Title:
Platelets and P-selectin control tumor cell metastasis in an organ-specific manner and independently of NK cells

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Abstract

The pro-metastatic role of platelets has long been recognized with proposed mechanisms of action including shielding tumor cells from 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 pro-metastatic 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. Additionally, the pro-metastatic 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 anti-metastatic activity of NK cells was equally effective in the presence or absence of platelets and chronologically distinct from the pro-metastatic role of platelets. Lastly, 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.
Introduction

Platelets have long been implicated in hematogenous metastasis with the first demonstrated 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 αIIbβ3; (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 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). However, since platelets promote the extravasation of metastasising tumor cells, it is unlikely that the pro-metastatic role of platelets is limited to the inhibition of NK cell effector function. In this paper we examined whether the pro-metastatic role of platelets is global or organ specific, the relationship between the pro-metastatic activity of platelets and the anti-metastatic 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-XL mutant Plt20 and c-mpl mutant BC219 BALB/c mice (Benjamin Kile, WEHI, Melbourne, Australia); P-selectin deficient (P-sel-/-) C57BL/6 mice (Michael Hickey, Monash University, Melbourne, Australia) and NOD.SCID/γc-/- mice (Jackson Laboratories, USA) 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 αIIbβ3 (Barry Coller, Rockefeller University, 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.

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 - 2 x 10^5 tumor cells and organs removed 14 days later. For spontaneous metastasis assay mice were injected s.c. with 1 x 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 cells interactions with CFSE-labelled platelets in-vitro**

C57BL/6, BALB/c and P-sel-/ mice were injected i.v. with CFSE as described previously (8) to label platelets in-vivo. Approximately 18 hr later, 200 μL blood was collected into acid-citrate dextrose and washed fluorescent platelets added to tumor cell suspensions (ratio ~1000:1) for 15 min at room temperature prior to 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 x 10^6/ml) were incubated with rat mAbs specific for murine αv-integrins (biotinylated, BioLegend; clone RMV-7;) and tissue factor (Genentech; clone 1H1; (9)) or isotype controls for 30 min at 4°C, washed and, respectively, APC Streptavidin (BD Pharmingen) or a PE conjugated mAb specific for rat IgG2a
(BD Pharmingen) added prior to analysis by flow cytometry.

**Thrombin pre-treatment of tumor cells**

B16F1 melanoma cells resuspended in Hanks buffered saline solution (HBSS) (5 x 10^6/ml) were incubated with or without thrombin (2.5 U/ml) at 37°C for 15 min, gently agitated 5 minutely and washed 4 times in HBSS prior to i.v. injection.

**Statistics**

Comparisons of metastases between experimental groups were performed using the Student’s t-test when variance was equal between groups, as determined by the F-test, and the Welch’s 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 1-3 hr prior to i.v. tumor cell injection with the αIIbβ3-specific mAb, 1B5, resulting in >95% reduction in platelet numbers within 10 minutes and lasting 24-48 hr 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 pro-metastatic role of platelets, metastasis experiments were undertaken in c-mpl-/- C57BL/6 (lack the thrombopoietin receptor, ≤15% normal platelet numbers) and Plt20 BALB/c mice (non-functional anti-apoptotic protein, Bcl-XL, ~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 WT BALB/c mice (Fig. 1E). When C57BL/6 mice were administered 2 μg of the 1B5 mAb to achieve a similar platelet number reduction as Plt20 mutant mice (i.e., ~30% normal platelet numbers), no significant reduction in lung metastases was seen compared to control mice (Fig. 1E). Collectively these data indicate that the level of platelet reduction required to negatively impact upon
metastasis is substantial (∼85%) as ∼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/γc-/- mice, genetically NK cell deficient (also B and T cell deficient), were utilized. Initial experiments showed that splenic NK cells were depleted by ∼80% 1 hr following injection of an anti-asialo GM1 pAb whereas NKT, T and B cell splenic numbers were not affected (Fig. S1), a similar depletion of NK cells (77%) being observed 48 hr 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 to untreated control mice (Fig. 2A). Similarly, NOD.SCID/γc-/- 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 prior to 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 prior to 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 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 to the 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 prior to 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 significantly slower in platelet-deficient than in WT mice (Supp Fig 2). 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 while all WT mice had observable metastases (Fig 3D).

