

ORIGINAL RESEARCH

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## Phorbol ester-induced neutrophilic inflammatory responses selectively promote metastatic spread of melanoma in a TLR4-dependent manner

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### ABSTRACT

Increased neutrophil counts both in tumor tissue and peripheral blood correlate with poor clinical outcome in melanoma patients suggesting a pro-tumorigenic role of neutrophils for the pathogenesis of malignant melanoma. Recently, we discovered that neutrophilic skin inflammatory responses induced by UV exposure promote metastatic spread of primary cutaneous melanomas in genetically engineered Hgf-Cdk4(R24C) mice. We hypothesized that other pro-inflammatory stimuli that induce neutrophilic inflammatory responses also promote the development and progression of melanomas. In the current study, we therefore investigated how the most potent and frequently used tumor promoter 12-O-Tetradecanoylphorbol-13-acetate (TPA) affects the development and progression of carcinogen-induced melanomas in Hgf-Cdk4(R24C) mice. Local and systemic neutrophilic inflammatory responses induced by TPA also selectively increase the metastatic spread of melanoma cells to draining lymph nodes and lungs. Using a highly metastatic Hgf-Cdk4(R24C) melanoma skin transplant we could show that TPA enhances systemic spread of melanoma cells which was depended on intact TLR4 signaling in recipient mice and on the presence of neutrophils. Altogether, our experimental results support an important mechanistic role of TLR4-driven neutrophilic inflammation for melanoma progression.

**Abbreviations:** Cdk4, Cyclin-dependent kinase 4; DMBA, 7,12-Dimethylbenzanthracene; Hgf, hepatocyte growth factor/scatter factor; TPA, 12-O-Tetracarbonyl-13-acetate.

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Inflammation; melanoma; metastasis; neutrophils; TLR4; tumor microenvironment; TPA; UV-irradiation


### Introduction

Inflammatory responses in the tumor microenvironment can either inhibit or promote the development and progression of malignant diseases.<sup>1</sup> In recent years increased attention has been given to the role of neutrophil granulocytes in tumor-associated inflammation. For example, the infiltration of primary melanomas with neutrophils is associated with ulceration and early metastatic spread.<sup>2</sup> In line with this observation, recent combined transcriptomic and histopathologic analyses of primary melanomas found an association of ulceration and poor prognosis with the expression of pro-inflammatory cytokines and the infiltration of myeloid immune cells.<sup>3,4</sup> Increased numbers of neutrophils in the peripheral blood of metastatic melanoma patients have also been associated with a poor prognosis.<sup>2,5,6</sup> Taken together, these clinical observations suggest a pro-tumorigenic role for neutrophils in melanoma pathogenesis.

Most of our molecular knowledge how inflammatory responses contribute to tumor progression *in vivo* was generated using the classical DMBA-TPA two step skin carcinogenesis protocol that induces epithelial tumors in mice.<sup>7</sup> In this model, tumor development is initiated by a single application of the carcinogen 7,12-Dimethylbenzanthracene (DMBA) followed by repeated applications of the most common tumor

promotor TPA, also known as phorbol-12-myristate-13-acetate (PMA). The fact that mice deficient for the pro-inflammatory cytokine Tnf were resistant to the induction of TPA-induced inflammation and the subsequent development of epithelial tumors provided first experimental evidence for the tumor promoting role of inflammation *in vivo*.<sup>8</sup> Several studies have now demonstrated that TPA treatment can induce a neutrophil-rich inflammatory response that resembles an inflammatory wound reaction. It has been shown that TLR4, IL1R, MyD88, TNF, and CXCR2 are critically involved in this process.<sup>8-11</sup> Such inflammatory responses are also thought to play a role in the development and progression of melanoma.

Recently, we provided experimental evidence that neutrophilic skin inflammatory responses induced by UV exposure promote metastatic spread using our genetically engineered Hgf-Cdk4(R24C) mouse melanoma model.<sup>12</sup> In this model, primary cutaneous melanomas and spontaneous metastases are driven by deregulated receptor tyrosine kinase signaling due to transgenic overexpression of hepatocyte growth factor (Hgf) and impaired p16Ink4a-dependent cell cycle regulation due to an oncogenic Cdk4(R24C) germline mutation.<sup>13-15</sup> Melanoma genesis can be accelerated and synchronized by a single

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epicutaneous application of DMBA.<sup>14,16,17</sup> Repetitive UV irradiation of DMBA-induced melanomas selectively increased the numbers of lung metastases associated with increased local and systemic inflammation. We identified that Hmgb1 released from UV-damaged keratinocytes initiates a TLR4/Myd88-dependent myeloid-driven inflammatory response that fosters metastatic spread of melanoma cells. Depletion of neutrophils or inhibition of their activation and recruitment via Hmgb1 or TLR4 was able to significantly reduce the number of lung metastases.<sup>12</sup> These experimental data indicate that neutrophils are important players in UV-irradiation induced inflammation contributing to melanoma metastasis.

