Clinical & Experimental Metastasis (2018) 35:679–689
https://doi.org/10.1007/s10585-018-9924-8

RESEARCH PAPER

Platelet deficiency in *Tpo*$^{-/-}$ mice can both promote and suppress the metastasis of experimental breast tumors in an organ-specific manner

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Received: 8 June 2018 / Accepted: 19 July 2018 / Published online: 24 July 2018

Abstract

Platelets are thought to play an important role in metastasis formation, although the mechanisms involved remain incompletely understood. Here we studied the influence of platelet numbers on organ-specific metastasis to the lungs and lymph nodes using *Tpo* deficient mice that have low platelet counts. After tail vein injection of 4T1 breast cancer cells, the number of lung metastases was significantly lower in *Tpo*$^{-/-}$ mice compared to *Tpo*$^{+/+}$ mice. The same was true for the bone-tropic 4T1.2 derivative. In spontaneous orthotopic metastasis assays, 4T1 and 4T1.2 primary tumor growth was not affected by the genotype of the mice. However, the number of 4T1.2 lung metastases was significantly lower in *Tpo*$^{-/-}$ mice compared to *Tpo*$^{+/+}$ mice, whereas the number of 4T1 lung metastases was unaffected. Moreover, in mice bearing 4T1 tumors, lymph node metastases were larger in the *Tpo*$^{-/-}$ background, and lymph node metastasis frequency was higher in *Tpo*$^{-/-}$ mice bearing 4T1.2 tumors compared to that in wild-type mice. Enhanced lymph node metastasis in *Tpo*$^{-/-}$ mice was not associated with changes in peritumoral lymphatic vessel density in the primary tumors. Together, our data indicate that platelets do not affect primary tumor growth in this breast cancer model, but can differentially influence site-specific metastasis to lymph nodes and lungs.

Keywords Platelets · Metastasis · Lymph node · *Tpo* deficient mice · Tumor growth

Introduction

The majority of carcinoma patients succumb to their disease as a consequence of the metastases they develop [1]. Once a tumor has metastasized to distant sites, treatment options are limited and rarely curative. Therefore, a better understanding of the metastatic process and the development of novel therapies remain major challenges.

Physiologically, platelets are mainly involved in blood clotting, wound closure and wound healing. In the context of tumors, platelets have been implicated in the development of metastases, as was first demonstrated 50 years ago [2]. Since then, platelets have been suggested to play various roles in the metastatic process, ranging from prevention of anoikis [3], protection against shear-stress [4], shielding from Natural Killer cell (NK) attack [5–9], induction and maintenance of EMT [10], adhesion [11–13], spreading [14], recruitment of CD11b^{+} monocytes (e.g. [15, 16]), to the induction of proliferation of bone metastases [17]. However, despite the advances in the field, a comprehensive

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10585-018-9924-8) contains supplementary material, which is available to authorized users.

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framework that explains the role of platelets in metastasis has yet to be established.

Platelets are derived from megakaryocytes that undergo differentiation and maturation in a process called megakaryopoiesis. Major sites of platelet production are the bone marrow, spleen and the lung [18]. Megakaryopoiesis is dependent on the hormone thrombopietin (TPO). Loss of TPO or its cognate receptor cMPL in mice results in significantly reduced numbers of megakaryocytic precursors and megakaryocytes, reduced megakaryocyte ploidy, and, as a consequence, substantially reduced platelet counts [19, 20].

The murine mammary carcinoma cell line 4T1 readily forms secondary tumors in syngeneic mice, in both spontaneous (orthotopic tumor cell inoculation) and experimental (intravenous tumor cell inoculation) metastasis assays. The 4T1.2 cell line is a sub-clone of the 4T1 cell line that was selected for increased metastasis to the bone, but which also metastasizes to the lymph nodes and the lung [21]. Data from a recent study suggest that metastasis of 4T1.2 mammary carcinoma cells to the bone is enhanced in *Tpo* deficient mice [22].

To investigate the influence of platelets on breast cancer growth and metastasis to organs other than the bone, we employed the 4T1 and 4T1.2 cells lines and focused on metastasis of the cells to the lung and lymph nodes in wild-type and $Tpo^{-/-}$ mice. *Tpo* deficiency led to reduced lung colonisation by 4T1 and 4T1.2 tumor cells in experimental metastasis assays. Primary tumor growth was unaffected in spontaneous metastasis assays. Although metastasis of 4T1 tumor cells to the lung was similar in $Tpo^{-/-}$ and $Tpo^{+/+}$ mice in these assays, the number of 4T1.2 lung metastases was significantly lower in $Tpo^{-/-}$ compared to wild-type mice. However, we found increased lymph node metastasis in $Tpo^{-/-}$ mice for both cell lines. Enhanced lymph node metastasis in $Tpo^{-/-}$ mice was not associated with differences in tumor-associated lymphatic vessel density, suggesting that this phenomenon is independent of peritumoral lymphangiogenesis. Together these data indicate that platelet numbers can differentially influence site-specific metastasis to lymph nodes and lungs.

Materials and methods

Experimental mice and genotyping

BALB/cJ; $Tpo^{-/-}$ mice bearing homologous recombination and insertion of a neomycin resistance gene into the third coding exon [19] were provided by Dr. Andrea Mastro (The Pennsylvania State University, Pennsylvania, USA). The mice were bred and maintained on a BALB/cJ background and genotyped as previously described [22]. Briefly, genomic DNA was used as a template for PCR. For the detection of wild-type *Tpo* alleles, "p.TPO Sac I wt" (ggt gaa tgt aac ctg gga taa) and "p.TPO Sal I wt" (gtc gac cct ttg tct atc cct) primers were used in PCR reactions (95 °C, 4 min; 95 °C, 1 min; 55 °C, 30 s; 72 °C, 1 min; 30 cycles; 72 °C, 10 min), resulting in an amplicon of 330 nucleotides. Detection of a neomycin insert, indicating a knock out allele, was achieved by using "Neo 1" (ggg ttc ttt ttg tca aga c) and "Neo 2" (atc ctc gcc gtc ggg cat gc) primers in PCR reactions creating an amplicon of 416 nucleotides with the following conditions: 95 °C, 4 min; 95 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min; 35 cycles; 72 °C, 10 min.

