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Tumor cell intrinsic Toll-like receptor 4 signaling promotes melanoma progression and metastatic dissemination

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Abstract

Most melanoma-associated deaths result from the early development of metastasis. Toll-like receptor 4 (TLR4) expression on nontumor cells is well known to contribute to tumor development and metastatic progression. The role of TLR4 expression on tumor cells however is less well understood. Here we describe TLR4 as a driver of tumor progression and metastatic spread of melanoma cells by employing a transplantable mouse melanoma model. HCmel12 melanoma cells lacking functional TLR4 showed increased sensitivity to tumor necrosis factor α induced cell killing in vitro compared to cells with intact TLR4. Interestingly, TLR4 knockout melanoma cells also showed impaired migratory capacity in vitro and a significantly reduced ability to metastasize to the lungs after subcutaneous transplantation in vivo. Finally, we demonstrate that activation of TLR4 also promotes migration in a subset of human melanoma cell lines. Our work describes TLR4 as an important mediator of melanoma migration and metastasis and provides a rationale for therapeutic inhibition of TLR4 in melanoma.

KEYWORDS
dedifferentiation, melanoma, metastasis, migration, TLR4

What's new?

Toll-like receptor 4 (TLR4) expression on non-tumour cells is well known to contribute to tumour development and metastatic progression. The role of TLR4 expression on tumour cells, however, remains unclear. Here, the authors show that TLR4 drives two processes that might contribute to increased metastatic potential in melanoma—increased resistance to inflammatory stimuli and enhanced cell migration. The work highlights TLR4 as an important mediator of melanoma migration and metastasis and provides a rationale for therapeutic inhibition of TLR4 in melanoma. Moreover, the results support the view that innate inflammatory pathways are essential for malignant tumour progression.

Abbreviations: LPS, lipopolysaccharide; SKCM, skin cancer melanoma; TCGA, The Cancer Genome Atlas; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor α.

Meri Rogava and Andreas Dominik Braun contributed equally to this study.

1 | INTRODUCTION

Melanoma accounts for the vast majority of skin cancer-related deaths due to the ability of a subset of primary tumors to form distant metastases early in the course of disease.[1,2] Despite recent therapeutic advances, metastatic melanoma remains challenging to treat and is frequently incurable. Investigations have revealed several common mechanisms of the metastatic process, including the detachment of single cells from the primary tumor with infiltration into the surrounding tissue, the entrance into the vasculature with survival in the bloodstream, the extravasation and survival in the new environment and finally the reactivation of proliferative pathways and the formation of macrometastases.[2] Recent work has provided evidence that the later stages, that is, the colonization and outgrowth of cells at metastatic sites, are the rate limiting steps in this developmental cascade.[3]

Protumoral inflammatory responses in the microenvironment have been shown to drive metastatic progression of cancer.[4] A well-described molecular mechanism involves activation of the transcription factor NF-κB, which can promote tumor cell dedifferentiation and foster migration and metastasis.[5] Moreover, inflammatory activation of tumor cells increases their resilience to hypoxia and cellular stress, enabling them to survive at the site of metastasis prior to vascularization of the new environment.[6,7] Protumoral inflammation also creates premetastatic niches for tumor cells in distant organs.[8] In melanoma, intense intermittent ultraviolet-irradiation represents a major environmental risk factor that has been shown to promote metastatic spread via Toll-like receptor 4 (TLR4)-dependent neutrophilic inflammation.[9]

TLR4 is a pattern recognition receptor involved in host defense and maintenance of tissue homeostasis upon tissue injury.[10] It recognizes a variety of pathogen-associated molecular patterns[11] and damage-associated molecular patterns, which are released upon cell damage. Upon stimulation, TLR4 initiates an inflammatory response which promotes tissue repair and plays an important role in tumor pathogenesis.[12-14] Furthermore, it has been shown that the chemotherapeutic agent paclitaxel, a direct TLR4 ligand, enhances tumor cell survival and metastatic progression in a breast cancer model.[15] High TLR4 expression in the tumor microenvironment correlates with tumor progression in melanoma patients and it has become clear that in vitro stimulation of TLR4 can enhance tumor cell migration.[16-18]

In our current work, we hypothesized that TLR4 expression on tumor cells promotes metastatic tumor progression. To experimentally test this hypothesis, we generated Clustered regularly interspaced short palindromic repeats (CRISPR)-mediated knockouts and following reconstitution of TLR4 via transgenic overexpression in the spontaneously metastasizing mouse melanoma cell line HCmel12. We demonstrate a decreased migratory capacity and fewer metastases of TLR4 knockout cells and connect TLR4-signaling in human melanoma cell lines with a migratory phenotype. Our data support a prometastatic function of TLR4 in melanoma cells and warrant further investigation toward the inhibition of innate immune sensors in melanoma.