Consequently, we hypothesized that other pro-inflammatory stimuli that induce neutrophilic inflammatory responses also promote the development and progression of melanomas. In the current study, we therefore investigated how the most potent and frequently used tumor promoter TPA affects melanoma pathogenesis in Hgf-Cdk4(R24C) mice. We show that TLR4-dependent neutrophilic inflammatory responses induced by repetitive TPA treatment selectively increase metastatic spread of primary and transplanted melanomas to the draining lymph nodes and lungs. Our experimental results support clinical findings indicating that increased numbers of neutrophils in tumor tissue and peripheral blood are associated with a poor prognosis in melanoma patients.

## Results

### ***Stimulation of a TLR4-dependent neutrophilic skin inflammatory response following application of TPA in Cdk4(R24C) and Hgf-Cdk4(R24C) mice***

In initial experiments we comparatively investigated the ability of short-term TPA exposure to induce an acute skin inflammatory response in Hgf-Cdk4(R24C) and Cdk4(R24C) C57BL/6 mice (Fig. 1A). Although Hgf has been described as immunosuppressive,<sup>18</sup> TPA treatment led to infiltration of the upper dermis with CD45<sup>+</sup> immune cells and the development of epidermal hyperplasia in a dose-dependent manner in both Hgf-Cdk4(R24C) mice and Cdk4(R24C) littermate controls (Fig. 1B). Consistent with previously published results,<sup>9</sup> we found that the TPA-induced acute skin inflammatory response was largely abrogated in mice genetically lacking TLR4 (Fig. 1C). TPA treatment predominantly induced the systemic expansion and local recruitment into the skin of CD11b+Ly6C+Ly6G+ neutrophils in a TLR4-dependent manner (Fig. 1D). This was associated with a significant increase in the expression of proinflammatory genes including *S100A8*, *Il-1 $\beta$* , *Tnf $\alpha$* , *Cxcl2*, and *Mmp2* that are known to be potentially upregulated upon TPA treatment (Fig. 1E).

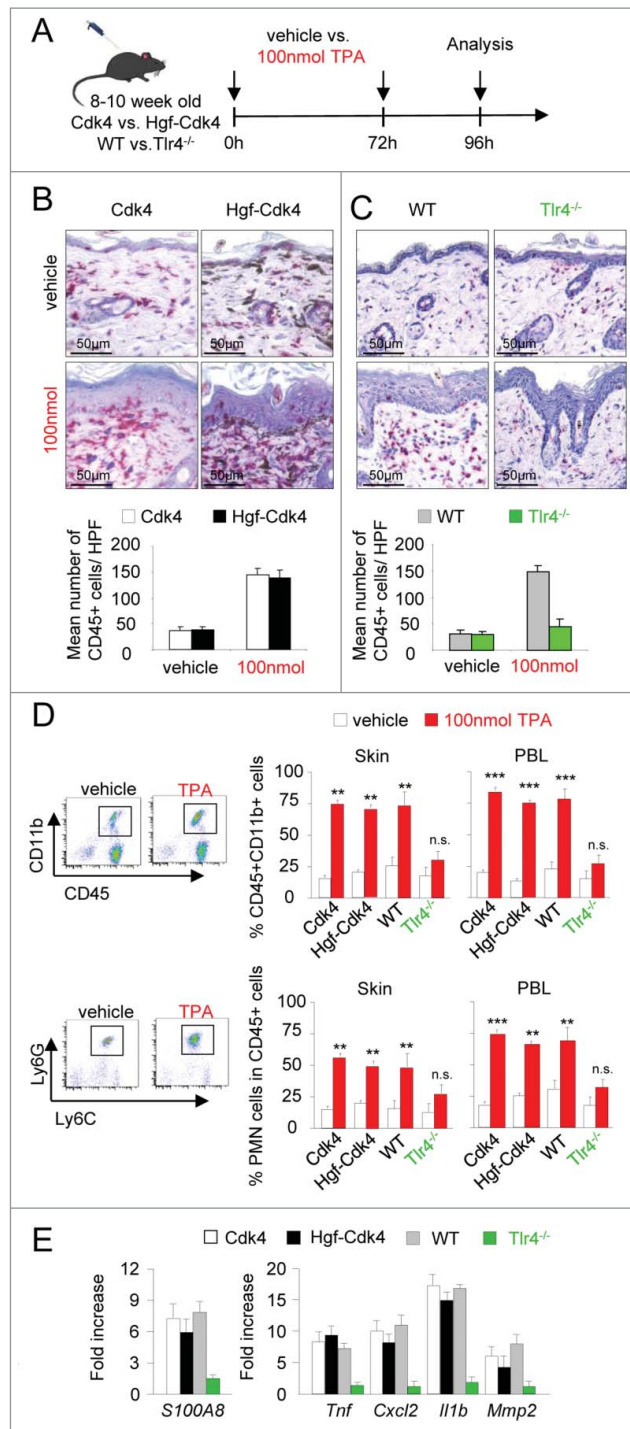
### ***Selectively increased metastatic spread of DMBA-induced primary Hgf-Cdk4(R24C) mouse melanomas following long-term epicutaneous application of TPA***

In our previous work, we found that Hgf-Cdk4(R24C) mice are highly sensitive to the carcinogen DMBA and develop multiple rapidly growing and spontaneously metastasizing

