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Carcinoma Produced Factors Activate Myeloid Cells via TLR2 to Stimulate Metastasis

Sunhwa Kim[1], **Hiroyuki Takahashi**[1], **Wan-Wan Lin**[1,2], **Pascal Descargues**[1], **Sergei Grivennikov**[1], **Youngjun Kim**[1,3], **Jun-Li Luo**[1,4], and **Michael Karin**[1]

[1] Department of Pharmacology and Cancer Center, School of Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0723, USA

[2] Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Republic of China

Abstract

Metastatic progression depends on genetic alterations intrinsic to cancer cells as well as the inflammatory microenvironment of advanced tumors[1,2]. To understand how cancer cells affect the inflammatory microenvironment, we conducted a biochemical screen for macrophage activating factors secreted by metastatic carcinomas. Amongst the cell lines screened, Lewis lung carcinoma (LLC)[3] were the most potent macrophage activators leading to production of IL-6 and TNF-α through activation of the Toll-like receptor family members[4] TLR2 and TLR6. Both TNF-α and TLR2 were found to be required for LLC metastasis. Biochemical purification of LLC conditional medium (LCM) led to identification of the extracellular matrix proteoglycan versican, which is upregulated in many human tumors including lung cancer[5,6], as a macrophage activator that acts via TLR2 and its co-receptors TLR6 and CD14. By activating TLR2:TLR6 complexes and inducing TNF-α secretion by myeloid cells, versican strongly enhances LLC metastatic growth. These results explain how advanced cancer cells usurp components of the host innate immune system, including bone marrow-derived myeloid progenitors[7], to generate an inflammatory microenvironment hospitable for metastatic growth.

Distant site metastases are the leading cause of cancer-associated mortality and depend on genetic and/or epigenetic alterations that are intrinsic to cancer cells or extrinsic factors provided by the tumor microenvironment[1]. For instance, cytokines produced by inflammatory cells can enhance metastatogenesis by repressing the metastasis suppressor maspin within primary prostate carcinoma cells[8]. Furthermore, tumor progression and metastasis positively correlate with presence of infiltrates containing myeloid and lymphoid cells[2,9]. It was shown that certain carcinomas secrete factors that upregulate fibronectin and recruit vascular endothelial growth factor receptor 1 (VEGFR1)-positive hematopoetic progenitors to sites of

Correspondence and requests for materials should be addressed to M.K. (e-mail: karinoffice@ucsd.edu, Tel: 858-534-0872, and Fax: 858-534-8158).
[3]Present address: Department of Applied Biochemistry, Konkuk University, 322 Danwol-dong, Chungju-City, Chungbuk, 380-701, Korea
[4]Present address: Department of Cancer Biology, The Scripps Research Institute, 5353 Parkside Dr., Jupiter, FL 33458, USA

Author contributions
S.K., H.T., W.L. and M.K. conceived the project, planned experiments and analyses, that were carried out by S.K., H.T. and W.L. Y.K. and J.L. helped with protein purification and tail vein injection of cancer cells and tumor analysis, respectively. P.D. and S.G. analyzed M2 macrophages and tissue versican content and effect of TNF-α neutralization on lung metastasis. M.K. oversaw the entire project and wrote the manuscript together with S.K.