2 | MATERIAL AND METHODS

2.1 | Cell culture and treatments

The melanoma cell line HCmel12 was established from a primary melanoma derived from the Hgf-Cdk4 mouse model as previously described.[9] Human melanoma cell lines were kindly provided by D. Schadendorf (Essen) and T. Wölfel (Mainz): Ma-Mel-15 (RRID: CVCL_A151), MZ-MEL-7 (RRID: CVCL_1436), Ma-Mel-54a (RRID: CVCL_A189) and Ma-Mel-65 (RRID: CVCL_A200). All cells were routinely maintained in RPMI 1640 medium (Life Technologies, Carlsbad, California) supplemented with 10% fetal calf serum (FCS; Biochrome, Berlin, Germany), 2 mM L-glutamine, 1 mM sodium-pyruvate, 10 mM nonessential amino acids, 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; all from Thermo Fisher, Waltham, Massachusetts), 20 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, Missouri), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen). All cells were routinely maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS (Biochrome), 2 mM L-glutamine, 1 mM sodium-pyruvate, 10 mM nonessential amino acids, 1 mM HEPES (all from Thermo Fisher), 20 μM 2-mercaptoethanol (Sigma-Aldrich), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, California). The human embryonic kidney cell line HEK293T (RRID: CVCL_0063) was maintained in Dulbecco's modified eagle medium (DMEM) medium supplemented with 10% FCS, 2 mM L-glutamine (Thermo Fisher), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen). All cell lines were routinely tested for mycoplasma by Polymerase chain reaction (PCR). All human melanoma cell lines used in this study were authenticated using Short tandem repeats (STR) profiling in 2020 (Microsynth, Balgach, Switzerland). Human and mouse cell lines were treated with recombinant mouse or human tumor necrosis factor α (TNF-α) (1000 IU/mL, both from Peprotech, Rocky Hill, New Jersey) or with ultra-pure lipopolysaccharide (LPS)-EB (1 μg/mL, or indicated amounts) (InvivoGen, San Diego, California) for indicated duration. For TLR4 signaling inhibition, cells were treated with CLI-095 (InvivoGen) at a concentration of 1 μg/mL 2 hours prior to further treatment, which has been shown to selectively inhibit TLR4.[19]

2.2 | Generation of CRISPR/Cas9 constructs

pX330-U6-Chimeric_BB-CBh-hSpCas9 (further referred to as pX330) (Addgene plasmid #42230) was linearized by restriction digest with the enzyme BpiI (Thermo Scientific). Subsequently, DNA oligonucleotides encoding for single guide RNAs (sgRNAs) targeted against the exons 1 and 2 of the mouse Tlr4-gene (Microsynth) were cloned into the linearized pX330 plasmid. Sequences of DNA oligonucleotides are listed in Table S1.

2.3 | Generation of HCmel12 CRISPR/Cas9 knockout cell line

A total of 5×10^5 HCmel12 melanoma cells were seeded to 12-well plates 1 day prior to transfection. The cells were transfected with

1.6 μg pX330-TLR4-sgRNA and 0.4 μg pRP enhanced green fluorescent protein (pRP-EGFP) plasmid using Fugene HD transfection reagent (Promega) according to the manufacturer's protocol. After 48 hours, green fluroescent protein (GFP) positive cells were sorted (BD FACSAria II Cell Sorter; BD Biosciences). Genetic disruption of Tlr4-gene was identified by next generation sequencing on a MiSeq