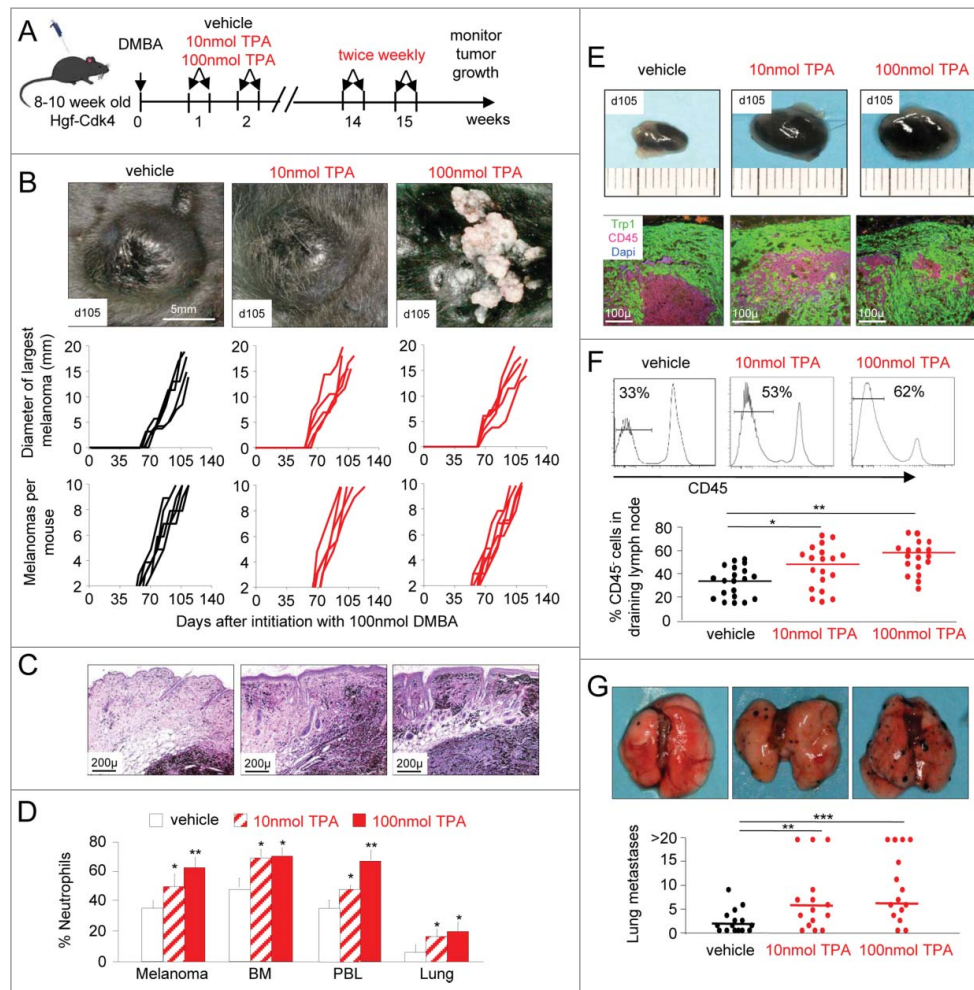
nodular melanomas in the skin within 3 mo after a single low-dose exposure with 100nmol DMBA.<sup>16</sup> To address whether and how a chronic skin inflammatory response induced by TPA affects the appearance and progression of such nodular melanomas, we first treated 6–8 week old groups of Hgf-Cdk4(R24C) mice once with 100nmol DMBA on the shaved back skin. Mice were then randomly assigned to cohorts receiving vehicle alone, 10nmol or 100nmol TPA twice weekly for 15 weeks (Fig. 2A). Hundred and five days after carcinogen exposure all mice carried rapidly growing nodular melanomas greater than 6mm in diameter and the only observable difference between the three treatment cohorts was the additional appearance of papillomas in mice receiving 100nmol TPA (Fig. 2B, upper panel). We found similar total numbers of nodular melanomas (Fig. 2B, middle panel) and similar growth kinetics of the largest nodular melanomas (Fig. 2B, lower panel) in the cohorts receiving vehicle alone, 10nmol or 100nmol TPA. TPA-treated mice did show a dose-dependent diffuse expansion of heavily pigmented, epitheloid melanoma cells in the papillary dermis in addition to the large nodular melanomas composed of both epitheloid and spindle-shaped pigmented cells, which are typical for DMBA-induced primary cutaneous melanomas (Fig. 2C). This was associated with a dose-dependent increase of neutrophils both locally in the tumor microenvironment as well as systemically in the bone marrow, the peripheral blood and the lungs (Fig. 2D). The draining inguinal lymph nodes of DMBA-exposed and TPA-treated Hgf-Cdk4(R24C) mice were considerably enlarged (Fig. 2E, upper panel) and showed an increased infiltration with Trp1<sup>+</sup> CD45<sup>-</sup> melanoma cells (Fig. 2E, lower panel) when compared to DMBA-exposed and vehicle-treated controls. Accordingly, CD45-negative cells in lymph nodes, which correspond to the number of melanoma cells, revealed a dose-dependent and significant increase (Fig. 2F). Most importantly, we also observed a significantly increased number of macroscopically visible lung metastases in DMBA-exposed and TPA-treated Hgf-Cdk4(R24C) mice compared to DMBA-exposed and vehicle-treated controls (Fig. 2G). Taken together, these results demonstrated that a TPA-induced chronic inflammatory response selectively increased metastatic spread of primary DMBA-induced melanomas in Hgf-Cdk4(R24C) mice.