Tissue culture

4T1 cells and 4T1.2 cells (provided by Dr. Andrea Mastro) were cultivated in DMEM supplemented with 10% FCS and 1% penicillin–streptomycin.

Animal experiments

All animal experiments were approved by the local regulatory authorities (license number: AZ 35-9185.81/G-136/15), and were performed according to German legal requirements. In spontaneous metastasis assays, tumor size was measured with a micrometer caliper. Once a tumor reached the approved limit of 2 cm in one dimension or a mouse became moribund, the animal was killed and an autopsy was performed. The volume of the draining ipsilateral axillary lymph nodes was assessed as a measure of lymph node metastasis. Contralateral lymph nodes served as a control. In addition, longitudinal transverse paraffin-embedded sections were taken from the central portion of the lymph nodes to ensure that corresponding regions of the lymph nodes were compared. Sections were hematoxylin and eosin (H&E) stained, analyzed for the presence of tumor cells with a BX51 light microscope equipped with a 4× Plan S Apo objective, aperture 0.16 (Olympus, Münster, Germany), and photographed with a UC90 Camera (Olympus). The tumor-infiltrated area was quantified using Cell Sens Standard 1.17 software (Olympus). The lung was examined for the occurrence of metastases, and nodules were counted under a stereo microscope. The tumors and surrounding tissue were isolated and embedded in paraffin for histological studies.

Quantification of lymphatic vessel density

Pieces of tumor were formalin-fixed and embedded in paraffin wax. Paraffin wax-embedded sections (5 µm thick) were boiled in 10 mM citric acid buffer, pH 6.0 in a microwave oven for 10 min to retrieve antigens, then blocked with 10% goat serum in PBS and incubated with primary biotinylated antibodies directed against LYVE-1 (Reliatech, Wolfenbüttel, Germany) overnight at 4 °C. Binding of the primary

antibody was visualized using Alexa-coupled fluorescent streptavidin. Cell nuclei were counterstained with DAPI. Specimens were examined using a light microscope (LEICA, Wetzlar, Germany), and images were captured with a digital camera. The number of positively stained lymphatic microvessels in the peritumoral region was evaluated in five independent fields (1 mm^2 each) for each of the tumors using ImageJ software.

Statistics

Testing for statistical significance was performed either by 2-tailed unpaired *t* tests or Fisher Exact Tests (frequency of lymph node metastasis).

Results

The number of 4T1 and 4T1.2 lung metastases is significantly lower in *Tpo* deficient mice in experimental metastasis assays

Tpo deficient mice were originally generated on a C57Bl/6 background [19]. The $Tpo^{-/-}$ animals used in this present study were backcrossed onto a BALB/cJ background to allow implantation of syngeneic mammary carcinoma cell lines derived from BALB/cJ mice. As the genetic background can significantly influence the phenotype exhibited by genetically modified mice, we first analysed platelet counts in the BALB/cJ; $Tpo^{-/-}$ mice to ensure that robust reduction of platelet counts is retained in *Tpo* deficient mice on a BALB/cJ background. As expected, platelet counts were significantly lower in $Tpo^{+/-}$ and $Tpo^{-/-}$ mice in comparison with $Tpo^{+/+}$ mice, and the reduction was comparable to that reported for $Tpo^{-/-}$ mice on a C57Bl/6 background (Supplementary Fig. 1) [19]. These results indicate that a BALB/cJ genetic background does not affect the impact of *Tpo* deficiency on platelet counts.

TPO exerts its functions via interaction with its receptor cMPL that is expressed on hematopoietic and megakaryocytic cells. cMPL has also been reported to be expressed on colorectal carcinoma cells, and its activation by TPO can increase metastasis [23]. To rule out the possibility that *Tpo* deficiency might directly modify the metastatic behavior of 4T1 or 4T1.2 mammary carcinoma cells due to expression of the cMPL receptor on these cells, we performed flow cytometry to determine cMPL expression on their surface. Both cell lines were negative for the receptor (Supplementary Figs. 2a, b).

In order to assess the impact of low platelet numbers on metastatic lung colonization, 4T1 and 4T1.2 mammary carcinoma cells were injected into the tail vein of BALB/cJ; $Tpo^{+/+}$, $Tpo^{+/-}$ and $Tpo^{-/-}$ mice. After 20 (4T1) or 13 (4T1.2) days, respectively, the animals were sacrificed, the lungs were removed, and the number of surface lung metastases was quantified. The number of lung metastases was significantly lower in $Tpo^{-/-}$ mice compared with $Tpo^{+/-}$ ($p < 0.001$) and $Tpo^{+/+}$ ($p < 0.01$ and $p < 0.001$, respectively) mice (Fig. 1). Thus loss of TPO impairs the ability of 4T1 and 4T1.2 tumor cells to colonize the lungs in experimental metastasis assays.