Author information
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future metastatic growth, termed the pre-metastatic niche[7]. To examine whether cancer cells secrete factors that directly activate myeloid cells to produce tumor promoting cytokines[10], we collected serum free conditioned medium (CM) from different cancer cell lines, derived mainly from C57BL6 mice, and applied it to bone marrow (BM)-derived macrophages (BMDM), which were assayed for production of IL-1β, IL-6 and TNF-α. The screen included 1C1C7 and TrampC1, which are liver and prostate cancer cell lines, respectively, with little or no metastatic activity, and two metastatic breast and lung carcinomas, 4T1 and LLC, respectively. CM from metastatic cells, especially LLC, induced higher amounts of IL-6 and TNF-α secretion than CM from non-metastatic cells (Fig. 1A). IL-1β secretion was undetectable and the CM did not contain IL-6 or TNF-α (data not shown). LLC-CM (LCM) also induced expression of *Il1β*, *Il6* and *Tnfα* mRNAs, whereas serum free medium (SFM) and NIH3T3 CM were inactive (Fig. 1B and data not shown). We investigated the metastatogenic function of some of the LCM-induced cytokines by tail vein injection of LLC into age- and sex-matched *Tnfα* and *Il6* knockout mice and wild-type (WT) controls. $Tnf\alpha^{-/-}$ mice exhibited markedly ($p<0.001$) reduced mortality relative to WT mice after inoculation with 1×10^6 LLC cells (Fig. 1C) and showed an even greater survival advantage when given a smaller LLC inoculum (Suppl. Fig. 1A). Similar differences were seen in lung tumor multiplicity (Fig. 1D). By contrast, there was little difference in survival of $Il6^{-/-}$ and WT mice inoculated with 1×10^6 LLC cells (Suppl. Fig. 1B). Thus, TNF-α but not IL-6 is important for LLC metastasis.

We explored the involvement of TLR family members in sensing LCM components. BMDM from mice deficient in TLR2, TLR3, TLR4 or TLR9 or their adaptor proteins, Myd88 and TRIF (which is inactivated by the *Lps2* mutation: $Trif^m$)[4], were examined for production of IL-6, a convenient BMDM activation marker. LCM-induced IL-6 was fully dependent on TLR2 and Myd88 but not on TLR3, TLR4, TLR9 or TRIF (Fig. 2A). $Tlr2^{-/-}$ BMDM were also defective in LCM-induced *Il1β* and *Il6* mRNA expression and LCM did not induce anti-tumorigenic type I interferon (IFN) genes (Suppl. Fig. 2A), which are readily induced upon TLR3 or TLR4 engagement[11]. TLR2 was also required for LCM-induced of IL-6 and TNF-α secretion by alveolar macrophages, which produced 10-fold more TNF-α than BMDMs (Suppl. Fig. 2B). TLR2 was required for optimal LCM-induced activation of mitogen-activated protein kinases (MAPK) and IκB kinase (IKK) or IκBα degradation (Fig. 2B). TLR2 uses TLR1, TLR6 or CD14 as co-receptors[12]. LCM-induced IL-6 production was dependent on TLR2, TLR6 and CD14 but not on TLR1 (Fig. 2C). By contrast, the response to Pam3CSK4, a bacterial lipoprotein analog[4], depended on TLR2 and TLR1 but not on TLR6 and CD14. These results rule out possible contamination with bacterial lipoproteins. Furthermore, anti-mycoplasma treatment of LLC had no effect on LCM activity (data not shown).

To examine the *in vivo* role of TLR2, we inoculated sex- and age-matched $Tlr2^{-/-}$ and WT mice with 2×10^5 LLC cells via the tail vein and measured mRNAs encoding cytokines and chemokines in their lungs. LLC-induced lung inflammation was previously described[13], but its mechanism was unknown. *Tnfα*, *Il1β*, *Il6* and inflammatory chemokine mRNAs were induced 5 days after LLC inoculation in WT lungs, peaking at 9 days post-inoculation (Fig. 2D). None of these mRNAs was induced in lungs of $Tlr2^{-/-}$ mice, whose basal content of *Mip1β*/*Ccl4*, *Mip2*/*Cxcl2* and *Kc*/*Cxcl1* mRNAs was higher than WT lungs (Suppl. Fig. 3). In addition, $Tlr2^{-/-}$ and WT mice were subcutaneously (SC) inoculated with LLC cells and examined for lung macrophage infiltration and inflammatory cytokine gene expression 15 days later. Although no difference was observed in primary SC tumor growth, macrophage infiltration and inflammatory cytokine gene expression were greatly reduced in $Tlr2^{-/-}$ relative to WT mice (Suppl. Fig. 4A–C).

