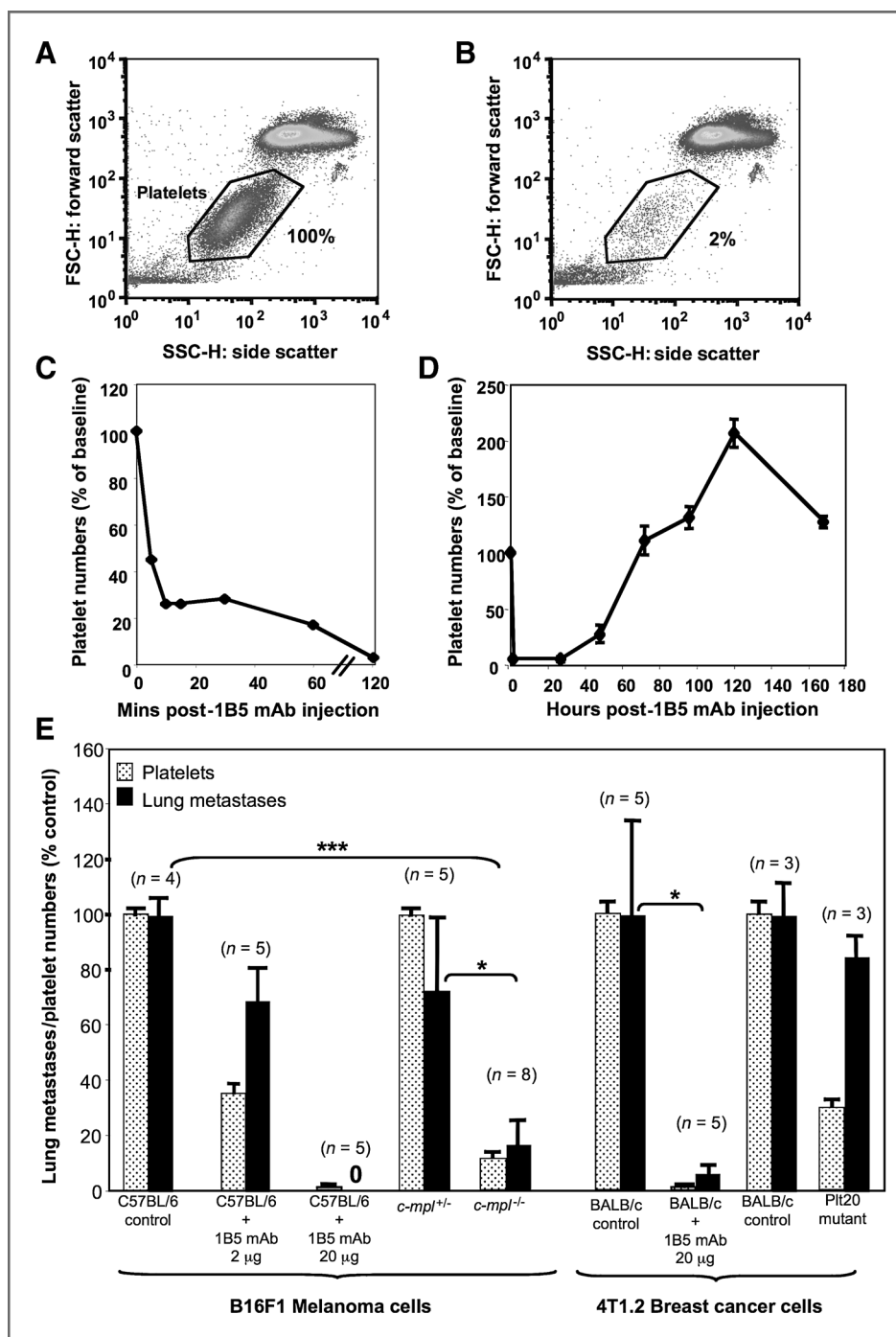


Figure 1. Antibody and genetic depletion of platelets shows that few platelets are required to promote lung metastasis. Flow cytometry detection of C57BL/6 platelets at baseline (A) and 2 hours following i.v. injection of 20 μ g of 1B5 mAb specific for platelet α IIb β 3 (B). Characteristic log forward- and side-scatter identified platelet populations and inclusion of reference beads allowed enumeration. C and D, C57BL/6 mice were injected with the 1B5 mAb and platelet numbers monitored for 60 minutes (C) or 7 days (D; $n = 3$). E, C57BL/6 and BALB/c mice depleted of platelets (1B5 mAb) 1 to 3 hours prior. C57BL/6 *c-mpl*^{-/-} mice (~15% of normal platelet numbers) and their *c-mpl*^{+/+} littermates (normal platelet numbers), and BALB/c Plt20 mutant mice (~30% normal platelet numbers) were injected i.v. with 1 to 2×10^5 B16F1 melanoma or 4T1.2 cells. Metastasis expressed as percentage control as data pooled from 5 experiments. Columns, mean; bars, SEM; significant reductions in metastases relative to control. *, $P \leq 0.05$ and ***, $P \leq 0.0005$.

depleted of both NK cells and platelets had a 48% decrease in lung metastases compared with mice depleted of NK cells alone ($P < 0.05$), accompanied by a 72% increase in liver

metastases ($P < 0.05$; Fig. 3A). Similar results were seen in the second B16F1 model, where *c-mpl*^{-/-} mice depleted of NK cells had an 83% reduction in lung metastases compared with the