Tpo deficiency does not impact on primary breast tumor growth, but differentially affects lung and lymph node metastasis from orthotopic sites

Experimental metastasis assays simulate major events in the process of metastasis, such as survival in the

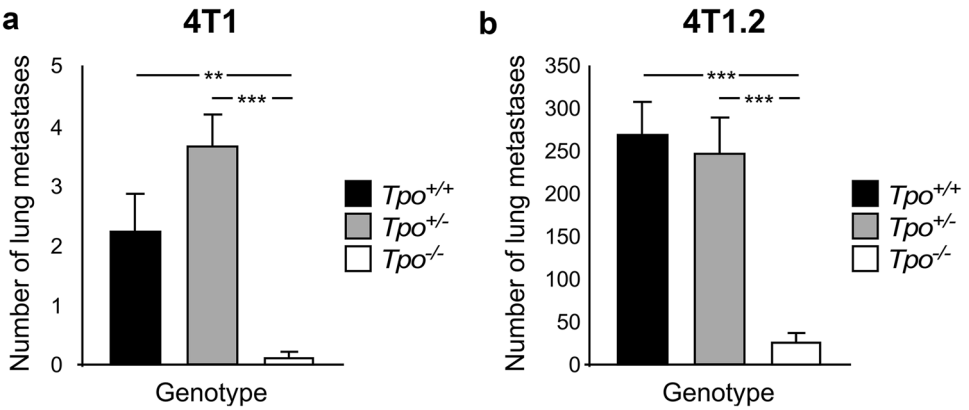
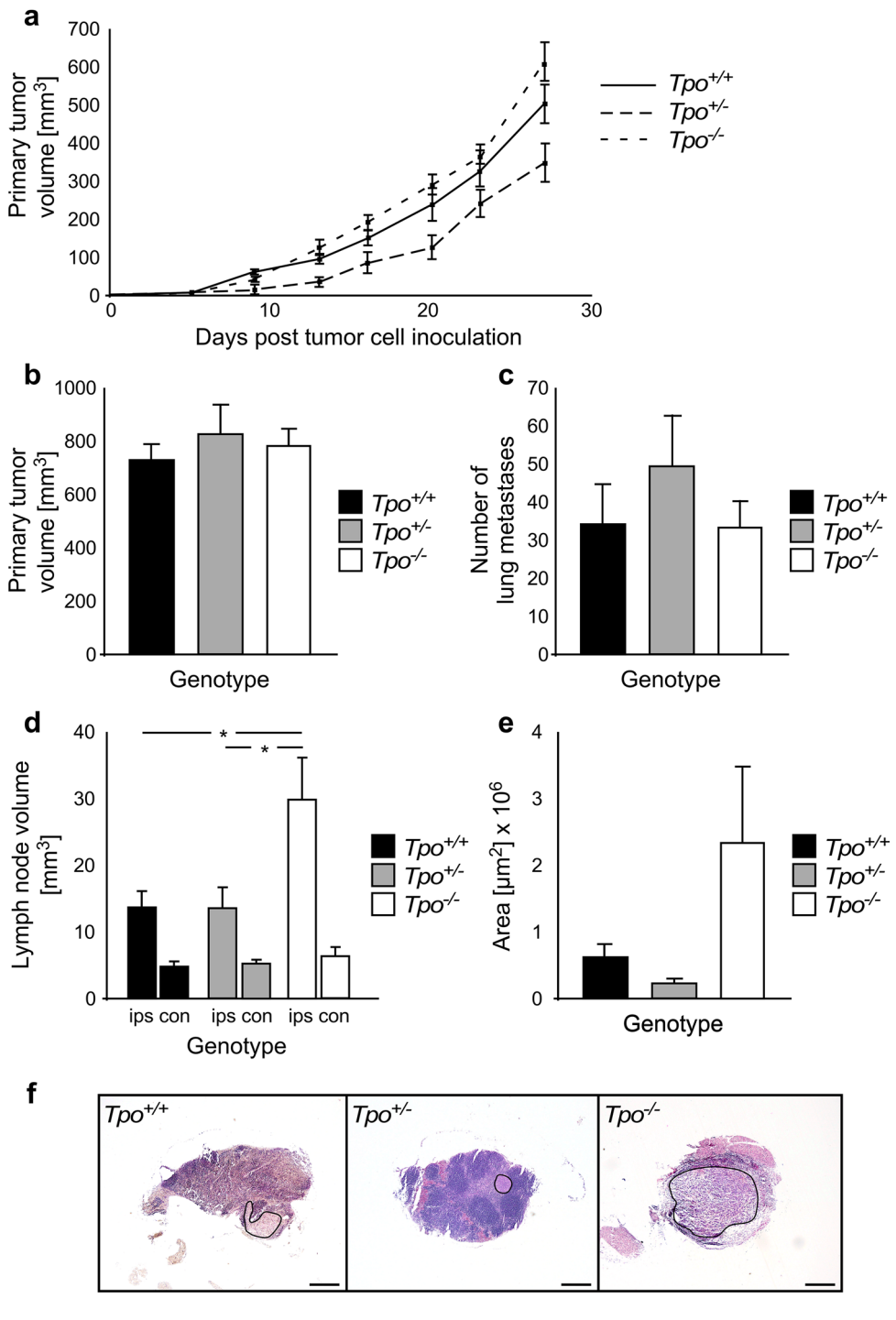


Fig. 1 The number of superficial 4T1 and 4T1.2 lung metastases is significantly reduced in *Tpo* deficient mice in experimental metastasis models. 4T1 mammary carcinoma cells (1×10^4 each animal) (**a**) and 4T1.2 mammary carcinoma cells (8×10^5 each animal) (**b**) were injected into the tail vein of syngeneic $Tpo^{+/+}$, $Tpo^{+/-}$ and $Tpo^{-/-}$ mice. The animals were sacrificed 20 days (4T1) or 13 days (4T1.2) post tumor cell injection. The lungs were removed and the number of superficial lung metastases was quantified. (4T1.2: $Tpo^{+/+}$: N = 8; $Tpo^{+/-}$: N = 8; $Tpo^{-/-}$: N = 7) (**$p < 0.01$; ***$p < 0.001$). Error bars represent SE