To investigate whether TLR2 signaling contributes to LLC metastatogenesis, we inoculated age-and sex-matched $Tlr2^{-/-}$ and WT mice with dsRed-labeled LLC cells via the tail vein and examined their lungs for micrometastases. WT but not $Tlr2^{-/-}$ lungs showed small clusters of

DsRed-LLC cells with adjacent $CD11b^+$ and $CD11c^+$ myeloid cells (Fig. 3A). We also detected a few $CD3^+$ cells (T cells) in micro-metastases of WT lungs (data not shown). Importantly, *Tlr2*$^{-/-}$ mice exhibited significantly greater ($p<0.02$) survival than WT mice after LLC inoculation and their lungs contained fewer and smaller tumor nodules (Fig. 3B, C). Tumor nodules in WT mice contained more $CD11b^+/Gr1^+$ inflammatory monocytes/myeloid suppressors and IL-10^{high}/$F4/80^+$ M2 macrophages (Suppl. Fig. 5). *Tlr2*$^{-/-}$ mice exhibited significantly fewer lung and liver tumor nodules than WT mice and lower incidence of adrenal gland metastasis after SC implantation of LLC cells (Fig. 3D). To investigate whether TLR2 is acting in BM-derived cells, we examined survival of LLC-inoculated chimeric mice. Mice reconstituted with *Tlr2*$^{-/-}$ BM (WT/*Tlr2*$^{-/-}$) exhibited markedly improved ($p<0.04$) survival relative to mice reconstituted with WT BM (WT/WT) (Fig. 3E). WT/WT and WT/*Tlr2*$^{-/-}$ mice were also inoculated with 2×10^5 LLC cells followed by intraperitoneal injections of LCM or SFM. Lung and liver tumor loads were significantly higher ($p<0.05$) in WT/WT mice receiving LCM vs. those receiving SFM along with the LLC inoculum (Fig. 3F). The pro-metastatic effect of LCM was dependent on TLR2 activation, as little or no metastatic enhancement was seen in WT/*Tlr2*$^{-/-}$ mice. These results strongly suggest that LCM contains TLR2-activating factors that enhance metastatogenesis.

To identify the nature of these factors, we collected large amounts of LCM and separated it on a mono-Q anion exchange column and monitored column fractions for their ability to induce IL-6 in BMDM. Fractions with IL-6 inducing activity were pooled and separated on a Superdex 200 sizing column (Suppl. Fig. 6). Most of the IL-6 inducing activity eluted in a few high molecular weight (HMW) (>400 kDa) fractions that contained several polypeptides larger than 200 kDa. These fractions were pooled, deglycosylated and subjected to mass spectrometry (MS), resulting in identification of several peptides derived from the extracellular matrix (ECM) proteins: versican v1, laminin β1, thrombospondin 2 and procollagen type III α1 (Fig. 4A). To examine which protein accounts for induction of inflammatory cytokines, we incubated LCM with individual neutralizing antibodies prior to BMDM stimulation and measurement of cytokine production. Incubation of LCM with antibodies to versican, laminin β1 or procollagen type III α1, but not thrombospondin 2, reduced IL-6 and TNF-α production (Suppl. Fig. 7). A control antibody to HMGB1, an inflammatory mediator released by necrotic cells[14], did not inhibit cytokine induction.

To investigate the role of these proteins in LCM-enhanced metastatogenesis, we generated stable LLC cell lines containing shRNAs specific to versican v1 (Vers), laminin β1 (Lb) or procollagen III α1 (Proco). Silencing efficiency was approximately 90 % or higher (Fig. 4B). The silenced cells and LLC cells transduced with a control shRNA were injected into mice and lung tumor nodules and survival were monitored. Silencing of versican v1 significantly reduced ($p<0.001$) tumor multiplicity, but silencing of laminin β1 resulted in only a modest inhibition whereas procollagen III α1 silencing slightly enhanced tumor multiplicity (Fig. 4C). Furthermore, silencing of versican v1 reduced lung nodule multiplicity by 4-fold (Suppl. Fig. 8A). Tumors isolated from mice inoculated with versican-silenced LLC cells displayed very low versican expression (Suppl. Fig. 8B), whereas lung tumors from LLC-inoculated mice expressed much versican than normal lung (Suppl. Fig. 8C). Importantly, mice inoculated with versican-silenced LLC cells exhibited significantly improved ($p<0.05$) survival (Fig. 4C). Silencing of versican also reduced metastatic spread to lung, liver and adrenal glands in the SC implantation model (Fig. 4D). To ascertain the proinflammatory and premetastatic functions of versican, we used the low metastasic LLC variant, P29-LLC[15]. CM from P29-LLC did not induce IL-6 in BMDM and contained very little versican (Suppl. Fig. 9). Ectopic expression of human versican in P29-LLC cells increased lung tumor multiplicity after tail vein injection (Fig. 4E).