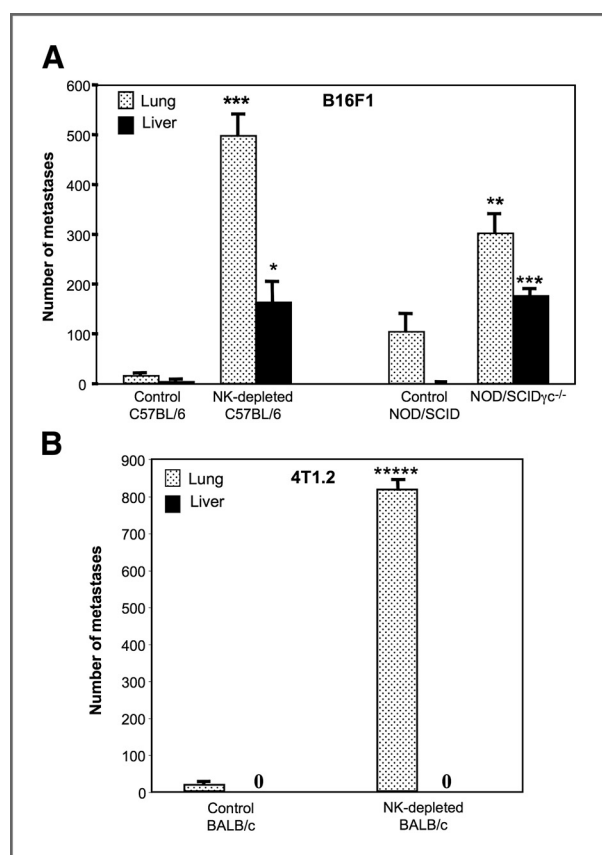


Figure 2. NK cells inhibit B16F1 melanoma and 4T1.2 breast cancer metastasis. **A**, B16F1 melanoma metastases in C57BL/6 mice depleted of NK cells 48 hours before tumor cell injection and in NOD/SCID^{γc-/-} mice ($n = 5$) genetically deficient in NK cells. **B**, tumor metastases in BALB/c mice ($n = 5$), depleted of NK cells 48 hours before the injection of 4T1.2 breast cancer cells. Columns, mean; bars, SEM; significant changes in metastasis numbers relative to controls. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$; and ****, $P \leq 0.00005$.

NK-depleted C57BL/6 controls ($P < 0.05$) with a concomitant 200% increase in liver metastases ($P < 0.05$; Fig. 3B). In the 4T1.2 model when BALB/c mice were depleted of platelets, NK cells or both before i.v. injection of 4T1.2 cells, virtually identical results were obtained as with the B16F1 melanoma. Thus, while NK cell depletion dramatically increased the number of lung metastases, a significant reduction in lung metastases occurred with the depletion of platelets both in the presence ($P < 0.05$) and absence of NK cells ($P < 0.05$; Fig. 3C). Overall these studies imply that NK cells dramatically reduce the metastatic potential of tumor cells and that platelets assist lung but not liver metastasis.

Platelets promote spontaneous lung metastasis independent of NK cells

To determine if spontaneous metastasis was also platelet-dependent, WT and platelet-deficient (BC219) BALB/c mice were injected s.c. with 4T1.2 tumor cells and depleted of NK cells when it was considered that metastasis from the primary tumors would have commenced. Tumor growth was signifi-

cantly slower in platelet-deficient than in WT mice (Supplementary Fig S2). Subgroup analysis of platelet-deficient ($n = 5$) and WT ($n = 4$) mice with equivalent tumor sizes revealed a total absence of lung metastases in the platelet-deficient mice whereas all WT mice had observable metastases (Fig. 3D).

The prometastatic effect of platelets occurs early and the antimetastatic effect of NK cells occurs later and independent of platelets

To resolve the relationship between the prometastatic action of platelets and the antimetastatic role of NK cells, platelets and NK cells were depleted at varying times before and after i.v. injection of B16F1 melanoma cells (Fig. 4A). These experiments showed that depletion of platelets 3 hours before tumor cell injection resulted in an almost total absence of lung metastases, whereas platelet depletion 1, 3, and 24 hours following tumor cell injection did not reduce the number of lung metastases (Fig. 4A). In contrast, when NK cells were depleted 48 hours before or 1 hour after i.v. tumor cell injection a comparable dramatic enhancement in lung and liver metastasis occurred. This enhancement gradually declined when the NK cell depleting pAb was given 3, 6, and 24 hours after the tumor cells (Fig. 4B). These data imply that NK cells exert their action after tumor cells lodge in the vasculature of target organs. As NK cell depletion is rapid (<1 hour, Supplementary Fig. S1), it seems that NK cells exert their antimetastatic effect between 1 and 6 hours following tumor cell injection. Additional studies determined whether the kinetics of NK cell inhibition of tumor metastasis changes in the absence of platelets. Mice were NK cell depleted 48 hours before and 1 or 24 hours following injection of B16F1 melanoma cells or mice were additionally depleted of platelets 24 hours before tumor cell injection (Fig. 4C). Similar results were obtained whether platelets were present or absent (Fig. 4C), supporting the view that the antimetastatic effect of NK cells is independent of platelets.