### ***TPA treatment of mice bearing serial Hgf-Cdk4(R24C) melanoma skin transplants promotes spontaneous lung metastases in a manner dependent on host TLR4***

To further substantiate a selective effect of the TPA-induced, TLR4-dependent inflammation on the metastatic spread of melanomas in the skin, we utilized serial Hgf-Cdk4(R24C) melanoma transplants that spontaneously develop lung metastases upon intracutaneous injection (Fig. 3A). In three independent experiments we found that twice weekly treatment with 10nmol TPA did not affect the growth kinetics of the Hgf-Cdk4(R24C) melanoma transplant HcMel12 in the skin of wild-type mice or TLR4-deficient C57BL/6 mice (Fig. 3B). Flow cytometric analyses confirmed the TLR4-dependent increase of neutrophils both



**Figure 1.** TPA-treatment stimulates a TLR4-dependent neutrophilic skin inflammatory response in mice. (A) Experimental protocol: The skin of Cdk4(R24C), Hgf-Cdk4 (R24C), wild type (C57BL/6) and Tlr4-deficient mice was treated twice with 100nmol TPA or vehicle (acetone). 24h after the second treatment skin samples were collected and immune cell infiltration as well as expression of proinflammatory genes analyzed. (B) Top: Representative immunohistochemical stainings showing CD45<sup>+</sup> immune cells infiltrating the skin of Cdk4(R24C) (left) and Hgf-Cdk4(R24C) mice (right) treated as indicated (40 $\times$  magnification). Bottom: Mean number of CD45<sup>+</sup> immune cells per high power-field (HPF, 20 $\times$  magnification, n = 9 in each group). (C) Top: Corresponding images of wild type (right) and Tlr4-deficient mice (left). Bottom: Mean number of CD45<sup>+</sup> immune cells per high power-field (HPF, 20 $\times$  magnification, n = 9 in each group). (D) Flow cytometry gating strategy to analyze myeloid inflammatory cells following TPA treatment shown as representative dot plots of peripheral blood lymphocytes (PBL) of wildtype mice (left panels) and cumulative results for skin and PBL (middle and right panels, n = 9 in each group, mean percentage  $\pm$  s.e.m.; unpaired two-tailed student's t-test; n.s. = not significant; \*\*p < 0.01; \*\*\*p < 0.001). (E) Corresponding qRT-PCR data shown is the fold increase of indicated genes in the skin of Cdk4(R24C), Hgf-Cdk4(R24C), wild type or TLR4-deficient mice induced upon TPA treatment compared to vehicle.



**Figure 2.** TPA-induced chronic skin inflammation selectively increases the metastatic spread of DMBA-induced primary Hgf-Cdk4(R24C) mouse melanomas. (A) Experimental protocol to evaluate the effect of a chronic skin inflammation on DMBA-induced melanomas. Adult Hgf-Cdk4(R24C) mice received a single dose of 100nmol DMBA on the shaved back skin followed by a twice weekly application of vehicle (acetone), 10nmol or 100nmol TPA. (B) Representative macroscopic pictures (upper panel), growth kinetics of the largest nodular melanoma (middle panel) and number of nodular melanomas over time (lower panel) in individual DMBA-exposed Hgf-Cdk4 mice treated with vehicle, 10 nmol or 100 nmol TPA ( $n = 5$ ). (C) Corresponding representative H&E-stained sections of nodular primary melanomas ( $n = 15$  in each group). (D) Flow cytometric analyses showing the percentage of CD45+CD11b+Ly6G+Ly6C+ neutrophils in melanomas, bone marrow (BM), PBL and lungs of Hgf-Cdk4 (R24C) mice treated as indicated ( $n = 15$  in each group, mean percentage  $\pm$  s.e.m.; unpaired two-tailed student's t-test  $*p < 0.05$ ;  $**p < 0.01$ ). (E) Representative macroscopic picture (upper panel) and corresponding immunofluorescence analysis (lower panel) showing Trp1+CD45- melanoma cells in draining lymph nodes of mice treated as indicated. (F) Flow cytometry gating strategy to analyze CD45- melanoma cells in draining lymph nodes of Hgf-Cdk4(R24C) mice treated as indicated. Representative histogram (upper panel) and cumulative data for individual mice (lower panel;  $n = 15$  in each group, bar indicates the mean; unpaired two-tailed Mann-Whitney-U test  $*p < 0.05$ ;  $**p < 0.01$ ). (G) Macroscopic appearance of lung metastases (upper panel) and number of macroscopically visible pigmented metastases in individual mice (lower panel;  $n = 15$  in each group, bar indicates the mean; unpaired two-tailed Mann-Whitney-U test  $**p < 0.01$ ;  $***p < 0.001$ ).

locally in the tumor microenvironment and systemically in the peripheral blood, the lungs and the spleens (Fig. 3C). However, the number of macroscopically visible lung metastases was significantly increased in wild-type mice bearing serial Hgf-Cdk4(R24C) melanoma skin transplants (Fig. 3D, left). These pro-metastatic effects of TPA treatment were abrogated in TLR4-deficient mice (Fig. 3D, right).