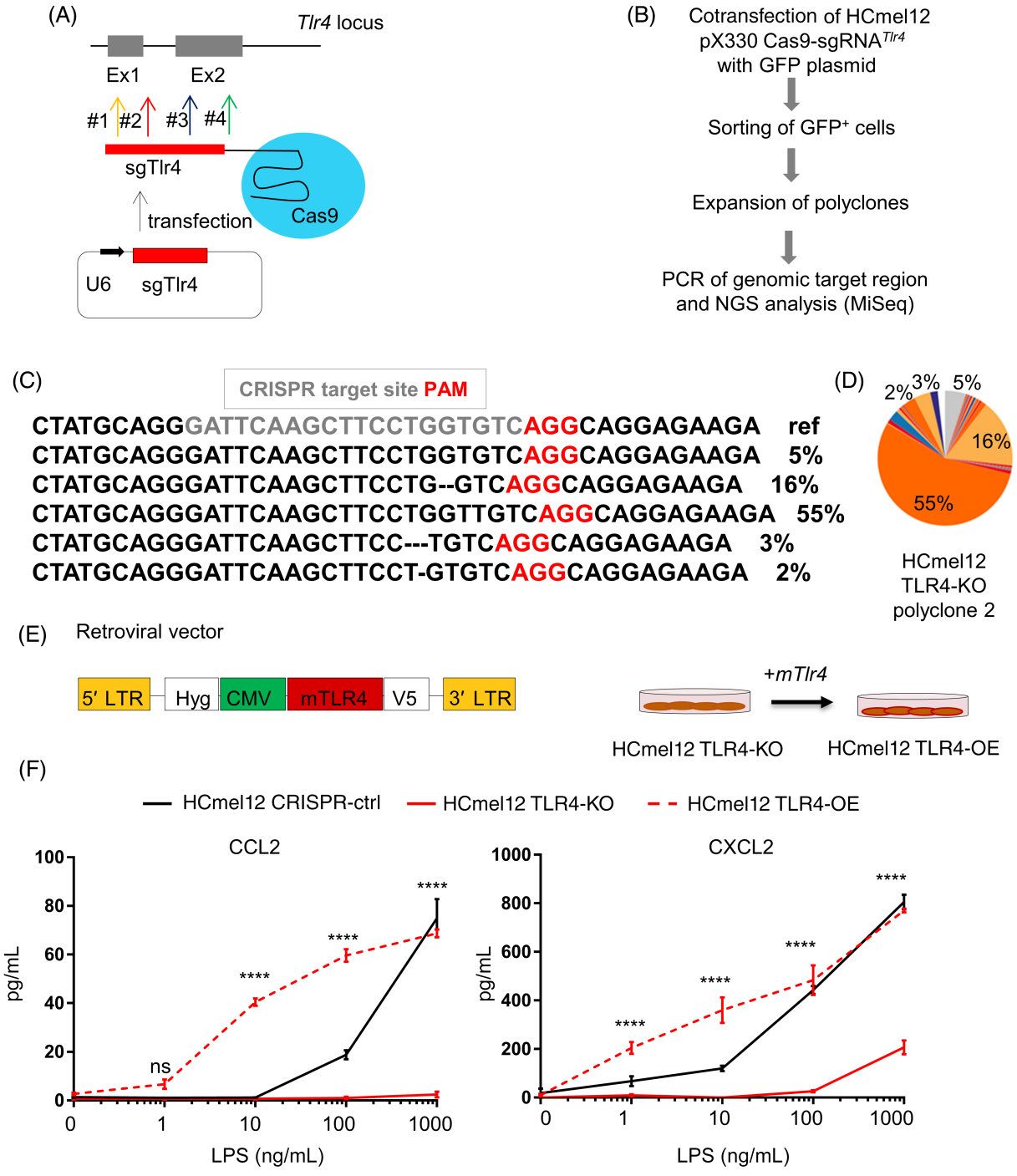
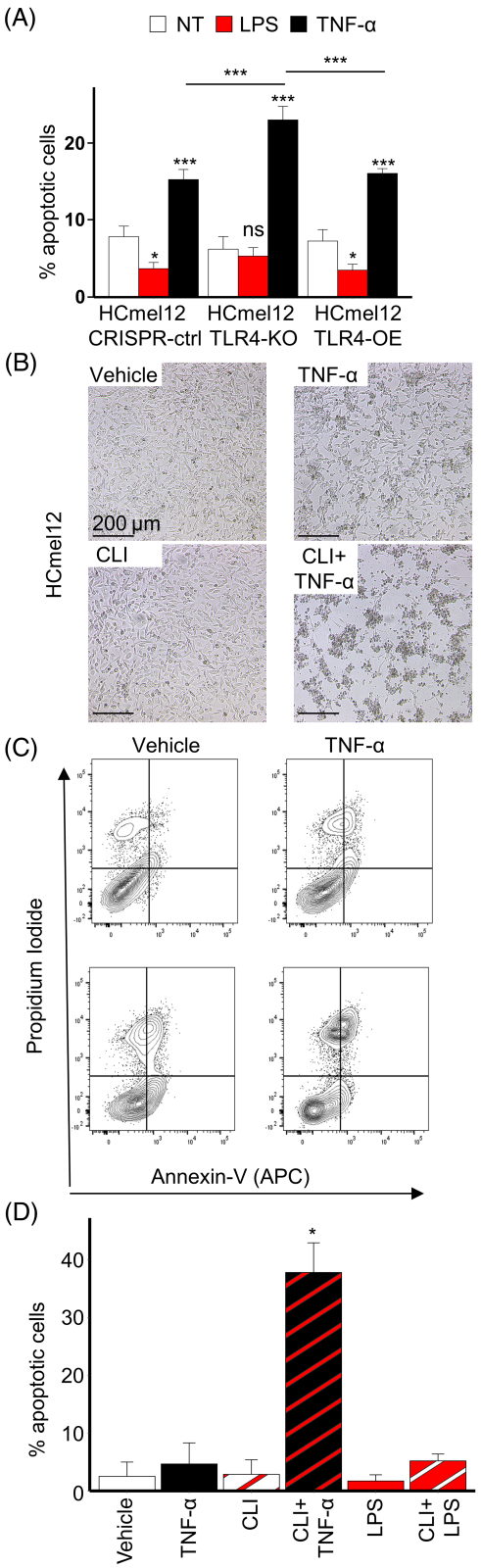


FIGURE 1 Generation and validation of HCmel12 TLR4-KO and HCmel12 TLR4-OE cells. (A) Schematic representation of the targeting strategy of the mouse Tlr4 genetic locus. (B) Outline of the CRISPR/Cas9 genome editing workflow. (C,D) Composition of the HCmel12 TLR4-KO polyclone 2 used in the following experiments. Depicted is the result from an amplicon based NGS with local alignment to the Tlr4 genetic locus. Most frequent variant sequences are shown in (C), proportion of in-frame indels (blue) and out-of-frame indels (orange/red) are shown in (D). (E) Schematic representation of the TLR4 overexpression construct and used workflow. (F) Sandwich-ELISA of supernatants from HCmel12 CRISPR-ctrl, HCmel12 TLR4-KO and HCmel12 TLR4-OE cells treated with LPS for 24 hours. Shown is the absolute amount of protein measured (mean ± SEM, two-way ANOVA with Tukey-test to correct for multiple testing). Data in (E) are representative for one out of two independently performed experiments with two biological replicates. ns, nonsignificant; *P < .05; **P < .01; ***P < .001; ****P < .0001. ANOVA, Analysis of variance; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; NGS, Next generation sequencing; TLR4, Toll-like receptor 4

Sequencer (Illumina) and following analysis using the Outknocker tool (http://www.outknocker.org). All experiments with HCmel12 TLR4 knockout cells were performed with polyclone 2 cultivated no longer than 4 weeks to avoid selection of isolated subclones.



2.4 | Generation of retroviral transduced mouse melanoma cells

The TLR4 overexpression plasmid pRH-TLR4 was generated by replacing the puromycin resistance gene with a hygromycin resistance gene of the plasmid pRP (Addgene plasmid #41841, kindly provided by E. Latz) and the subsequent subcloning of mouse Tlr4-cDNA. Retroviral particles were generated by transfection of HEK293T cells with pRH-TLR4 plasmid together with the retroviral helper plasmids (gag-pol [Addgene plasmid #14888] and pCMV VSV-G [Addgene plasmid #14887], kindly provided by E. Latz) by calcium phosphate transfection according to standard protocols. Supernatant containing retroviral particles was filtered through 0.45 µm syringe filters and added onto target cells. Antibiotic selection with puromycin (10 µg/mL) or hygromycin B (100 µg/mL) was started 48 hours after transduction and performed for 5 days.