Endothelial P-selectin plays a critical role in lung and liver metastasis

As platelets do not promote tumor metastasis via inhibition of NK cell function, other roles of platelets in the metastatic process were examined. P-selectin, an adhesion protein expressed on both activated platelets and endothelial cells, has been shown to be involved not only in leukocyte extravasation, but also able to promote lung metastasis (10–12). We confirmed these previous findings, lung metastases in *P-selectin*^{-/-} mice being 63% lower than WT mice ($P < 0.0005$, Fig. 5A).

As P-sel is expressed by both platelets and endothelial cells, to ascertain the relative contributions of platelet and endothelial P-sel to lung metastasis, P-sel bone marrow chimeras were generated in 2 separate formats (see Supplementary section). The selective absence of platelet P-selectin substantially (77% Format 1, 78% Format 2) and significantly ($P < 0.005$ and $P < 0.05$, respectively) reduced the number of lung metastases compared with control bone marrow chimeras (Fig. 5B). Similarly, the selective absence of endothelial P-selectin resulted in a comparable (69% Format 1, 74% Format 2) and significant ($P < 0.005$ and $P < 0.005$) reduction in B16F1 lung

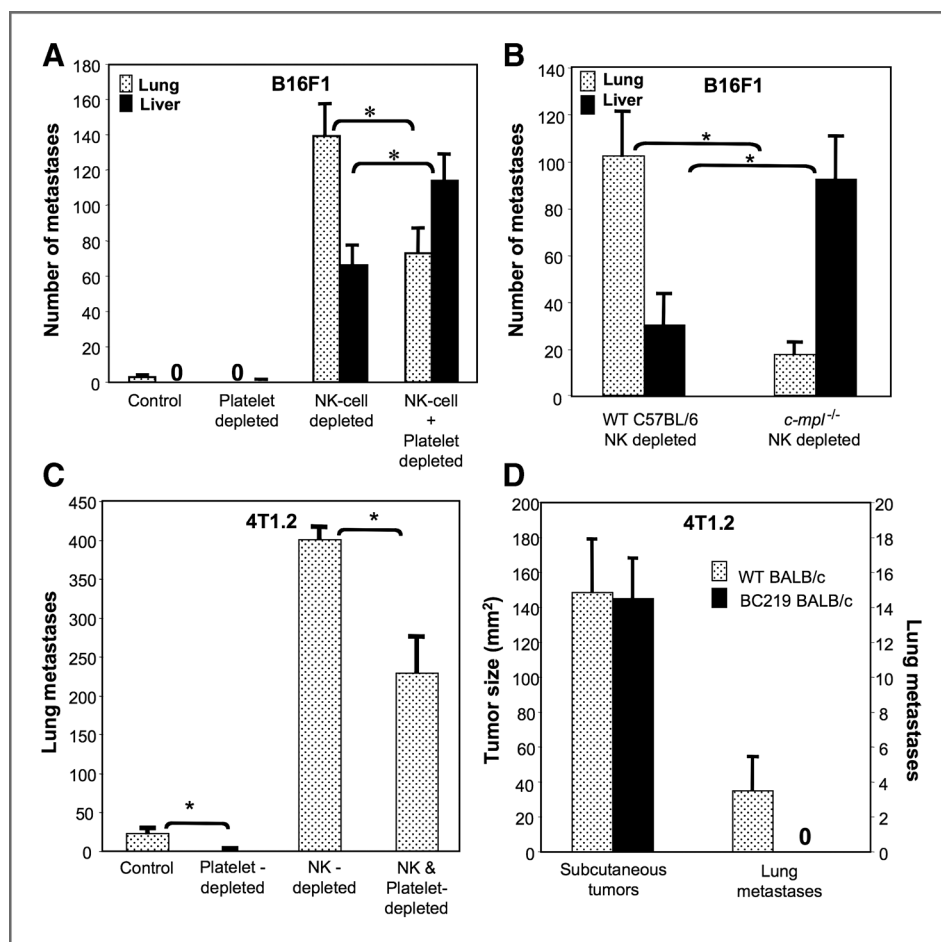


Figure 3. Interplay between platelets and NK cells on B16F1 melanoma lung and liver metastasis and 4T1.2 lung metastasis. A, C57BL/6 mice ($n = 5$) were depleted of NK cells 48 hours before and/or platelets 1 day before i.v. injection of B16F1 melanoma cells. B, WT C57BL/6 mice ($n = 5$) and *c-mpl*^{-/-} mice ($n = 5$) were depleted of NK cells 48 hours before B16F1 melanoma cells. C, BALB/c mice ($n = 5$) were depleted of NK cells 48 hours before and/or platelets 1 day before i.v. injection of 4T1.2 breast cancer cells. D, lung metastasis in NK-depleted WT ($n = 4$) and platelet-deficient (BC219) BALB/c mice ($n = 5$) with equivalent sized s.c. 4T1.2 tumors (see Supplementary Fig S2). Columns, mean; bars, SEM; significant changes in metastasis numbers. *, $P \leq 0.05$.

metastasis (Fig. 5B). Collectively these data indicate that both platelet and endothelial P-selectin are required for the lung metastasis of B16F1 melanoma cells.