### Critical role of neutrophils for the TPA-induced increase of spontaneous lung metastases in mice bearing serial Hgf-Cdk4(R24C) melanoma skin transplants

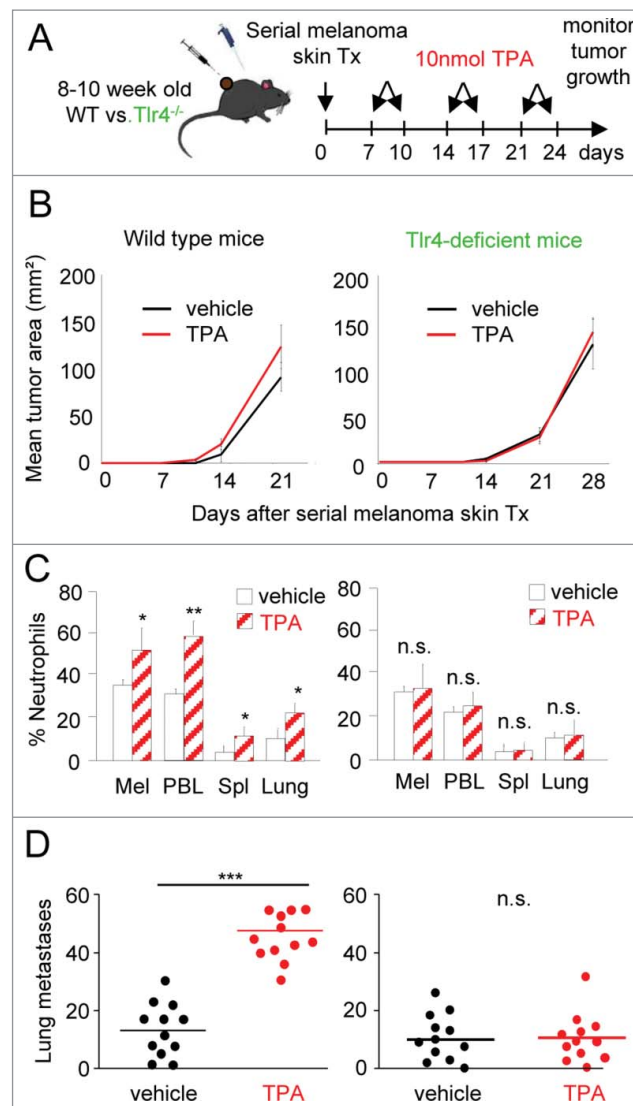
In subsequent experiments we evaluated the impact of antibody-mediated depletion of Ly6G+ neutrophils on the growth of serial Hgf-Cdk4(R24C) melanoma transplants in the skin and the development of spontaneous metastases in the lungs of

mice treated with TPA (Fig. 4A). Injections of the Ly6G-specific mAb 1A8 did not affect the local growth but significantly reduced the number of lung metastases when compared to injections with control IgG mAb (Fig. 4B, C). In conclusion, these data indicate a critical role of neutrophils for melanoma metastasis upon TPA treatment.

## Discussion

Here, we show that chronic local and systemic neutrophilic inflammation elicited by repetitive application of the tumor promoter TPA selectively increased the number of spontaneous lung metastasis of primary DMBA-induced Hgf-Cdk4(R24C) melanomas. Using the highly metastatic Hgf-Cdk4(R24C) melanoma skin transplant HcMel12 we could show that the ability of TPA to recruit neutrophils and enhance systemic melanoma