2.5 | Enzyme-linked immunosorbent assay

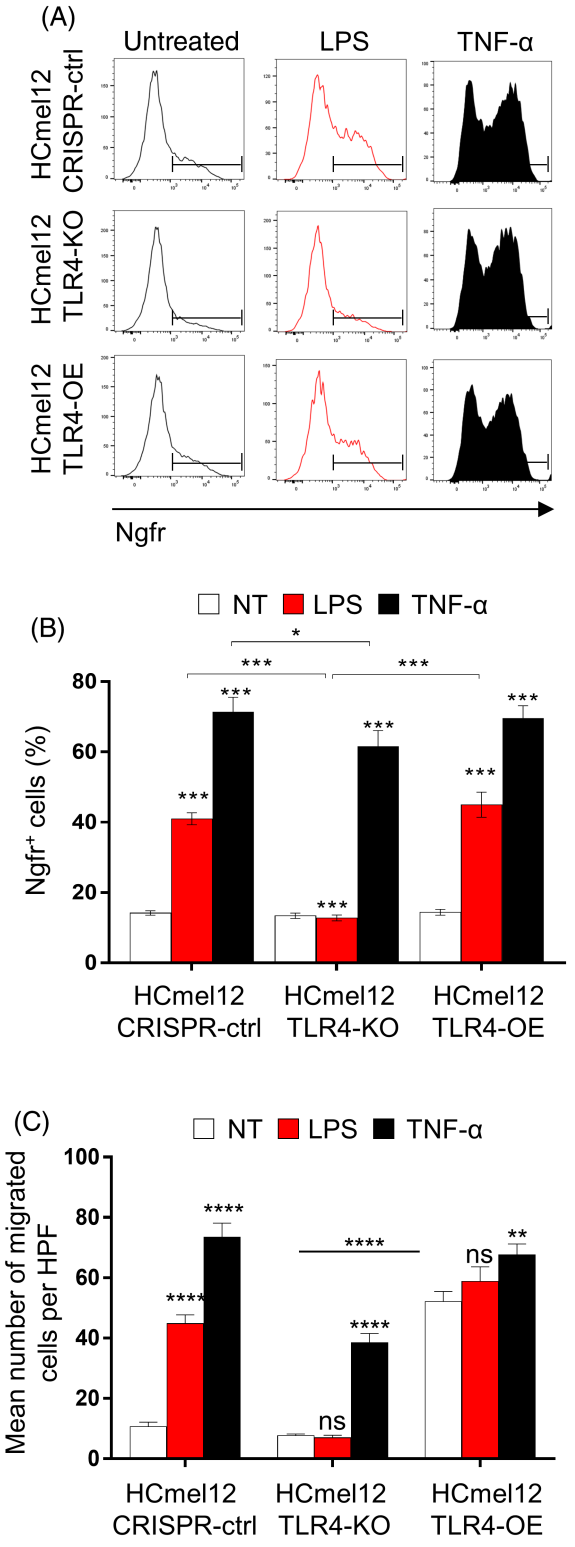
A total of 5×10^4 cells were plated in 96 well plates 16 hours before treatment with LPS as indicated for 24 hours. The supernatant was collected and cleared of cells by centrifugation at 350*g* for 5 minutes. Chemokines were determined by sandwich enzyme-linked immunosorbent assay using kits for mouse CCL2 and CXCL2 as well as human CXCL1 and CXCL8 (R&D) according to manufacturer's protocol. The Epoch Microplate Spectrophotometer (BioTek) was used to measure absorbance at 450 nm with a background subtraction of 570 nm.

2.6 | Flow cytometry

Melanoma cells were stained with goat polyclonal anti-mouse Ngfr biotinylated antibody (R&D) followed by Streptavidin-APC (BD Pharmingen), according to standard protocols. For the

FIGURE 2 Inhibition of TLR4 sensitizes HCmel12 cells to TNF-α induced apoptosis. (A) HCmel12 CRISPR-ctrl, HCmel12 TLR4-KO and HCmel12 TLR4-OE cells were treated with LPS or TNF-α for 72 hours. Apoptosis was measured by flow cytometry using costaining for Annexin-V and PI. Shown are representative bar plots showing the sum of Annexin-V+/PI+ and Annexin-V+/PI− cells (mean ± SEM, two-way ANOVA with Tukey-test to correct for multiple testing). (B-D) HCmel12 cells were treated with CLI and TNF-α for 72 hours. HCmel12 cells treated with vehicle only served as control. Shown are representative phase contrast pictures (B). Apoptosis was measured via flow cytometry by costaining for Annexin-V and PI (B,C). Shown are representative contour plots (C) and bar plots (D) where bar plots show the sum of Annexin-V+/PI+ and Annexin-V+/PI− cells (mean ± SEM, two-way ANOVA with Tukey-test to correct for multiple testing). Data in (A-D) are representative for one out of three independently performed experiments with two biological replicates. ns, nonsignificant; **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001. ANOVA, Analysis of variance; CLI, Chemical TLR4 signaling inhibitor; LPS, lipopolysaccharide; PI, Propidium iodide; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor α [Color figure can be viewed at wileyonlinelibrary.com]

apoptosis assay, Annexin V-APC (BD PharMingen) and Propidium Iodide (Sigma) were used according to manufacturer's instructions. Data were acquired with a FACS Canto flow cytometer (BD Biosciences) and analyzed with the FlowJo software (TreeStar, V7.6.5 for Windows).