As liver metastases were undetectable in the P-sel chimera experiments, to ascertain the role of P-selectin in B16F1 melanoma liver metastasis, WT and *P-sel*^{-/-} mice were depleted of NK cells before i.v. B16F1 cell injection. As seen with NK cell sufficient mice (Fig. 5A), lung metastases in NK cell depleted *P-sel*^{-/-} mice were reduced by 49% compared with NK cell depleted WT controls ($P < 0.005$). Liver metastases in NK cell depleted *P-sel*^{-/-} mice were also substantially (37%) and significantly ($P < 0.05$) reduced (Fig. 5C).

Our data suggests that platelets are not required for B16F1 liver metastasis (Fig. 3A and B), implying that platelet P-sel does not play a role in liver metastasis. The question remained whether endothelial P-sel is required for liver metastasis. To examine this possibility, WT and *P-sel*^{-/-} mice were depleted of NK cells and platelets 48 and 2 hours, respectively, before the i.v. injection of B16F1 melanoma cells. The platelet-depleted *P-sel*^{-/-} mice had significantly fewer liver (52%, $P < 0.05$) and lung (65%, $P < 0.05$) metastases than their platelet-depleted WT controls (Fig. 5D), indicating that endothelial P-selectin is just as important for B16F1 melanoma liver metastasis as lung metastasis.

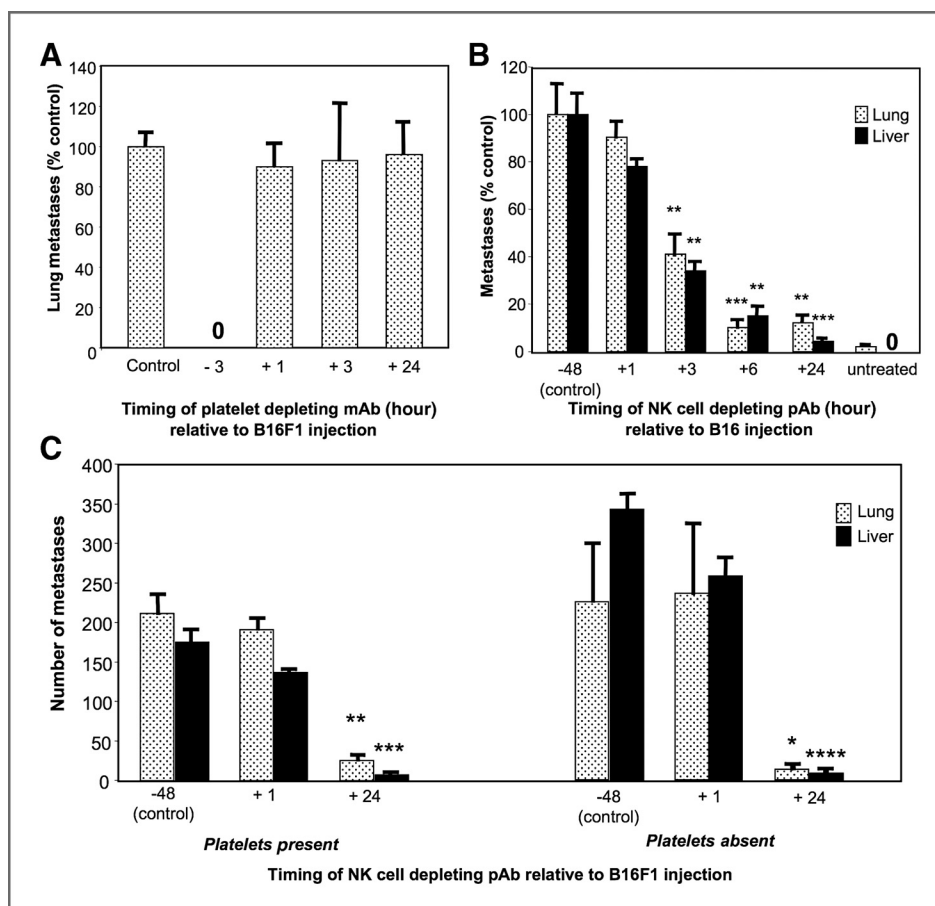
Interaction of B16F1 and 4T1.2 tumor cells with platelets *in vitro*

As platelets clearly are prometastatic, the capacity of B16F1 melanoma and 4T1.2 tumor cells to bind platelets was analyzed *in vitro* using fluorescently (CFSE) labeled platelets. Both tumor cell lines were capable of directly binding platelets (Fig. 6A and B), with platelets from WT and *P-sel*^{-/-} exhibiting comparable, although a subpopulation of melanoma cells failed to bind *P-sel*^{-/-} platelets. This small *in vitro* effect of P-selectin deficiency on platelet binding is not surprising as there was extensive platelet processing and the *in vitro* experimental conditions were vastly different from those occurring *in vivo* (Fig. 6A). As the integrin $\alpha v \beta 3$ has recently been shown to mediate tumor cell binding to platelets via $\alpha IIb \beta 3$ (13), B16F1 melanoma and 4T1.2 breast cancer cells were analyzed for αv -integrin expression. Both tumor cell lines express αv -integrins at high levels (Fig. 6D and E) αv -integrin-specific mAb used did not block binding of CFSE-labeled platelets to B16F1 melanoma cells (data not shown).