Genotype	$Tpo^{+/+}$	$Tpo^{+/-}$	$Tpo^{-/-}$
Frequency	2/8	3/8	3/7

circulation, intravascular arrest, extravasation and outgrowth. Spontaneous metastasis assays recapitulate additional events, including intravasation into the blood stream, lymphatic vessel invasion, and formation of lymph node metastases. To test whether spontaneous metastasis is affected in *Tpo* deficient mice, we implanted 4T1 and 4T1.2 cells orthotopically into the mammary fat pad of three groups of syngeneic mice that were genetically either $Tpo^{+/+}$, $Tpo^{+/-}$ or $Tpo^{-/-}$. Animals were sacrificed once the tumors reached the legal limit of 2 cm in one

◂**Fig. 2** *Tpo* deficiency does not affect tumor growth or the number of superficial lung metastases after orthotopic implantation of 4T1 mammary carcinoma cells, but the size of lymph node metastases is larger in *Tpo* deficient mice compared to wild-type mice. 4T1 cells (1×10^6 each animal) were injected orthotopically into the mammary fat pads of $Tpo^{+/+}$, $Tpo^{+/-}$ and $Tpo^{-/-}$ mice (8 animals per group). **a** Tumor growth was monitored regularly, and the animals were sacrificed as soon as the tumors reached the legal limit of 2 cm in one dimension, or when the animals became moribund. The final measurement shown was made when the first animal had to be taken out of the experiment for these reasons. Tumor volume was calculated assuming spherical geometry. The volumes of the primary tumors are plotted against time. Error bars represent SE. **b** Final volumes of the primary tumors measured when the animals were sacrificed. Error bars represent SE. **c** Post-mortem examinations were performed, and the number of superficial lung metastases was counted. Error bars represent SE. **d** The dimensions of the axillary lymph nodes were measured, and their volume calculated, assuming spherical shape. Contralateral lymph nodes (con) served as internal controls. *ips* ipsilateral. Error bars represent SE. (*$p < 0.05$). **e** Histological sections of the lymph nodes were analysed for the presence of tumor cells, and the area occupied by tumor cells in the lymph nodes was quantified. Mean values of metastatic area of metastasis-containing ipsilateral axillary lymph nodes ($Tpo^{+/+}$: N = 2; $Tpo^{+/-}$: N = 3; $Tpo^{-/-}$: N = 3) are shown. Error bars represent SE. **f** Pictures of representative lymph node sections (metastatic areas are encircled; scale bars: 500 µm). The table shows the frequency of metastases in the ipsilateral axillary lymph nodes

dimension, or when the animals became moribund. Post-mortem examinations were performed, and the lungs and axillary lymph nodes were removed. The number of metastases on the surface of the lung lobes was counted, the volume of the axillary lymph nodes was calculated, and the area occupied by tumor cells in the lymph nodes was quantified.

No significant difference in 4T1 tumor growth was observed between $Tpo^{+/+}$, $Tpo^{+/-}$ and $Tpo^{-/-}$ mice (Fig. 2a, b). There was also no significant difference in the number of superficial lung metastases between the groups (Fig. 2c). However, we found that the volume of the ipsilateral axillary lymph nodes that drain the primary tumors was significantly ($p < 0.05$) larger in $Tpo^{-/-}$ mice compared to $Tpo^{+/-}$ and $Tpo^{+/+}$ mice (Fig. 2d). Histological examination of sections of the lymph nodes for the presence of tumor cells by a trained pathologist revealed that tumor cells were present in the ipsilateral axillary lymph nodes of 2 out of 8 $Tpo^{+/+}$ mice, 3 out of 8 $Tpo^{+/-}$ mice and 3 out of 7 $Tpo^{-/-}$ mice (Fig. 2f). Thus the incidence of lymph node metastases was not affected by TPO loss. However, metastatic cells occupied a larger area of the lymph node from $Tpo^{-/-}$ mice compared to $Tpo^{+/-}$ and $Tpo^{+/+}$ mice (Fig. 2e). Our results therefore suggest that in contrast to the experimental metastasis model, the number of 4T1 lung metastases and the incidence of lymph node metastases in spontaneous metastasis assays was not affected by the loss of TPO, whereas the size of 4T1 lymph node metastases was larger in *Tpo* deficient mice compared to heterozygous and wild-type mice.

Next, 4T1.2 tumor cells were implanted orthotopically into the mammary fat pad of $Tpo^{+/+}$, $Tpo^{+/-}$ and $Tpo^{-/-}$ mice. Similarly to our observations for 4T1 tumors, 4T1.2 primary tumor growth was not affected by *Tpo* deficiency (Fig. 3a, b). However, the number of lung metastases was significantly ($p < 0.01$) lower in $Tpo^{-/-}$ mice compared to $Tpo^{+/+}$ mice (Fig. 3c). Conversely, primary tumor-draining axillary lymph node volumes were significantly ($p < 0.01$) larger in $Tpo^{-/-}$ mice compared to $Tpo^{+/+}$ mice (Fig. 3d). Consistently, histopathological examination of lymph node sections for the presence of tumor cells showed that tumor cells were present in the ipsilateral axillary lymph nodes of two out of eight $Tpo^{+/+}$ mice, three out of eight $Tpo^{+/-}$ mice and 6 out of 7 $Tpo^{-/-}$ mice (Fig. 3f). The difference between $Tpo^{+/+}$ mice and $Tpo^{-/-}$ mice was statistically significant ($p = 0.04$; Fisher Exact Test), indicating that the incidence of lymph node metastasis increased in response to *Tpo* deficiency. Quantification of the area of the lymph node occupied by metastatic cells showed no significant differences between $Tpo^{-/-}$, $Tpo^{+/-}$ and $Tpo^{+/+}$ mice, and there was a tendency that lymph node metastases were larger in $Tpo^{-/-}$ than in $Tpo^{+/+}$ mice (Fig. 3e). These data suggest that lung metastasis by 4T1.2 is reduced in *Tpo* deficient mice, whereas lymph node metastasis is enhanced.