2.7 | Transwell migration assays

Human and mouse melanoma cells were labeled with enhanced green fluorescent protein (EGFP) or mCherry by retroviral transduction as described in previous sections. A total of 2×10^4 cells were pretreated with 1 μg/mL LPS or 1000 U/mL TNF-α for 72 hours, harvested, washed and resuspended in 200 μL complete RPMI medium supplemented with 1% FCS, and seeded in the upper chamber of uncoated transwell inserts (BD Biosciences, 8 μm pore size). Migration of melanoma cells was observed along a FCS gradient by filling the lower chamber with complete RPMI medium containing 10% FCS. Cell were incubated at 37°C 5% CO_2 overnight. Cells on the lower surface of the transwell were counted in five high power fields using an AxioVert A1 microscope (Zeiss). Experiments were repeated at least three times.

2.8 | Analysis of published data sets

Gene expression data (RNA-seq) and corresponding clinical information of The Cancer Genome Atlas (TCGA) melanoma cohort (SKCM) were retrieved through the cBioportal package (http://www.cbioportal.org) using the R package CGDS-R and following TCGA guidelines for the use of TCGA data (https://cancergenome.nih.gov). We used RSEM-normalized values for the TLR4 gene expression. Data set GSE51221 containing log 2-transformed and vsn-normalized expression values was retrieved from the gene expression omnibus via the R package GEOquery.

2.9 | Mice

Wild-type C57BL/6 mice were purchased from Janvier. All animal experiments were 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). Age and sex matched cohorts of mice were randomly allocated to the different experimental groups for each experiment.

FIGURE 3 Genetic disruption of TLR4 impairs LPS-induced inflammatory dedifferentiation and migration of HCmel12 melanoma cells. (A,B) FACS analysis of HCmel12 CRISPR-ctrl, HCmel12 TLR4-KO and HCmel12 cells treated with LPS or TNF-α for 72 hours. Shown are representative histograms (A) and bar plots (B) (mean ± SEM, two-way ANOVA with Tukey-test to correct for multiple testing). (C) Transwell migration assay of HCmel12 CRISPR-ctrl, HCmel12 TLR4-KO and HCmel12 cells treated with LPS or TNF-α for 72 hours. Depicted are absolute numbers of cells detected per high powered field (HPF) as counted in three HPF (mean ± SEM, two-way ANOVA with Tukey-test to correct for multiple testing). Data in (A-C) are representative for one out of three independently performed experiments with two biological replicates. ns, nonsignificant; *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$. ANOVA, Analysis of variance; FACS, Flurorescene Activated Cell Sorting; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor α [Color figure can be viewed at wileyonlinelibrary.com]

2.10 | Transplantation experiments

Mice were intracutaneously transplanted with 2×10^5 mCherry-transduced HCmel12 CRISPR-ctrl, HCmel12 TLR4-KO and HCmel12 TLR4-OE cells, respectively, into the right flank. Tumor size was measured at least twice weekly using a vernier caliper and recorded as mean tumor diameter. Mice were sacrificed when tumors exceeded 20 mm in diameter or when signs of illness were observed. Lung metastases were counted macroscopically by inspection and by fluorescence microscopy using an Axio ZoomV16 Stereo Zoom microscope (Zeiss).

2.11 | Statistical analysis

All statistical tests were performed using GraphPad Prism 7. Statistical tests are specified in the corresponding legends of the article. *P* values less than .05 were considered significant.

3 | RESULTS

3.1 | Generation and functional validation of *Tlr4* gene knockout HCmel12 melanoma cells

We and others have previously shown that host TLR4 signaling plays an important role in metastasis formation.[9,17,20-22] To investigate whether tumor cell intrinsic TLR4 signaling also contributes to metastatic dissemination, we performed a CRISPR/Cas9-mediated TLR4 gene knockout in the mouse melanoma cell line HCmel12 (Figure 1A,B). Disruption of the TLR4 gene was analyzed via next generation sequencing (Figure 1C). For further functional experiments, we selected a polyclone with the highest frequency of frameshift mutations (Figure 1D). Furthermore, we generated a retroviral vector for reconstitution of TLR4 expression of TLR4 in HCmel12 TLR4-KO cells (Figure 1E). HCmel12 TLR4-KO cells secrete only very low levels of the chemokines CCL2 and CXCL2 upon stimulation with the TLR4 ligand LPS (Figure 1F). Chemokine secretion was restored following retroviral reconstitution in HCmel12 TLR4-OE cells. Interestingly, TLR4 overexpression in reconstituted cells conveyed a significantly increased sensitivity to low concentrations of LPS. Taken together, these experiments functionally validate the successful CRISPR/Cas9-mediated TLR4 gene knockout in HCmel12 TLR4-KO cells.