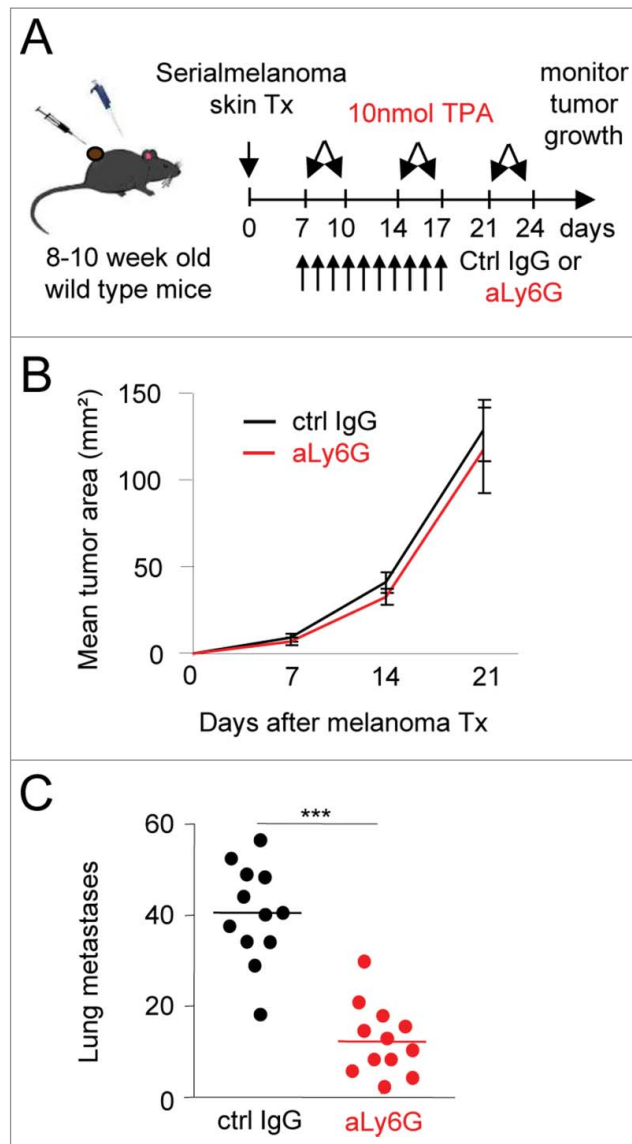


**Figure 3.** TPA treatment of mice bearing serial Hgf-Cdk4(R24C) melanoma skin transplants promotes spontaneous lung metastases in a manner dependent on host TLR4. (A) Experimental protocol. Wild type (left column) and TLR4-deficient (right column) mice were intracutaneously injected with a spontaneously metastasizing serial Hgf-Cdk4(R24C) melanoma skin transplant (Tx, HcMel12) in the flank. Vehicle or 10nmol TPA was applied twice weekly for three weeks on the back skin including the transplantation site and tumor development was monitored. (B) Tumor growth kinetics. Shown is the mean tumor area in mm<sup>2</sup> ( $\pm$  s.e.m.) of wild type (left) and TLR4-deficient (right) mice treated as indicated (n = 12 in each group). (C) Corresponding flow cytometric quantification of neutrophils in melanomas (Mel), PBL, spleens (Spl) and lungs of wild type (left) and TLR4-deficient (right) mice (n = 12 in each group, mean percentage  $\pm$  s.e.m.; unpaired two-tailed student's *t*-test; n.s. = not significant; \**p* < 0.05; \*\**p* < 0.01). (D) Number of macroscopically visible pigmented metastases in wild type (left) or TLR4-deficient (right) mice treated as indicated (lower panel; n = 12 in each group, bar indicates the mean; unpaired two-tailed Mann-Whitney-U test; n.s. = not significant; \*\*\**p* < 0.001).

cell dissemination depended on intact TLR4 signaling in recipient mice. These observations are in accordance with our previous work showing that UV-irradiation induces a neutrophilic inflammatory response in a TLR4/MyD88-dependent manner promoting metastatic spread of primary cutaneous DMBA-induced melanomas in Hgf-Cdk4(R24C) mice.<sup>12</sup> Therefore, TPA-treatment imitates the neutrophil-driven pro-metastatic effect of ultraviolet radiation in primary melanomas in the skin of Hgf-Cdk4(R24C) mice. Altogether, our data strongly support an important mechanistic role of TLR4/MyD88-driven neutrophilic inflammation for melanoma progression independent from the pro-inflammatory stimulus.

Our results add to a growing body of evidence that activation of the innate immune system can contribute to a pro-inflammatory microenvironment that promotes

metastasis.<sup>19-21</sup> It was previously shown that TPA treatment of the skin leads to the release of the endogenous TLR4 ligand Hmgb1 from epidermal keratinocytes triggering a TLR4-dependent inflammatory response.<sup>9</sup> Other potential endogenous TLR4 ligands such as serum amyloid A, S100A8/A9 and fragments of hyaluronan, which are known to be produced in inflamed skin tissue, also likely play a role in amplifying TLR4-driven pro-metastatic inflammatory responses. S100A8/A9 has been shown to recruit myeloid-derived suppressor cells (MDSC) including neutrophils to the tumor site and to enhance their immune suppressive qualities. As a result MDSCs can polarize antitumor immunity toward a tumor-promoting phenotype.<sup>22-24</sup> However, triggering of TLR4 also augmented cellular antitumor immunity following chemotherapy or irradiation.<sup>25,26</sup>



**Figure 4.** Depletion of neutrophils in mice bearing serial Hgf-Cdk4(R24C) melanoma skin transplants abrogates the TPA-induced increase of spontaneous lung metastases. (A) Experimental protocol. Wild type mice were intracutaneously injected with Hcmel12 melanoma cells in the flank. 10nmol TPA was applied twice weekly for three weeks on the back skin and mice received 10 consecutive injections of control (Ctrl IgG) or anti-Ly6G antibody (aLy6G) (i.p.), respectively. (B) Tumor growth kinetics. Shown as the mean tumor area in mm<sup>2</sup> ( $\pm$  s.e.m.) of wild type mice treated as indicated ( $n = 12$  in each group). (C) Number of macroscopically visible metastases in wild type mice treated as indicated (lower panel;  $n = 12$  in each group, bar indicates the mean; unpaired two-tailed Mann-Whitney-U test; \*\*\* $p < 0.001$ ).