Role of tissue factor and thrombin in platelet-dependent B16F1 melanoma metastasis

The mechanisms of platelet-assisted tumor cell metastasis were further explored by determining the expression status of

Figure 4. Platelet and NK cell effects on B16F1 metastasis occur at different time points and independently of each other. A and B, C57BL/6 mice ($n = 5$) were depleted of platelets (A) or NK cells (B) at time indicated relative to i.v. injection of B16F1 melanoma cells. C and D, C57BL/6 mice ($n = 5$) were depleted of NK cells at time indicated (C) and in a separate experiment, additionally depleted of platelets 24 hours before i.v. injection of B16F1 melanoma cells (D). A and B, metastasis expressed as percentage control as data pooled from 3 experiments. Columns, mean; bars, SEM; significant changes in metastasis numbers. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$; and ****, $P \leq 0.00005$.



tissue factor on B16F1 melanoma and 4T1.2 breast cancer cells. Both tumor cell lines were negative for tissue factor expression (Supplementary Fig. S3) and, therefore, are unable to initiate coagulation, via factor VII binding and activation, with subsequent thrombin generation.

As thrombin has been reported to increase the metastatic capacity of tumor cells via PAR signaling, the effect of thrombin pretreatment of B16F1 melanoma cells on lung and liver metastasis in platelet-sufficient and platelet-deficient mice was investigated. Thrombin pretreatment doubled the number of lung metastases ($P < 0.05$, Fig. 6C) but did not alter liver metastasis in platelet-sufficient mice. The increased lung metastatic potential, however, was eliminated in platelet-deficient mice ($P < 0.005$; Fig. 6C). Any platelet-independent effect of thrombin on B16F1 melanoma metastatic potential would, therefore, seem to be minimal in this model.

Discussion

This study provides several novel insights into the role of platelets, NK cells and P-selectin in tumor metastasis. First, it was confirmed that platelets enhance metastasis but the prometastatic effect of platelets is surprisingly brief, lasting ≤ 1 hour following tumor cell entry into the circulation. Second, NK cells have a profound effect on lung metastasis reducing

metastases by 30- to 50-fold and totally masking B16F1 liver metastases. Third, the antimetastatic action of NK cells occurs for a short period (1 to 6 hours) following tumor cell injection but clearly after the prometastatic actions of platelets and is not inhibited by platelets. Fourth, unlike B16F1 melanoma lung metastasis, platelets do not seem to be necessary for B16F1 melanoma liver metastasis. Fifth, endothelial P-selectin plays an important role in both B16F1 melanoma lung and liver metastasis, with it being confirmed that platelet P-selectin contributes to lung metastasis.

The susceptibility of B16F1 melanoma and 4T1.2 breast carcinoma metastasis to NK cell inhibition was dramatically shown using either the genetically NK cell deficient NOD.SCID/ $\gamma c^{-/-}$ mouse strain, or mice depleted of NK cells (Fig. 2). It was found in both these models, depletion of NK cells resulted in a very substantial increase in lung metastases (3- to 33-fold). Interestingly, liver metastases were not usually seen with B16F1 melanoma, however, in the absence of NK cells the number of liver metastases was dramatically increased. The appearance of B16F1 tumor metastases in the liver of NK depleted mice implies that a significant proportion of B16F1 melanoma cells pass through the lung capillary beds to the left cardiac ventricle from where they are dispersed to multiple organs including the liver. In NK cell sufficient mice, these tumor cells are efficiently eliminated from the liver, however, in