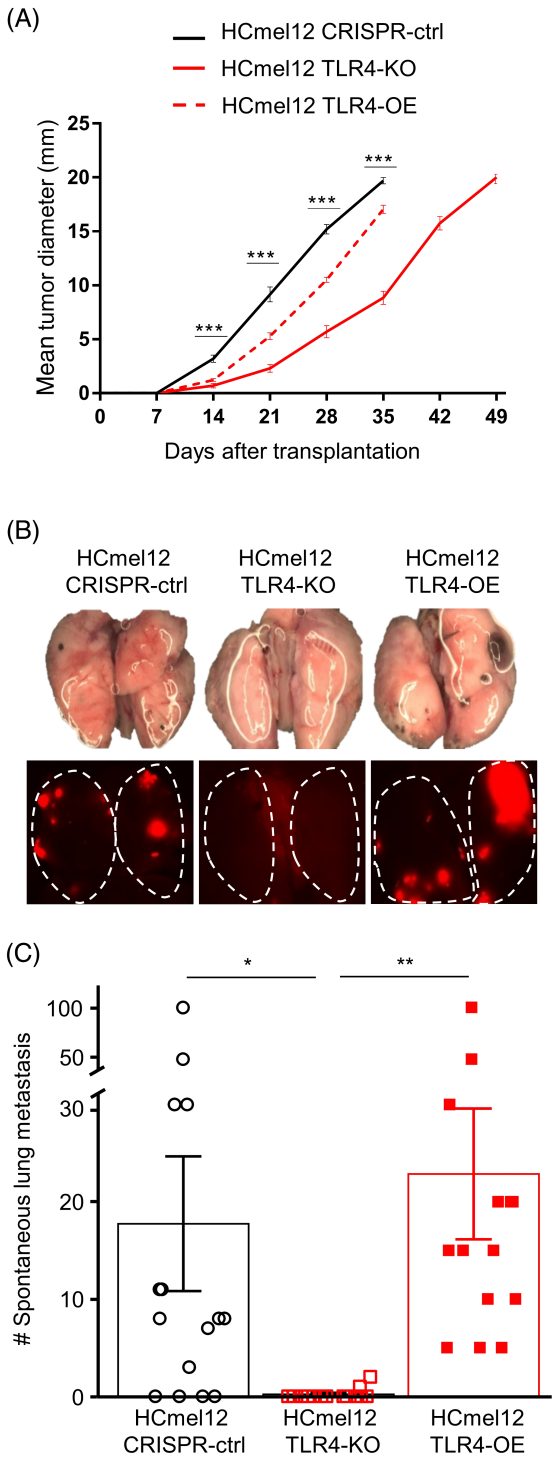


FIGURE 4 Tumor cell intrinsic Tlr4 signaling promotes melanoma growth and spontaneous metastatic spread in vivo. (A) Melanoma growth curves from mice intracutaneously transplanted with 200 000 mCherry labeled HCmel12 CRISPR-ctrl, HCmel12 TLR4-KO and HCmel12 TLR4-OE cells. Shown is the mean tumor diameter per group (n = 15 per group in all experiments, two-way ANOVA and Tukey-test for correction of multiple testing). (B) Representative macroscopic and immunofluorescence images of lungs. Lines recapitulate the shape of the lungs. (C) Absolute quantification of pulmonary metastases in mice. Box includes the interquartile range, whiskers show 1.5× the interquartile range. Markers depict the macroscopic number of lung metastasis per mouse (unpaired two-tailed Mann-Whitney *U* test). Data in (A-C) show cumulative data from three independently performed experiments. ns, nonsignificant; **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001. ANOVA, Analysis of variance [Color figure can be viewed at wileyonlinelibrary.com]

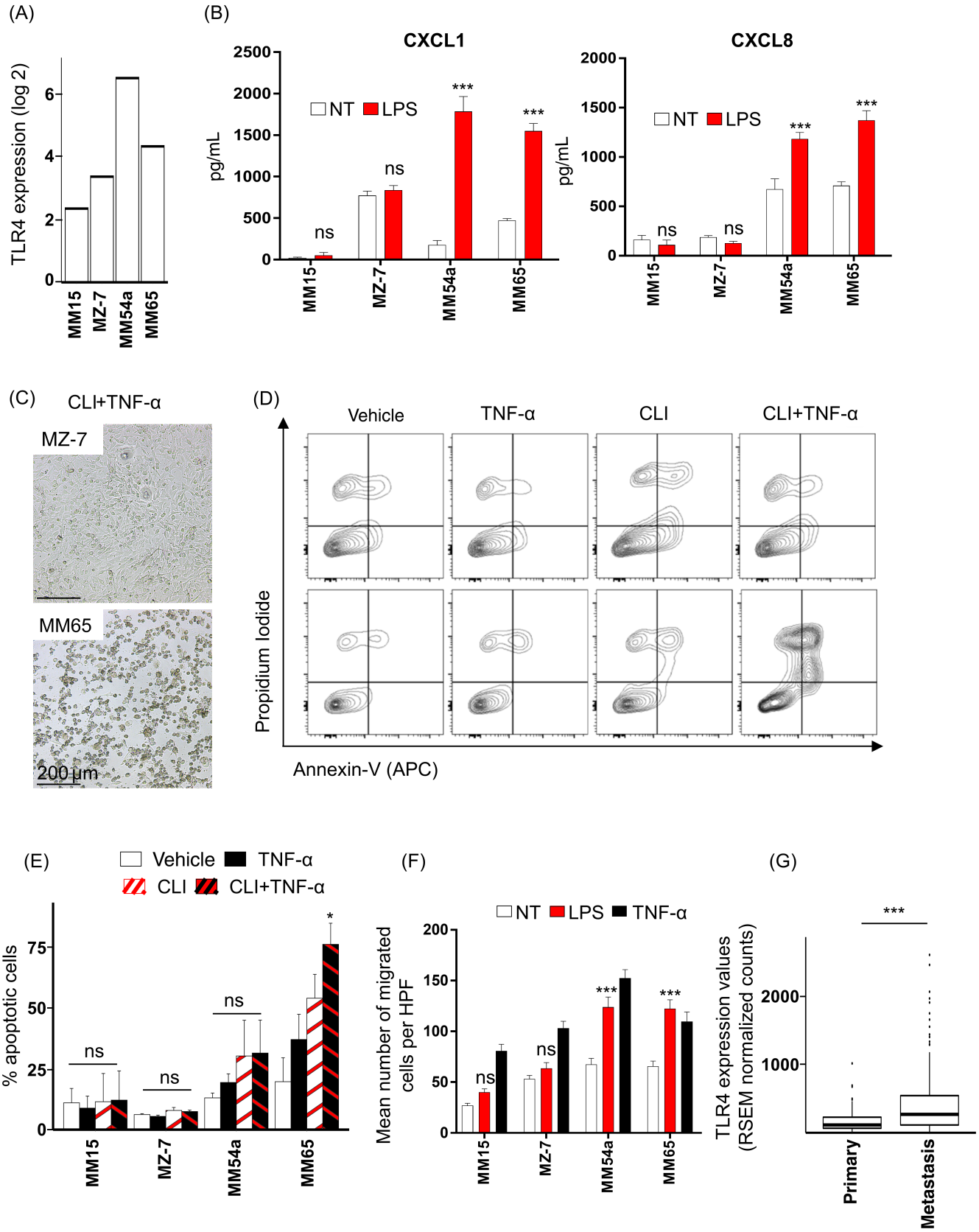


FIGURE 5 Legend on next page.