The pro-metastatic effect of platelets occurs early and the anti-metastatic effect of NK-cells occurs later and independent of platelets

To resolve the relationship between the pro-metastatic action of platelets and the anti-metastatic 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 hr prior to tumor cell injection resulted in an almost total absence of lung metastases, whereas platelet depletion 1, 3 and 24 hr following tumor cell injection did not reduce the number of lung metastases (Fig. 4A). In contrast, when NK cells were depleted 48 hr before or 1 hr 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 hr 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.
Since NK cell depletion is rapid (<1 hr, Fig. S1), it appears that NK cells exert their anti-metastatic effect between 1 and 6 hr 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 hr prior to and 1 or 24 hr following injection of B16F1 melanoma cells or mice were additionally depleted of platelets 24 hr prior to tumor cell injection (Fig. 4C). Similar results were obtained whether platelets were present or absent (Fig. 4C), supporting the view that the anti-metastatic effect of NK cells is independent of platelets.

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

Since 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 demonstrated 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-sel-/− mice being 63% lower than WT mice (P<0.0005, Fig. 5A).

As P-sel is expressed by both platelets and endothelial cells, in order to ascertain the relative contributions of platelet and endothelial P-sel to lung metastasis, P-sel bone marrow (BM) chimeras were generated in two 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 to control BM 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 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 prior to 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 hr and 2 hr, respectively, prior to 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**

Since platelets clearly are pro-metastatic, the capacity of B16F1 melanoma and 4T1.2 tumor cells to bind platelets was analysed in-vitro using fluorescently (CFSE) labelled 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 αvβ3 has recently been demonstrated to mediate tumor cell binding to platelets via -αIIbβ3 (13), B16F1 melanoma and 4T1.2 breast cancer cells were analysed 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-labelled 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 tissue factor (TF) on B16F1 melanoma and 4T1.2 breast cancer cells. Both tumor cell lines were
negative for TF expression (Supp. Fig. 3) 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 signalling, the effect of thrombin pre-treatment of B16F1
melanoma cells on lung and liver metastasis in platelet-sufficient and platelet-
deficient mice was investigated. Thrombin pre-treatment 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, appear 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 pro-metastatic 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 30-50
fold and totally masking B16F1 liver metastases. Third, the anti-metastatic
action of NK cells occurs for a short period (1-6 hr) following tumor cell
injection but clearly after the pro-metastatic actions of platelets and is not
inhibited by platelets. Fourth, unlike B16F1 melanoma lung metastasis,
platelets do not appear 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 demonstrated using either the genetically NK cell deficient NOD.SCID/γc-/- 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 (3-33 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 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 pro-metastatic effects of platelets and the anti-metastatic effects of NK cells for evidence of an interrelationship by depleting mice of platelets, NK cells or both prior to the i.v. injection of B16F1 melanoma and 4T1.2 breast cancer cells. Additionally, 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 48-83% reduction in lung metastases occurred compared to mice depleted of NK cells only. This result indicates, firstly, that platelets promote B16F1 melanoma and 4T1.2 lung metastasis in the absence of NK cells and, secondly, 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 42-66% 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 pro-metastatic effect of platelets was also demonstrated in a spontaneous metastasis model, with lung metastases reduced 100% in platelet-deficient, NK cell depleted, mice compared to NK cell depleted WT mice.
Studies on the kinetics of the pro-metastatic effect of platelets demonstrated 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 demonstrate that platelets neither impede nor inhibit NK cell activity in these metastasis models. Additionally, 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 anti-metastatic 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 Gαq, a protein critical for platelet activation, were used to assess the role of platelets and NK cells in tumour 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 tumour cell:platelet interactions, a factor that may explain why no difference was observed in residual tumour cells in the lungs between WT and Gαq-/- mice until 5-24 hr following tumour 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 tumour 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-sel-/- 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-sel mAb (15). Additional experiments revealed that B16F1 melanoma liver metastasis is also endothelial P-selectin dependent (Fig. 5).

Although difficult to demonstrate, 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 TF 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 TF expression (Supp. Fig. 2).