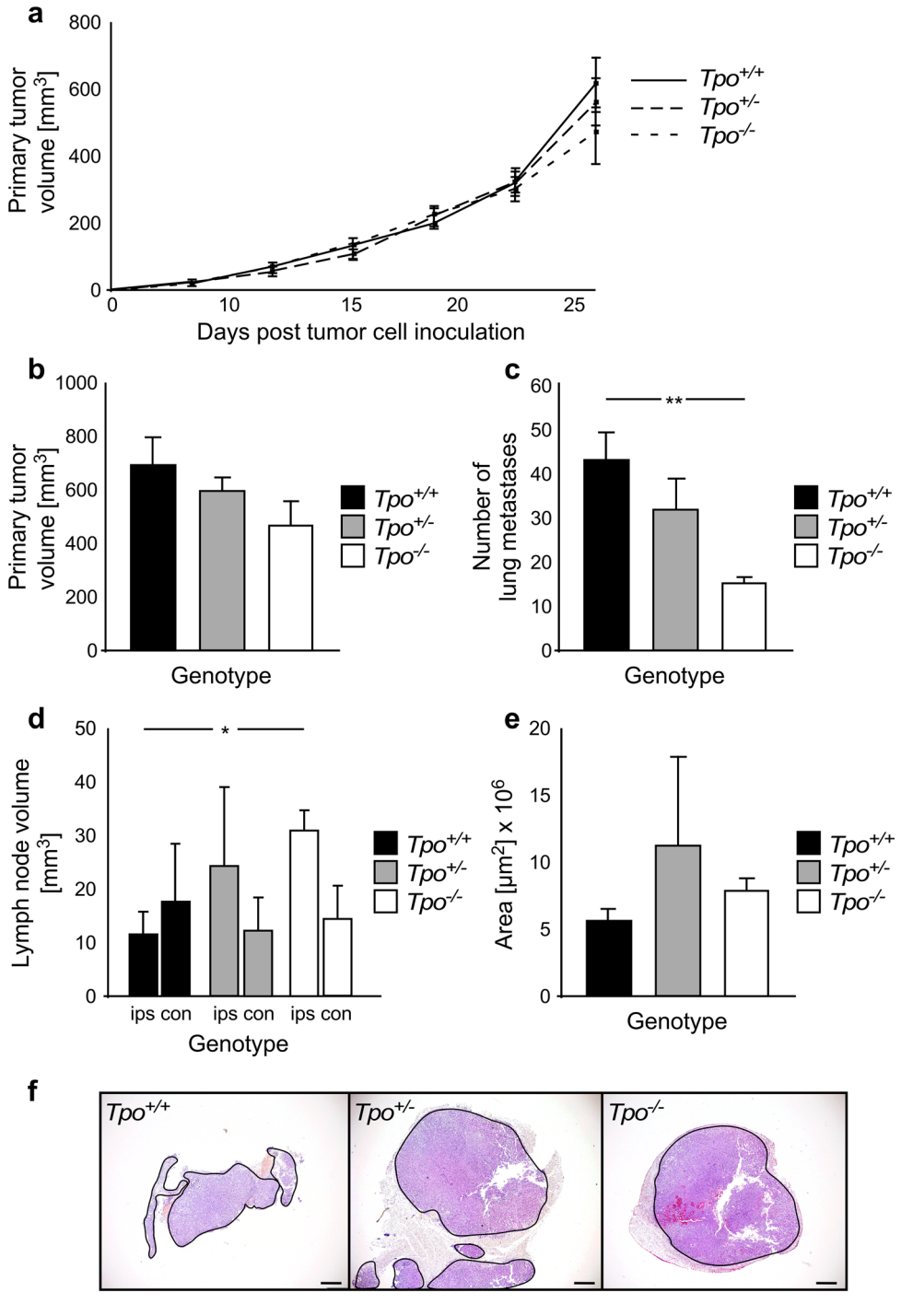
Peritumoral lymphatic vessel density is not increased in *Tpo* deficient mice

Our findings suggest that *Tpo* deficiency promotes lymph node metastasis. The induction of lymphangiogenesis in the primary tumor promotes metastasis formation, and the development of lymph node metastases correlates correspondingly with increased peritumoral lymphatic vessel density. We therefore investigated whether differences in peritumoral lymphatic vessel density according to the degree of *Tpo* deficiency might account for the differences in lymph node metastasis formation we observed.

To this end, we stained sections of 4T1 and 4T1.2 primary tumors for the lymphatic endothelial marker LYVE-1, and quantified the LYVE-1 positive area. No significant difference in peritumoral lymphatic vessel density was found between the different groups of animals (Fig. 4). These data therefore suggest that increased lymph node metastasis in *Tpo* deficient mice is not associated with changes in peritumoral lymphatic vessel density.