3.2 | Tumor cell intrinsic TLR4 signaling protects HCmel12 melanoma cells against TNF-α induced apoptotic cell death

We next sought to investigate the effect of tumor cell intrinsic TLR4 signaling on the induction of apoptosis in HCmel12 melanoma cells. Therefore, we treated HCmel12 CRISPR-ctrl, HCmel12 TLR4-KO and HCmel12 TLR4-OE cells with LPS of TNF-α and performed flow cytometric stainings for Annexin-V and PI. We observed a decrease of Annexin-V/PI double positive HCmel12 CRISPR-ctrl cells after treatment with LPS (Figure 2A). This effect was not observed in HCmel12 TLR4-KO cells. Interestingly, HCmel12 TLR4-KO was more susceptible to TNF-α induced apoptosis. This phenotype was reversed by reconstitution of TLR4 in HCmel12 TLR4-OE cells. In addition, pharmacologic inhibition of TLR4 with the cyclohexene derivative CLI-095 also enhanced TNF-induced apoptosis in HCmel12 cells (Figure 2B-D). These experimental results suggest that TLR4 activation of mouse melanoma cells provides protection against TNF-induced cell death.

3.3 | Tumor cell intrinsic TLR4 signaling promotes dedifferentiation and migration of HCmel12 melanoma cells

In previous work, we have described that the exposure to the pro-inflammatory cytokine TNF-α shifted mouse melanoma cells toward a more dedifferentiated, neural crest like phenotype indicated by the expression of Ngfr.[23] To assess the role of tumor cell intrinsic TLR4 signaling in this process, we treated HCmel12 TLR4-KO and HCmel12 TLR4-OE cells with LPS or TNF-α and evaluated the number of Ngfr expressing cells by flow cytometry. LPS induced an increase of $Ngfr^+$ HCmel12 CRISPR-ctrl and HCmel12 TLR4-OE cells, but not of HCmel12 TLR4 KO cells, while TNF-α also induced an increase of $Ngfr^+$ HCmel12 TLR4-KO cells (Figure 3A,B). Inflammatory activation of HCmel12 melanoma cells with TNF-α can also enhance their migratory capacity.[9] In transwell migration assays, LPS also enhances migration of HCmel12 CRISPR-ctrl and HCmel12 TLR4-OE but not of HCmel12 TLR4-KO cells, while TNF-α was active in all HCmel12 variant cells (Figure 3C). This demonstrates that TLR4 activation of mouse melanoma cells contributes to inflammation-induced dedifferentiation and migration.

3.4 | Tumor cell intrinsic TLR4 signaling supports local growth and metastatic progression of transplanted HCmel12 melanoma cells in vivo

Having established a functional role of tumor cell intrinsic TLR4 signaling in HCmel12 cells for inflammation-induced dedifferentiation, migration and protection against apoptosis, we hypothesized that TLR4 deficient melanoma cells would show impaired growth and metastatic dissemination following transplantation in vivo. To test this hypothesis, we injected mCherry-transduced fluorescent HCmel12 CRISPR-ctrl, HCmel12 TLR4-KO and HCmel12 TLR4-OE cells intracutaneously into the flanks of immunocompetent mice. HCmel12 TLR4-KO melanomas showed reduced growth in the skin when compared to HCmel12 CRISPR-ctrl or HCmel12 TLR4-OE melanomas (Figure 4A). Mice were killed when transplanted skin tumors reached a size of 20 mm in diameter. Macroscopic and fluorescence imaging analysis of lungs revealed a significantly reduced number of spontaneous pulmonary metastasis in mice bearing HCmel12 TLR4-KO cells when compared to mice bearing HCmel12 CRISPR-ctrl or HCmel12 TLR4-OE melanomas (Figure 4B,C). Together, these results support our hypothesis that tumor cell intrinsic TLR4 signaling enhances melanoma growth and metastatic dissemination.

3.5 | A subset of human melanomas is TLR4 signaling competent, which protects them against TNF-α induced apoptosis

In order to translate our findings into the human system, we analyzed the impact of TLR4 activation on a set of four human melanoma cell lines. We analyzed previously published transcriptomes for the expression of TLR4 in these cell lines and detected differing expression levels with highest expression of TLR4 in the cell line Ma-Mel54a (Figure 5A). In initial experiments, we evaluated whether LPS was able to promote the secretion of the pro-inflammatory cytokines CXCL1 and CXCL8/IL-8. The two more melanocytic cell lines Ma-Mel15 and MZ-7 did not respond to stimulation with LPS, whereas the two less differentiated cell lines Ma-Mel54a and Ma-Mel65 showed a significant increase in chemokine secretion (Figure 5B). Furthermore, pharmacologic inhibition of TLR4 with the cyclohexene derivative CLI-095 also enhanced TNF-induced apoptosis in