Tumour cells may express members of the protease-activated receptor family, PAR, of which the members 1, 3 and 4 are activated by thrombin and signalling via PARs has been demonstrated to promote tumour growth, invasion and metastasis (18). The effect of thrombin exposure on the capacity of B16F1 melanoma cells to metastasise to the lung and liver was studied and, although thrombin pre-treatment of B16F1 cells increased metastatic potential to the lung, this pro-metastatic effect was found to be highly reliant upon platelets. Liver metastasis was not affected by thrombin pre-treatment (Fig. 6E). It is possible that thrombin treatment of tumour cells activates membrane integrins thus promoting interactions with platelets. Indeed, PAR1 signalling was found to promote invasion and metastasis of melanoma
through integrin αvβ5 activation (19). Consistent with this hypothesis, both B16F1 and 4T1.2 tumor cells were demonstrated to express high levels of αv-integrin (Fig 6 C & D). Furthermore, recent studies demonstrated that integrin αvβ3 on melanoma cells binds αIIbβ3 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's not surprising the liver and lungs are susceptible to invasion by metastasising 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 demonstrated 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 pro-metastatic role
of platelets and the anti-metastatic 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 pro-metastatic roles of both platelet-derived and endothelial-derived P-selectin in lung metastasis, and endothelial-derived P-selectin in liver metastasis, were demonstrated. Thus, although previous investigators have proposed P-selectin as a target for the prevention of lung metastasis, our studies demonstrate for the first time in-vivo that P-selectin is an excellent target for preventing metastasis in multiple organs.
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FIGURE LEGENDS

Figure 1. Antibody and genetic depletion of platelets demonstrates that few platelets are required to promote lung metastasis. Flow cytometry detection of C57BL/6 platelets (A) at baseline and (B) 2 hr following i.v. injection of 20 μg of 1B5 mAb specific for platelet αIIbβ3. Characteristic log forward- and side-scatter identified platelet populations and inclusion of reference beads allowed enumeration. C-D, C57BL/6 mice were injected with the 1B5 mAb and platelet numbers monitored for (C) 60 mins or (D) 7 days (n=3). E, C57BL/6 and BALB/c mice depleted of platelets (1B5 mAb) 1-3 hr 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 1-2 x 10⁵ B16F1 melanoma or 4T1.2 cells. Metastasis expressed as % control as data pooled from 5 experiments. Columns, mean; Bars, SEM; Significant reductions in metastases relative to control *, P≤0.05 and ***, P≤0.0005.
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 hr prior to tumor cell injection and in NOD/SCIDγc-/- mice (n=5) genetically deficient in NK cells. B, tumor metastases in BALB/c mice (n=5), depleted of NK cells 48 hr prior to the injection of 4T1.2 breast cancer cells. Columns, mean; Bars, SEM; Significant changes in metastasis numbers relative to controls *, P≤0.05 **, P≤0.005, ***, P≤0.0005 and ****, P≤0.000005.

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 hr prior to and/or platelets 1 day prior to 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 hr prior to B16F1 melanoma cells. C, BALB/c mice (n=5) were depleted of NK cells 48 hr prior to and/or platelets 1 day prior to 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 Supp Fig 2). Columns, mean; Bars, SEM; Significant changes in metastasis numbers *, P≤0.05.

Figure 4. Platelet and NK cell effects on B16F1 metastasis occur at different time points and independently of each other. A, C57BL/6 mice (n=5) were depleted of platelets or (B) NK cells at time indicated relative to i.v. injection of B16F1 melanoma cells. C, C57BL/6 mice (n=5) were depleted of NK cells at
time indicated and (D) in a separate experiment, additionally depleted of platelets 24 hr prior to, i.v. injection of B16F1 melanoma cells. A and B; Metastasis expressed as % 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 ****, $P \leq 0.00005$.

**Figure 5.** Platelet- and endothelial P-selectin promote B16F1 melanoma lung metastasis whilst 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 % control as data pooled from 2 experiments. C, WT C57BL/6 and P-selectin-/- mice were depleted of NK cells 48 hr prior to injection of B16F1 melanoma cells. D, as in (C), however mice were additionally depleted of platelets 2 hr prior to 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-sel deficient; P+E- = endothelial P-sel deficient.

**Figure 6.** Ability of tumor cells to directly bind platelets in-vitro and effect of thrombin pre-treatment of tumor cells on their metastatic potential. A, CFSE-
labelled platelets from WT and P-sel-/- C57BL/6 mice were incubated with
B16F1 melanoma cells and bound platelets determined by flow cytometry. B,
CFSE-labelled 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 αv
subunit or isotype control and mAb binding quantified by flow cytometry. D,
4T1.2 breast cancer cells were assessed for αv- 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≤0.05, and **, P≤0.005.
Figure 4

A. Timing of platelet depleting mAb (hr) relative to B16F1 injection

B. Timing of NK cell depleting pAb (hr) relative to B16 injection

C. Number of Metastases

- Plates Present
- Plates Absent

Legend:
- Lung
- Liver
Platelets and P-selectin control tumor cell metastasis in an organ-specific manner and independently of NK cells

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