Protective immunity and regenerative inflammation needs to be tightly controlled and balanced to maintain tissue homeostasis and organismal integrity.<sup>21,27</sup>

A number of *in vitro* and *in vivo* studies on murine models provide experimental evidence how neutrophil associated chemokines and their receptors facilitate metastatic spread of melanoma cells.<sup>28</sup> For example the pro-inflammatory IL-8/CXCL8 or CXCL2 is produced by a variety of human or mouse melanoma cells attracting neutrophils and upregulates  $\beta 2$  integrin expression on melanoma cells, which aids anchoring of melanoma cells to the vascular endothelium to facilitate transendothelial migration and extravasation of melanoma cells to form new metastases.<sup>29</sup> Neutrophil-derived TNF can promote the ability of mouse and human melanoma cells to migrate toward endothelial cells *in vitro* indicating increased metastatic capacity through TNF enhanced endothelial and melanoma cell

interaction.<sup>12</sup> Neutrophil induced degradation of the extracellular matrix (ECM) via release of enzymes such as neutrophil elastase, proteinase 3 and MMP9 might contribute to invasion and metastasis of melanoma cells.<sup>30</sup> It has recently been shown that MMP-9 regulates rounded-amoeboid cell migration in a panel of human melanoma cell lines through regulation of actomyosin contractility via CD44, a receptor for hyaluronic acid and collagen, showing a direct effect of MMP-9 on melanoma cell migration.<sup>31</sup>

Activation of TLR4 signaling and recruitment of neutrophils are both potent stimulators of angiogenesis.<sup>32,33</sup> Activated neutrophils express high levels of pro-angiogenic factors like MMP-9 and VEGF that contribute to tumor angiogenesis in different experimental models including melanoma<sup>34-36</sup> and facilitate tumor intravasation.<sup>37</sup> Work of several research groups collectively show that hypoxia and the release of

endogenous pro-inflammatory mediators such as Hmgb1 and S100A8/A9 link TLR4 signaling, the expansion of neutrophils, the suppression of cellular immunity and the formation of a pre-metastatic niche in the lungs as a prerequisite for metastatic seeding.<sup>38-40</sup> It remains to be elucidated how these innate immune pathways intersect with other recently described mechanism of melanoma metastasis, which include melanoma-derived exosomes, neutrophil-derived extracellular DNA traps, localized changes in vascular permeability and the activation of the coagulation cascade.<sup>41-43</sup>

The presence of neutrophils within tumor tissue and their increase in the peripheral blood indicate a poor prognosis in melanoma patients.<sup>2,6</sup> Further analyses how TLR4-dependent neutrophilic inflammation affects the complex network of molecular and cellular mechanisms that orchestrate the metastatic cascade in melanoma<sup>44</sup> will provide the scientific basis for new treatment strategies such as targeted inhibition of TLR4 signaling and neutrophil functions.

## Material and methods

### Mice

Wild-type C57BL/6 (H-2<sup>b</sup>) mice were purchased from Charles River. Melanoma-prone Hgf-Cdk4<sup>R24C</sup> mice were bred as described previously.<sup>14,17,45</sup> TLR4-deficient mice on the C57BL/6 background (Hoshino et al., 1999) were kindly provided by A. Limmer. Age and sex matched cohorts of mice were randomly allocated to the different experimental groups at the start of each experiment. All animal experiments were performed under specific pathogen-free conditions at the Central Animal Facility of the University of Bonn and conducted according to the institutional and national guidelines for the care and use of laboratory animals with approval by the local government authorities (LANUV, NRW, Germany).

### Induction of an acute TPA-induced skin inflammatory response

An acute skin inflammation was induced in groups of 5 mice by epicutaneous applications of 10 or 100nmol TPA, Sigmasolved in 200 $\mu$ L acetone on day 0 and 3. Control groups of vehicle-treated mice received 200 $\mu$ L acetone only. Skin tissue and peripheral blood was harvested on day 4 for further morphological, flow cytometric and molecular analyses.

### Induction of primary melanomas in Hgf-Cdk4(R24C) mice

Primary cutaneous Hgf-Cdk4<sup>R24C</sup> melanomas were induced as described previously (Landsberg et al., 2012). Briefly, synchronous development of primary melanoma was initiated by treating cohorts of 8–10 week old Hgf-Cdk4<sup>R24C</sup> mice with a single epicutaneous dose of 100nmol 7,12-dimethylbenz(a)anthracene (DMBA, Sigma) solved in 200 $\mu$ L acetone on the shaved back skin. Tumor growth was monitored by inspection, palpation, and digital photography. The size of the largest tumor was measured weekly using a vernier caliper and recorded as mean diameter. Mice were killed when progressively growing melanomas exceeded 10mm in diameter and tissues were harvested

for further analyses. The number of macroscopically visible metastases on lung surfaces was counted by two independent investigators in a blinded fashion.

### Melanoma transplantation studies

The melanoma skin transplant Hcmel12, which spontaneously metastasized to the lung after intracutaneous injection in immunocompetent C57BL/6 mice, was established from a primary DMBA-induced Hgf-Cdk4(R24C) melanoma as described previously.<sup>12</sup> In brief, tumors were excised, dissociated mechanically, filtered through 70 $\mu$ m cell strainers (BD Biosciences) and washed in PBS.  $2 \times 10^5$  cells were injected intracutaneously into the flanks of mice. Tumor sizes were measured weekly and recorded as mean diameter. Mice with tumors exceeding 20 mm in diameter were killed and the number of macroscopically visible metastases on the lung surfaces counted as described above. All experiments were performed in groups of 5 mice and repeated independently at least twice.