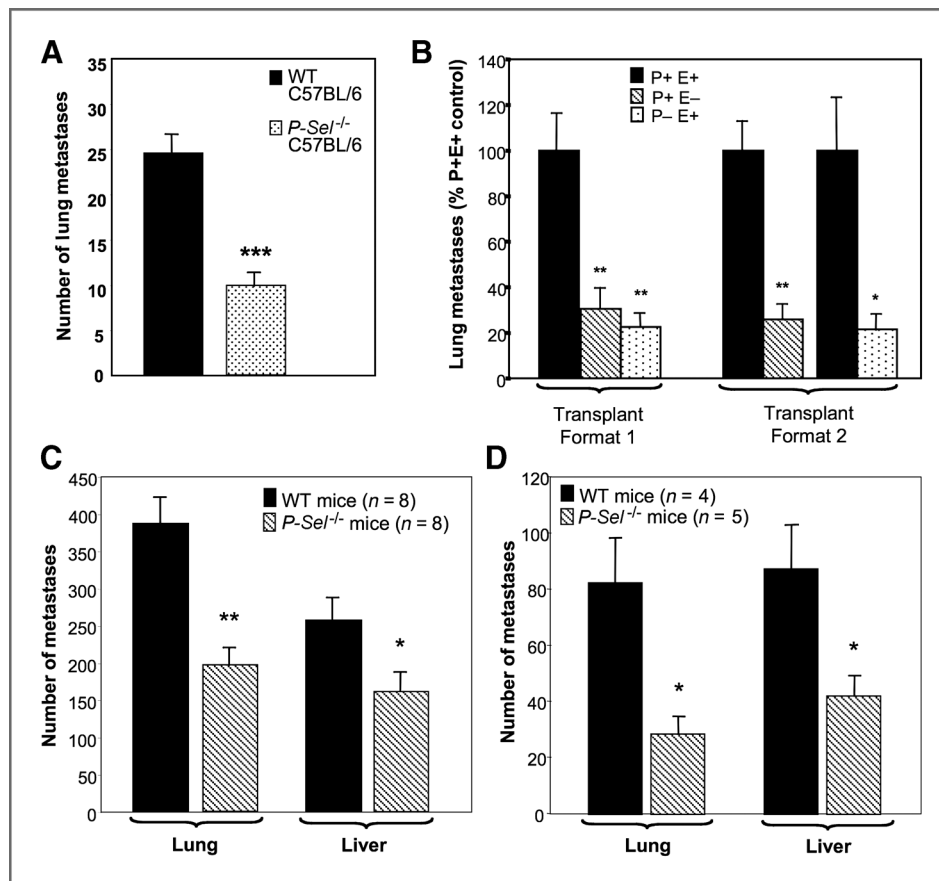


Figure 5. Platelet- and endothelial P-selectin promote B16F1 melanoma lung metastasis while endothelial P-selectin also promotes liver metastasis. **A**, WT C57BL/6 ($n = 24$) and *P-selectin*^{-/-} ($n = 22$) mice were injected i.v. with B16F1 melanoma cells. Data pooled from 3 separate experiments. **B**, P-selectin bone marrow chimeras ($n = 5$ /group) were generated in 2 experimental formats (see Supplementary Methods) to obtain platelet- or endothelial- P-selectin deficient mice. Following donor marrow engraftment, mice were injected i.v. with B16F1 melanoma cells. Metastasis expressed as percentage control as data pooled from 2 experiments. **C**, WT C57BL/6 and *P-selectin*^{-/-} mice were depleted of NK cells 48 hours before injection of B16F1 melanoma cells. **D**, as in **C**, however mice were additionally depleted of platelets 2 hours before injection of B16F1 melanoma cells. Columns, mean; bars, SEM; significant reductions in metastasis numbers relative to WT controls. *, $P \leq 0.05$; **, $P \leq 0.005$; and ***, $P \leq 0.0005$. P+E+ = WT controls; P-E+ = platelet P-selectin deficient; P+E- = endothelial P-selectin deficient.

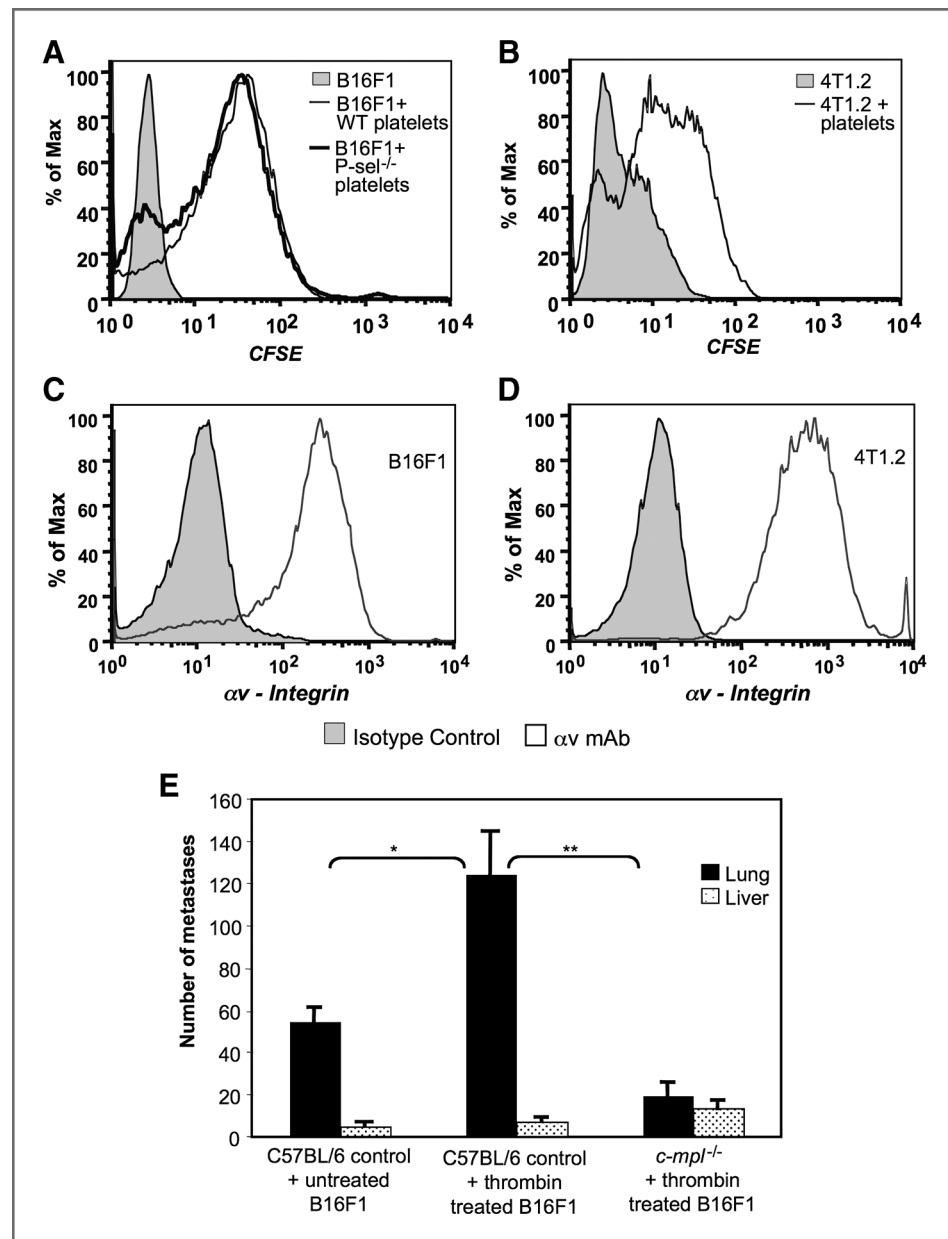
NK cell deficient mice the extent of tumor cell infiltration into hepatic tissue is revealed. Unlike B16F1 melanoma, liver metastases were not seen with 4T1.2 tumor cells in NK cell depleted mice. This observation suggests that 4T1.2 tumor cells are incapable of extravasating into, or surviving within, the hepatic environment and is consistent with previous studies on this cell line (14).