Discussion

Here, we employed $Tpo^{-/-}$ mice to study the influence of low platelet counts on metastasis of 4T1 and 4T1.2 mammary carcinoma cells to the lymph nodes and lung. We show that the ability of 4T1 and 4T1.2 tumor cells to colonize



Genotype	$Tpo^{+/+}$	$Tpo^{+/-}$	$Tpo^{-/-}$
Frequency	2/8	4/8	6/7

the lungs in experimental metastasis assays is impaired in *Tpo* deficient mice. In orthotopic implantation experiments, 4T1 and 4T1.2 primary tumor growth was unaffected by the genotype of the mice. However, lung metastasis from 4T1.2 tumors was lower in *Tpo* knockout mice compared to wild-type mice, while lung metastasis from 4T1 tumors was unaffected. In contrast, metastatic burden in the draining lymph nodes was increased for both 4T1 and 4T1.2 tumor-bearing mice, and the incidence of lymph node metastases was additionally increased as a consequence of TPO loss

◂**Fig. 3** *Tpo* deficiency does not affect the growth of orthotopically injected 4T1.2 mammary carcinoma cells, but the number of superficial lung metastases is lower in *Tpo* deficient mice than in wild-type mice while the frequency of lymph node metastases is higher in $Tpo^{-/-}$ mice than in $Tpo^{+/+}$ mice. 4T1.2 cells (1×10^6 each animal) were injected orthotopically into the mammary fat pad of $Tpo^{+/+}$, $Tpo^{+/-}$ and $Tpo^{-/-}$ mice. **a** Tumor growth was monitored regularly, and the animals were sacrificed as soon as the tumors reached the legal limit of 2 cm in one dimension or when the animals became moribund. The final measurement shown was made when the first animal had to be taken out of the experiment for these reasons. Tumor volume was calculated assuming spherical geometry. The volumes of the primary tumors are plotted against time. Error bars represent SE. **b** Final volumes of the primary tumors measured at the time when the animals were sacrificed. Error bars represent SE. **c** Post-mortem examinations were performed, and the number of superficial lung metastases was counted. ($Tpo^{+/+}$: N = 8; $Tpo^{+/-}$: N = 8; $Tpo^{-/-}$: N = 7). Error bars represent SE. $^{**}p < 0.01$. **d** The dimensions of the axillary lymph nodes were measured, and their volume calculated, assuming spherical shape. Contralateral lymph nodes (con) served as internal controls. *ips* ipsilateral. ($Tpo^{+/+}$: N = 8; $Tpo^{+/-}$: N = 8; $Tpo^{-/-}$: N = 7). Error bars represent SE. ($^{**}p < 0.01$). **e** Histological sections of the lymph nodes were analysed for the presence of tumor cells, and the area occupied by tumor cells in the lymph nodes was quantified. Mean values of metastatic area of metastasis-containing ipsilateral axillary lymph nodes ($Tpo^{+/+}$: N = 2; $Tpo^{+/-}$: N = 4; $Tpo^{-/-}$: N = 6) are shown. Error bars represent SE. **f** Pictures of representative lymph node sections (metastatic areas are encircled; scale bars: 500 µm). The table shows the frequency of metastases in the ipsilateral axillary lymph nodes. The difference between $Tpo^{+/+}$ and $Tpo^{-/-}$ mice was statistically significant ($p < 0.05$; Fisher Exact Test)

for 4T1.2 tumors. Augmented lymph node metastasis was not associated with changes in peritumoral lymphatic vessel density. These data indicate that reduced platelet numbers can both positively and negatively impact on metastasis formation in an organ-specific manner.

Since Gasic et al. first demonstrated a metastasis-promoting role for platelets five decades ago [2], a number of mechanisms have been suggested through which platelets might contribute to the metastasis process (reviewed in [24]). It has been proposed that upon intravasation into blood vessels, platelets become activated by tumor cells, and adhere to tumor cells to form a "cloak" around them [4, 25]. This cloak is thought to protect tumor cells from detrimental shear forces in the blood circulatory system [4], and to shield tumor cells from NK attack [5–9]. Furthermore, this platelet cloak may mediate tumor cell adhesion to damaged blood endothelium [11, 12], thereby contributing to extravasation of circulating tumor cells. Data supporting an alternative scenario suggest that tumor cells can adhere to blood endothelium without the involvement of platelets [14, 26–30], but that platelets get activated in the proximity of adhered tumor cells after tumor cell arrest, and subsequently stabilise tumor cell adhesion to the endothelium [14, 26, 27, 30–33], thereby promoting metastasis. Moreover, platelet-mediated recruitment of $CD11b^+$ monocytes increases the survival of entrapped tumor cells [15, 16]. Although tumor cells can extravasate in the absence of platelets [29, 31, 34–39], in vitro and in vivo evidence suggests that the efficiency of tumor cell diapedesis is enhanced in the presence of platelets [34, 40–42], possibly through induction and maintenance of EMT that is in part mediated by platelet-derived TGF-β1 [10]. Finally, platelets have been suggested to contribute to the outgrowth of bone metastases via release of lysophosphatidic acid upon activation [17].

Mechanistic evaluations of how platelets contribute to metastasis generally assume that platelets serve to promote metastasis. Consistent with this notion, we found that lung metastasis by 4T1 and 4T1.2 mammary carcinoma cells in *Tpo* deficient mice that display low platelet counts is strongly impaired in experimental metastasis assays (Fig. 1a, b), which corroborates previous reports [2, 5, 43–50]. However, we also found conversely that lymph node metastasis formation and outgrowth is increased in *Tpo* deficient mice with low platelet counts (Fig. 2d,e and 3d,f). Similarly, bone metastasis formation by 4T1.2 cells was accelerated in *Tpo* knockout mice [22]. These data suggest that platelets can both enhance and inhibit metastasis formation in an organ-specific manner.

A limitation of this current study is that the results were obtained with only two cell lines. Although 4T1 and 4T1.2 cells recapitulate the metastatic behaviour of human mammary carcinomas [51], it is currently unclear how far the results we have obtained can be generalized more broadly. Future experiments with additional cell lines will address this issue.