### Induction of a chronic skin inflammatory response in melanoma bearing mice

Hgf-Cdk4(R24C) mice previously exposed to 100nmol DMBA were subsequently treated with 200 $\mu$ L acetone as vehicle control, 10nmol or 100nmol TPA on the shaved back skin twice weekly for 15 weeks. Wild-type or TLR4-deficient C57BL/6 mice received serial Hgf-Cdk4(R24C) melanoma skin transplants in the flank. The back skin including the transplantation site was subsequently treated with vehicle alone or with 10nmol TPA solved in 200 $\mu$ L acetone twice weekly for three weeks starting at day 7 after injection.

### Histopathology and immunostaining

Tissues e.g. tumors, skin and lymph nodes were harvested when mice were killed. Tissue specimens were immersed in a zinc-based fixative (DAKO), embedded in paraffin, and routinely stained with hematoxylin and eosin. To confirm the melanocytic origin of tumor cells infiltrating the draining lymph node, sections were analyzed by an immunofluorescence double staining using the Trp1-specific polyclonal rabbit antibody (PEP7, a kind gift from Vincent Hearing, NIH, Bethesda) and a monoclonal rat anti-CD45 antibody (Clone 30-F11, BD Bioscience) followed by appropriated enzyme-conjugated secondary antibodies and the LSAB-2 color development system 8 (DAKO) or by fluorochrome-conjugated secondary antibodies, anti-rabbit Alexa488 and anti-rat Alexa594 (Jackson ImmunoResearch). Heavily pigmented lymph nodes were bleached by incubation in 3% H<sub>2</sub>O<sub>2</sub> and 0.5% KOH at 37°C for 20 min. Slides were placed into 1% acetic acid for 20 sec and washed in TRIS buffer for 5 min. Antigen-retrieval was done by incubation in citrate buffer pH = 6,0 and steaming for 20 min. CD45+ immune cells were counted in three sequential high-power-fields (20 $\times$  magnification) and mean number of immune cells were expressed as CD45+ immune cells/3 high-power fields. Stained sections were examined with a Leica DMLB immunofluorescence microscope and images were acquired with a JVC digital camera KY-75FU.

## Flow cytometry

Skin, melanomas, lymph nodes, spleens and lungs were harvested and dissociated mechanically before incubation with 1mg/mL type collagenase D (Roche, Penzberg, Germany) in PBS containing 5 % FBS (Biochrom, Berlin, Germany) for 30 min at 37°C under constant stirring. EDTA (2mM) was added to the mixture for 10 additional min. The cell suspension was filtered through 70µm cell strainers (BD Bioscience) and washed three times in PBS before erythrocyte lysis. Single cell suspensions, blood and bone marrow samples were incubated 10 min with erythrocyte lysis buffer and stained with fluorochrome-conjugated monoclonal antibodies specific for mouse CD45 (Clone 30-F11), CD11b (Clone M1/70), Ly6G (Clone 1A8) and Ly6C (CloneAL-21; all from BD PharMingen) according to standard protocols. Data were acquired with a FACSCanto flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, V7.6.5 for Windows).

## Expression analysis

Tissues were harvested and immediately snap frozen in liquid nitrogen. Total RNA was isolated using TRI Reagent (Sigma) and purified using NucleoSpin RNA (Macheray & Nagel). RNA was reversely transcribed using Superscript II (Invitrogen) and oligo-dT18 primers (Invitrogen). Quantitative real time PCR was performed by using cDNA and Fast SYBR Green Master Mix (ABI) to determine the expression. The PCR program contained an annealing temperature of 60°C and 35 repeating steps. Relative expression to the reference gene Ubc was calculated with the delta-Ct method using the following equations:  $\Delta Ct(\text{Sample}) = Ct(\text{Target}) - Ct(\text{Reference})$ ; relative quantity =  $2^{-\Delta Ct}$ .

*S100a8* F: TGCCACACCCACTTTTATCA; R: GAGTGTCC TCAGTTTGTGCAG;

*Tnf* F: GATTATGGCTCAGGGTCCAA; R: ACAGTCCAG GTCAGTGTCCC;

*Cxcl2* F: CGCTGTCAATGCCTGAAG; R: GGCGTCACA CTCAAGCTCT;

*Il-1b* F: GGGCCTCAAAGGAAAGAATC; R: TTCTTTGG GTATTGCTTGGG;

*Mmp2* F: TGGGGGAGATTCTCACTTTG; R: TGAAC AGGAAGGGGAAGTTC;

*Ubc* F: AGGCAAGACCATCACCTTGGACG; R: CCATC ACACCCAAGAACAAGCACA;

## Antibody-mediated neutrophil depletion

Antibody-mediated depletion of Ly6G+ immune cells was performed by daily intraperitoneal injections of 100 µg/mouse anti-Ly6G (Clone 1A8) or control-IgG2a (Clone 2A3, both from BioXcell) from day 7 to 16 in TPA-treated mice bearing serial HcMel12 melanoma transplants

## Statistical analyses

Statistically significant differences between data groups were calculated with the two-tailed unpaired student's t test if the values were normally distributed and had similar variances. Numbers of melanoma metastasis on lung surfaces were tested

with a non-parametric Mann-Whitney-U test. The statistical tests were performed with the GraphPad Prism 4 and are specified in the respective figure legends including their test direction (one- or two-sided).

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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