It has been suggested that platelets promote metastasis either through shielding tumor cells from NK cell lysis or by molecular inhibition of NK cell function (4–6). We therefore probed the prometastatic effects of platelets and the antimetastatic effects of NK cells for evidence of an interrelationship by depleting mice of platelets, NK cells or both before the i.v. injection of B16F1 melanoma and 4T1.2 breast cancer cells. In addition, the platelet-deficient mouse strain, *c-mpl*^{-/-}, was used to corroborate the results obtained with the Ab-mediated platelet depletion model (Fig. 3A–C). We found that in mice deficient/depleted of both platelets and NK cells, a 48% to 83% reduction in lung metastases occurred compared with mice depleted of NK cells only. This result indicates, first, that platelets promote B16F1 melanoma and 4T1.2 lung metastasis in the absence of NK cells and, second, that many tumor cells could extravasate in the absence of platelets, a result only revealed when NK cells were also depleted, and may be due to the involvement of endothelial adhesion molecules or physical entrapment within small vessels. Furthermore, in the absence

of platelets and NK cells, a concomitant 42% to 66% increase in B16F1 melanoma metastases was observed in the liver, probably due to many more tumor cells passing through the lungs from a reduced ability to bind to the endothelium without platelet assistance. The NK cell-independent prometastatic effect of platelets was also shown in a spontaneous metastasis model, with lung metastases reduced 100% in platelet-deficient, NK cell depleted, mice compared with NK cell depleted WT mice.

Studies on the kinetics of the prometastatic effect of platelets shown the time frame during which platelets are required for metastasis is very short in duration and early in the metastatic process. Essentially, platelets only act in the first hour following entry of tumor cells into the bloodstream (Fig. 4A). These results support the broadly accepted model of the role of platelets in metastasis, namely, that platelets mediate the initial interaction of tumor cells with the blood vessel wall via platelet adhesion proteins binding to their endothelial ligands (2). In contrast, studies on the kinetics of the profound negative effect of NK cells on B16F1 lung and liver metastasis also indicated a surprisingly short timeframe, between 1 and 6 hours following tumor cell entry into the blood stream, but more importantly, additional experiments showed the timeframe of the NK cell effect on metastasis was unchanged whether platelets were present or absent (Fig. 4). Collectively, these results unequivocally show that platelets neither impede

Figure 6. Ability of tumor cells to directly bind platelets *in vitro* and effect of thrombin pretreatment of tumor cells on their metastatic potential. **A**, CFSE-labeled platelets from WT and *P-selectin*^{-/-} C57BL/6 mice were incubated with B16F1 melanoma cells and bound platelets determined by flow cytometry. **B**, CFSE-labeled platelets from WT BALB/c mice were incubated with 4T1.2 tumor cells and tumor cell binding assessed as in **A**. **C**, B16F1 melanoma cells were incubated with a biotinylated mAb against murine integrin α v subunit or isotype control and mAb binding quantified by flow cytometry. **D**, 4T1.2 breast cancer cells were assessed for α v-integrin expression as in **C**. **E**, B16F1 melanoma cells were incubated with thrombin, washed thoroughly, and injected into WT or platelet-deficient C57BL/6 mice. Lung and liver metastases were counted at 14 days. Columns, mean; bars, SEM; significant reductions in metastasis numbers. *, $P \leq 0.05$ and **, $P \leq 0.005$.



nor inhibit NK cell activity in these metastasis models. In addition, our experiments suggest that platelets play little or no role in B16F1 melanoma liver metastasis.

Our findings contrast with some prior *in vivo* studies examining the role of platelets and NK cells in lung metastasis (4, 5). As the antimetastatic effect of NK cells is so profound, the level of experimental variation in the acute metastasis model may have previously masked the true NK cell-independent effect of platelets (4). In a separate study mice deficient in $G\alpha_q$, a protein critical for platelet activation, were used to assess the role of platelets and NK cells in tumor cell survival within the lungs as measured by residual radioactivity. This model, however, did not account for adhesion proteins expressed on resting platelets that may be involved in initial tumor cell:

platelet interactions, a factor that may explain why no difference was observed in residual tumor cells in the lungs between WT and $G\alpha_q$ ^{-/-} mice until 5 to 24 hours following tumor cell injection (5). Several *in vitro* studies have been conducted examining the molecular mechanisms of platelet inhibition of NK cell function, however, in the *in vitro* setting the very significant effect of the endothelium on platelet and immune cell function is removed and platelet processing dramatically alters the phenotype of platelets from their circulating state. Discrepancies between *in vivo* and *in vitro* results, therefore, commonly occur. A relevant example of this is whether MHC Class I transferred from platelets to tumor cells inhibits NK cell-tumor cell killing function. This was found to be the case *in vitro* (7) but not *in vivo* (4).