In the context of bone metastasis, it has been speculated that reduced numbers of megakaryocytes in the bone marrow of *Tpo* knockout mice leads to modification of hematopoietic niches that then offer a more permissive prometastatic environment for disseminated cells, and that megakaryocytes in the bone marrow play a protective role against bone metastasis formation [22]. As lymph nodes do not contain megakaryocytes that can protect them from metastasis, other mechanisms must be operative in the lymph nodes that result in suppression of metastasis by platelets in these organs. Peritumoral lymphangiogenesis is a common mechanism how metastasis to the lymph nodes can be enhanced (reviewed in: [52]), but we found no statistically significant correlation between the genotype of the animals and peritumoral lymphatic vessel density (Fig. 4c, d) that could explain the differences in lymph node metastasis between $Tpo^{+/+}$ and $Tpo^{-/-}$ mice (Figs. 2d–f, 3d–f). Although other mechanisms such as immune modulation exist that can promote metastasis to the lymph nodes independently of lymphatic vessel density (reviewed in: [53]), no obvious differences were observed in H&E-stained lymph node sections by a trained pathologist. It therefore remains unclear through which mechanism lymph node metastasis is increased in *Tpo* knockout mice.

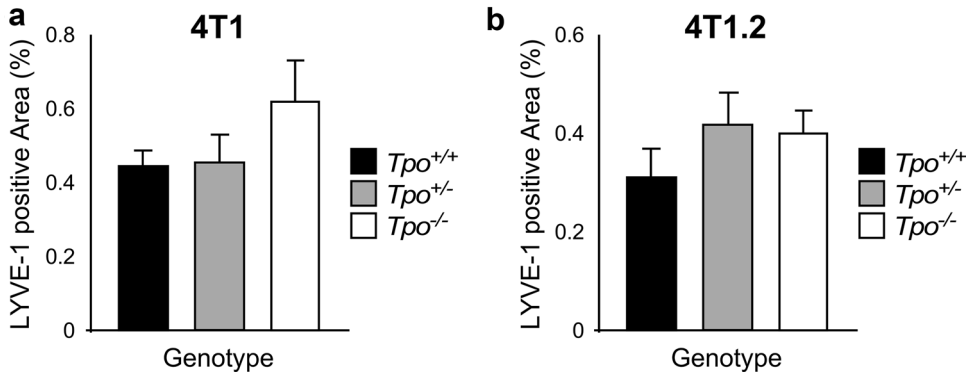


Fig. 4 Lymphatic vessel density is not increased around 4T1 and 4T1.2 primary tumors in *Tpo* knockout mice. Paraffin-embedded sections of 4T1 (**a**) and 4T1.2 (**b**) primary tumors were stained with fluorescently labelled antibodies specific for LYVE-1. Pictures were taken, and the percentage of the area positive for LYVE-1 was quantified in five independent quadrants per tumor. (4T1: $Tpo^{+/+}$: N = 7; $Tpo^{+/-}$: N = 8; $Tpo^{-/-}$: N = 7; 4T1.2: $Tpo^{+/+}$: N = 8; $Tpo^{+/-}$: N = 7; $Tpo^{-/-}$: N = 8). Error bars represent SE

In line with our findings, a previous report using footpad injection of B16 melanoma cells found that platelet depletion by anti-platelet serum slightly but not significantly increased tumor cell burden of the draining popliteal lymph nodes [13]. Although platelet depletion was robust (> 95%), and even more pronounced than in *Tpo* deficient mice (Supplementary Fig. 1), reduced platelet counts were not maintained throughout the whole duration of the experiment. It is therefore possible that prolonged platelet depletion and larger group sizes would have revealed a statistically significant increase in lymph node metastasis, similar to our findings.

Besides *Tpo* deficient mice, other genetic mouse models with reduced platelet counts exist, and metastasis to the lung has been investigated in some of them. For example, immunodeficient *Nf-e2*$^{-/-}$ mice have no detectable circulating platelets [54], and display significantly lower numbers of lung metastases upon tail vein injection of B16 F10 melanoma cells compared to wild-type mice [45]. Coupland and colleagues have used *c-mpl*$^{-/-}$ and Plt20 mice that have a deficiency in the TPO receptor cMpl or a nonfunctional anti-apoptotic protein, Bcl-X_L, respectively [46]. These mice display platelet counts reduced to 15 or 30%, respectively, of the wild-type levels. Plt20 and *c-mpl*$^{-/-}$ mice were used in experimental metastasis assays with syngeneic 4T1.2 mammary carcinoma or B16F10 melanoma cells [46]. The authors observed a statistically significant difference in lung metastases only for B16F10 cells in *c-mpl*$^{-/-}$ versus c-*mpl*$^{+/+}$ but not for 4T1.2 cells in Plt20 versus wild-type mice. However, with only 3 mice per group in the case of 4T1.2 cells and Plt20 mice, group sizes were too small to draw any robust conclusions. Notwithstanding, the authors conclude from the results of the study that a platelet reduction of more than 70% of the wild-type levels is necessary to significantly reduce lung metastasis [46]. In support of this notion, we found that in heterozygous $Tpo^{+/-}$ mice that have less than 50% of the platelet counts of $Tpo^{+/+}$ mice (Supplementary Fig. 1), metastasis was not significantly lower than in $Tpo^{+/+}$ animals (Figs. 1a, b, 2c, 3c). In further studies using these alternative genetic models of platelet deficiency, it will be interesting to explore whether lymph node and bone metastasis is enhanced, similar to the situation in $Tpo^{-/-}$ mice.