FIGURE 5 Inhibition of TLR4 sensitizes LPS-sensitive human melanoma cell lines to TNF-α induced apoptosis. (A) TLR4 expression levels for indicated melanoma cell lines from the GSE51221 data set. (B) Sandwich-ELISA of supernatants from a set of human melanoma cell lines treated with LPS for 24 hours. Shown is the absolute amount of protein measured (mean ± SEM, two-way ANOVA with Tukey-test to correct for multiple testing). (C) Phase-contrast microscopy images of MZ-7 and Ma-Mel 65 cells treated with CLI and TNF-α. (D,E) FACS analysis of human melanoma cells treated with CLI, TNF-α or both. Shown are representative contour plots (C) and bar plots (D) where bar plots show the sum of Annexin-V+/PI+ and Annexin-V +/PI− cells (mean ± SEM, two-way ANOVA with Tukey-test to correct for multiple testing). (F) Transwell migration assay of human melanoma cell lines treated with LPS or TNF-α for 72 hours. Depicted are absolute numbers of cells detected per high powered field (HPF) as counted in three HPF (mean ± SEM, two-way ANOVA with Tukey-test to correct for multiple testing). (G) RNAseq expression levels of TLR4 in TCGA skin melanoma samples extracted from cBioPortal as counts normalized with the RSEM tool where tissue source site was supplied (n = 403). Data in (B) are representative for one out of two independently performed experiments with three biological replicates. Data in (C-F) are representative for one out of three independently performed experiments with two biological replicates. ns, nonsignificant; *P < .05; **P < .01; ***P < .001; ****P < .0001. ANOVA, Analysis of variance; CLI, Chemical TLR4 signaling inhibitor; ELISA, enzyme-linked immunosorbent assay; FACS, Flurorescene Activated Cell Sorting; LPS, lipopolysaccharide; TCGA, The Cancer Genome Atlas; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor α [Color figure can be viewed at wileyonlinelibrary.com]

Ma-Mel65 cells (Figure 5C-E). In transwell migration assays, LPS could enhance the migration of Ma-Mel54a and Ma-Mel65 cells but of Ma-Mel15 and MZ-7, in agreement with the results of the chemokine secretion assay (Figure 5F). To assess if TLR4 also plays a role in human melanoma samples, we have analyzed TLR4 mRNA expression in the TCGA SKCM cohort and detected a significantly increased expression of TLR4 in samples from metastatic sites compared to primary tumors (Figure 5G). Taken together, this indicates that TLR4 activation also contributes to inflammation-induced activation and migration in a subset of human melanoma cells, suggesting that our observations in the mouse model are also relevant in the human system.

4 | DISCUSSION

In our current work, we demonstrate a functional role for tumor intrinsic TLR4 signaling in melanoma cells. We show that TLR4 knockout melanoma cells are more susceptible to TNF-α induced death, suggesting that TLR4 signaling can enhance melanoma cell survival in a hostile inflammatory environment. Various cancer treatments can enhance the intratumoral levels of TNF-α either directly by tumor cells or the surrounding microenvironment.[9,24,25] In agreement with our findings, TLR4 activation has been described to enhance prostate cancer cell survival under adverse environmental conditions.[26] Inhibition of NF-κB has been shown to increase TNF-induced apoptosis of melanoma cells.[27] Since TLR4 stimulation strongly activates the NF-κB pathway, we hypothesize that the increased TNF-induced apoptosis following genetic deletion or pharmacologic inhibition of TLR4 signaling is caused by decreased NF-κB-dependent pro-survival signals.

We also demonstrated that TLR4 signaling in HCmel12 melanoma cells promotes melanoma growth and metastatic progression in vivo. Interestingly, we have observed a decreased growth of HCmel12 TLR4-KO cells in vitro. One possible explanation for this seemingly contradictory function of TLR4 on melanoma growth is the induction of a neural-crest stem cell like, invasive phenotype with enhanced migratory potential but reduced proliferation.[28] Our current findings complement previous results implicating host TLR4 signaling in enhanced melanoma metastasis.[22] One possible mechanism described for TLR4 driven metastasis is the formation of a premetastatic niche after TLR4 stimulation.[29] This niche was constructed via a signaling cascade involving S100A8 and serum amyloid A3 (SAA3), both of which have been reported as physiological TLR4 ligands. Additionally, the high-mobility-group-protein B1 (HMGB1) is known as a driver of melanoma metastasis and putative TLR4 ligand.[9,20] Moreover, the inflammatory glycoprotein PTX3 has been shown to increase melanoma metastasis in a manner dependent on melanoma cell TLR4 signaling.[30] Melanoma cell TLR4 signaling with subsequent activation of STAT3 via MyD88 has also been linked to cell growth, angiogenesis, transition toward a more mesenchymal phenotype and metastasis.[31] Furthermore, TLR4 has been demonstrated to be crucial for the interaction of tumor cells and platelets in the bloodstream, where platelets are thought to protected circulating tumor cells from adverse environmental assaults.[20] Our results support this pro-metastatic role of tumor cell intrinsic TLR4 signaling and enhance the rationale of therapeutic TLR4 inhibition to combat metastasis.

Apart from tumor cell intrinsic roles of TLR4, its activation has also been shown to mediate antitumorigenic properties through microenvironmental effects. Agonistic antibodies against TLR4 have been shown to enhance the effect of an immune checkpoint blockade with anti PD1 antibodies.[32] In experiments with the chemotherapeutic agent paclitaxel, it has been shown that the therapeutic efficacy was dependent on the expression of TLR4 on macrophages.[33] However, TLR4 also plays a role in the reprogramming of myeloid cells toward a suppressive phenotype.[34] More research is required to understand these seemingly contradictory results.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All animal experiments were conducted on the C57BL/6J background 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, approval number: 87-51.04.2010. A339 and LVWA, Saxony-Anhalt, Germany, approval number: 203.6.3-42502-2-1374 UniMD_G).

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SUPPORTING INFORMATION

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