The adhesion protein P-selectin expressed by platelets and endothelial cells, has previously been shown to be involved in tumor metastasis although it has been generally assumed that platelet and not endothelial P-selectin is involved (10). Using the B16F1 melanoma experimental metastasis model in *P-selectin*^{-/-} mice we confirmed that P-selectin significantly promotes lung metastasis. However, using P-selectin deficient bone marrow chimeras we discovered, unexpectedly, that both endothelial-derived and platelet-derived P-selectin contribute to the metastatic process to a similar extent (Fig. 5). This confirms previous findings of the role of endothelial P-selectin in lung metastasis obtained in platelet P-selectin deficient chimeric mice administered a P-selectin mAb (15). Additional experiments revealed that B16F1 melanoma liver metastasis is also endothelial P-selectin dependent (Fig. 5).

Although difficult to show, it is generally believed that resting platelets express very low levels of P-selectin on their surface (personal communication, Dr Rodger McEver). Thus, the initial interaction between circulating tumor cells and platelets via P-selectin may not rely upon prior platelet activation. Some tumor cell types express tissue factor and are, therefore, able to generate thrombin and activate platelets thus dramatically increasing P-selectin numbers on the surface of platelets (16, 17). This is unlikely to have been the case in the studies described herein, however, as both tumor cell lines used were found to be negative for tumor factor expression (Supplementary Fig. S2).

Tumor cells may express members of the protease-activated receptor family, PAR, of which the members 1, 3, and 4 are activated by thrombin and signaling via PARs has been shown to promote tumor growth, invasion and metastasis (18). The effect of thrombin exposure on the capacity of B16F1 melanoma cells to metastasize to the lung and liver was studied and, although thrombin pretreatment of B16F1 cells increased metastatic potential to the lung, this prometastatic effect was found to be highly reliant upon platelets. Liver metastasis was not affected by thrombin pretreatment (Fig. 6E). It is possible that thrombin treatment of tumor cells activates membrane integrins thus promoting interactions with platelets. Indeed, PAR1 signaling was found to promote invasion and metastasis of melanoma through integrin $\alpha v \beta 5$ activation (19). Consistent with this hypothesis, both B16F1 and 4T1.2 tumor cells were shown to express high levels of αv -integrin (Fig. 6C and D). Furthermore, recent studies showed that integrin $\alpha v \beta 3$ on melanoma cells binds $\alpha IIb \beta 3$ on platelets and use of blocking mAbs to either of these proteins significantly decreased the interaction (13).

In contrast to platelets, liver and lung endothelium constitutively expresses P-selectin and at levels higher than all other organs (20, 21), possibly to promote leukocyte migration and surveillance as these organs are exposed to high levels of pathogens, either inhaled or ingested. It is not surprising the liver and lungs are susceptible to invasion by metastasizing tumor cells given their locations within the circulatory system, the constitutive expression of P-selectin, and the fact that many tumor cells express mucins containing sialyl Lewis^x and sialyl Lewis^a that bind P-selectin (22). Our studies suggest that in the lungs, platelets play a

significant role in anchoring tumor cells to the endothelium against the relatively high inherent blood flow velocity. In the liver, however, where blood flow is comparatively slow due to hepatic vessel anatomy, interactions between endothelial and tumor cell adhesion proteins are sufficient to arrest tumor cells enabling their subsequent extravasation, with platelet assistance not being required.

In summary, this study has shown that lung metastasis of both B16F1 melanoma and 4T1.2 breast cancer is promoted by platelets and inhibited by NK cells. Moreover, in the B16F1 metastasis model, the prometastatic role of platelets and the antimetastatic role of NK cells were shown to be chronologically distinct, and in both acute and spontaneous metastasis models platelets promote lung metastasis by a process that is independent of NK cells. Intriguingly, unlike B16F1 melanoma lung metastasis, B16F1 liver metastasis appeared independent of platelets as, while lung metastases decreased, liver metastases increased in the absence of platelets. These findings are of particular clinical importance as they suggest that the use of platelet inhibitors to prevent metastasis may result in the redistribution rather than diminution of metastatic spread. The prometastatic roles of both platelet-derived and endothelial-derived P-selectin in lung metastasis, and endothelial-derived P-selectin in liver metastasis, were shown. Thus, although previous investigators have proposed P-selectin as a target for the prevention of lung metastasis, our studies show for the first time *in vivo* that P-selectin is an excellent target for preventing metastasis in multiple organs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L.A. Coupland, B.H. Chong, C.R. Parish
Development of methodology: L.A. Coupland, B.H. Chong
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.A. Coupland
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.A. Coupland, B.H. Chong, C.R. Parish
Writing, review, and/or revision of the manuscript: L.A. Coupland, B.H. Chong, C.R. Parish
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.A. Coupland
Study supervision: C.R. Parish

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