There is an ongoing debate whether lymph node metastases are merely indicators that a tumor has gained metastatic competence or if they are functionally important [55]. Recent evidence suggest that lymph nodes can act as waystations in the metastatic cascade, and that tumor cells in lymph nodes can continue their journey via the blood stream to colonize the lung [56, 57]. Therefore, increased lymph node metastasis may promote metastasis to distant organs. We found that lung colonization by 4T1 cells was reduced in *Tpo* deficient mice in an experimental metastasis assay (Fig. 1a) which bypasses the lymph nodes, as tumor cells are injected directly into the blood stream, but was unaffected in a spontaneous metastasis assay (Fig. 2c), in which the lymph nodes are involved, and in which lymph node metastasis was increased (Fig. 2d, e). In the light of these findings it is tempting to speculate that a possible reason for the "net increase" in 4T1 metastasis in the spontaneous metastasis model in *Tpo* knock out animals might be a consequence of the enhanced lymph node metastasis we observed. However, this scenario would be dependent on additional, tumor cell-intrinsic parameters, as we did not observe the same effect on lung metastasis for the 4T1.2 cell line (Figs. 1b, 3c), although 4T1.2 lymph node metastasis was also promoted in $Tpo^{-/-}$ mice (Fig. 3d, f).

The 4T1.2 cell line was derived from an isolated 4T1 single cell clone [21]. The clone was selected due to its ability to form metastases in the bone after orthotopic implantation into the mammary fat pad, but also metastasizes to the lung and lymph nodes. Our data also show clear differences in the metastatic potential of 4T1.2 cells and the parental 4T1 line

to other organs, suggesting that tumor cell-intrinsic parameters that differ between 4T1 and 4T1.2 cells regulate patterns of metastasis by these lines. Mice bearing 4T1.2 tumors show increased levels of parathyroid hormone-related protein (PTHrP) that is considered to be important for metastasis formation in the bone [21]. However, PTHrP has not been implicated in lung metastasis formation, and is thus unlikely to be responsible for the difference in lung metastasis formation between 4T1 and 4T1.2 cells that were observed in our experiments. Further work is required to determine the underlying gene expression patterns that regulate differences in metastasis formation between the two cell lines.

Here we have employed mice with reduced platelet numbers. However, the exact constitution of platelets may also play an important role in the process of metastasis. Platelets can be "educated" by tumors (reviewed in [58]), for example through uptake of tumor-derived factors [59] and fusion of RNA-containing tumor-derived vesicles with platelets [60], suggesting that the analysis of platelets might be useful for cancer diagnostics [60, 61]. Although the role of this education with regard to metastasis formation is unclear, it is possible that not only the quantity but also the "quality" of platelets could be decisive for their influence on metastasis. While platelet counts are reduced in *Tpo* deficient mice, the morphology and performance of the remaining platelets is not impaired by *Tpo* deficiency [20, 62]. However, it is unclear whether platelets in *Tpo* deficient mice differ in terms of their ability to become educated by tumors.

Upon activation, platelets can release mitogenic and proangiogenic factors from their α-granules (reviewed e.g. in [24]), and can for example also mediate neovascularisation via TPO-induced platelet-derived SDF1α-mediated recruitment of $CXCR4^+/VEGFR\text{-}1^+$ hemangiocytes [63]. It has therefore been speculated that platelets may contribute to primary tumor growth [24, 64]. Indeed, platelet depletion in mice carrying subcutanous Lewis lung carcinoma tumors induced tumor hemorrhage, and was paralleled by reduced BrdU incorporation [65]. However, tumor volume and dry mass of the tumors remained unaffected [65]. Boucharaba et al. reported that the growth of subcutaneously injected mammary carcinoma xenografts can be enhanced by ectopic overexpression of lysophosphatidic acid receptor LPA_1 in the tumor cells [17]. However, while the authors show that platelets are a major source of lysophosphatidic acid, the ligand for LPA_1, the evidence provided for platelet involvement in primary tumor growth was only indirect [17]. In another study, platelet transfusion in ovarian tumor bearing mice increased the number of $KI67^+$ tumor cells, although neither the actual tumor volume nor the rate of apoptosis that might have counterbalanced tumor growth was assessed [66]. In contrast to the notion that platelets contribute to tumor growth, we found no significant differences between $Tpo^{-/-}$ and $Tpo^{+/+}$ mice in terms of the growth of either 4T1 or 4T1.2 tumors (Figs. 2a, 3a). In line with our findings, Lonsdorf and colleagues showed that co-culture of B16 melanoma cells with platelets has no effect on their proliferation in vitro, and also that platelet depletion by injection of anti-platelet serum had no effect on primary tumor growth upon foot pad injection of B16 melanoma cells in vivo [13]. Li et al. also reported that platelet depletion using anti-CD42b antibodies had no impact on the growth of B16 and 4T1 tumors in vivo [67]. The currently available data therefore do not support the notion that platelets play any significant role in primary tumor growth.

In conclusion, our data show that tail vein injection of 4T1 and 4T1.2 cells results in fewer lung metastases in *Tpo* deficient mice compared to wild type mice, consistent with the prevailing view that platelets promote lung colonization in experimental metastasis assays [2, 5, 43–50]. Furthermore, we observed that primary tumor growth is unaffected by low platelet counts, verifying in a genetic mouse model findings made using antibody-based platelet depletion [13, 67]. Moreover, we observed that while 4T1.2 and 4T1 lymph node metastasis was promoted in mice with low platelet counts, metastasis to the lung by 4T1.2 and 4T1 cells in these mice was reduced or unaffected, respectively. Together with the observation that bone metastasis formation is enhanced in mice with reduced platelet numbers [22], we conclude the platelets do not invariably promote metastasis, but can both promote and inhibit metastasis formation in an organ-specific manner.

Acknowledgements We gratefully acknowledge the expert technical assistance of Karim Badawi, Annette Gruber, Theresa Liepert, Amra Noa, Selma Huber and Sabine Müller. The authors thank Dr. Andrea Mastro for providing *Tpo* deficient mice and 4T1.2 cells.

Funding Funding was provived by Institutional funds.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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