To examine how versican activates macrophages, we produced His-tagged human (h) versican v1 in LLC cells and purified it on a Ni-chelate column. The purified protein induced IL-6 and TNF-α production in WT but not *Tlr2*$^{-/-}$ BMDM (Fig. 4F). We investigated whether versican interacts with TLR2 or its CD14 co-receptor. Immunoprecipitation of lysates of LCM-incubated Raw264.7 macrophages with versican-specific antibody, but not a control antibody, co-precipitated TLR2 and CD14 but not TLR4 (Fig. 4G). The versican antibody did not precipitate TLR2 unless the macrophages were first incubated with LCM.

Metastasis is the result of a complex process involving invasion of adjacent tissues, intravasation, circulatory transport, arrest at a distant site, extravasation, growth, survival and neoangiogenesis[16]. BM-derived cells, such as macrophages[2] and hematopoietic progenitors[7], are important participants in this process but how they are mobilized and activated to support metastasis is unclear. Our results indicate that versican secretion by LLC cells is necessary for metastatic spread to lung, liver and adrenal gland, a process that depends on TLR2-mediated myeloid cell activation and TNF-α production. Versican is an aggregating chondroitin sulfate proteoglycan that accumulates both in tumor stroma and cancer cells[5,6]. Versican can bind hyaluronan (HA) and both versican and HA are highly expressed in non-small cell lung cancer (NSCLC), especially in advanced disease with high recurrence rate, while versican in normal lung is rather low[6]. Versican or fragments thereof enhance tumor cell migration, growth and angiogenesis, processes that are of direct relevance to metastasis[17]. Versican also binds to several adhesion molecules expressed by inflammatory cells and has pro-inflammatory activity[18]. A related ECM proteoglycan, biglycan, was reported to activate both TLR2 and TLR4[19], but our results indicate that the pro-inflammatory activities of versican rely on TLR2 but not TLR4. TLR2 recognizes Gram positive bacteria-derived lipoteichoic acid and lipoproteins[4]. This activity mainly depends on TLR2:TLR1 dimers[20], but the response to versican requires TLR6 and not TLR1 as a co-receptor. Although TLR2 and versican interact, it is not clear whether this interaction is direct or depends on a versican ligand, such as HA. Indeed, HA fragments can activate macrophages through TLR2[21] and HA accumulation and the enzyme that converts large HA polymers to smaller fragments, hyaluronidase, have been linked to metastasis[22].

TLR2 on host myeloid cells and their product TNF-α are important positive modulators of LLC metastatic behavior but neither protein influences primary tumor growth of SC-implanted LLC. It appears that TNF-α is one of the major pro-metastatic factors produced by host myeloid cells. TNF-α can suppress the apoptosis of cancer cells and stimulate their proliferation through NF-κB activation[23]. In addition, by increasing vascular permeability[24], TNF-α can enhance recruitment of leukocytes as well as intravasation and extravasation of cancer cells. We suggest that versican, its interaction with TLR2 and production of TNF-α by activated myeloid cells, provide potential points for anti-metastatic intervention.

Methods Summary

A detailed Methods section is available in the Supplementary Information that accompanies this manuscript. Briefly, LLC cells were injected via the tail vein or SC implanted into 6–7 week old mice at 2×10^5 to 2×10^6 cells/mouse to measure metastases to lung, liver or adrenal gland. Metastasis enhancing factors were purified from LLC conditioned medium by column chromatography and identified on a QSTAR XL qQTOF mass spectrometer. Factor activity was determined by the ability to induce IL-6 production by BMDM. Gene and protein expression were monitored by Q-PCR and immunoblot analysis, respectively. Tumors and their composition were analyzed by immunohistochemistry and indirect immunofluorescence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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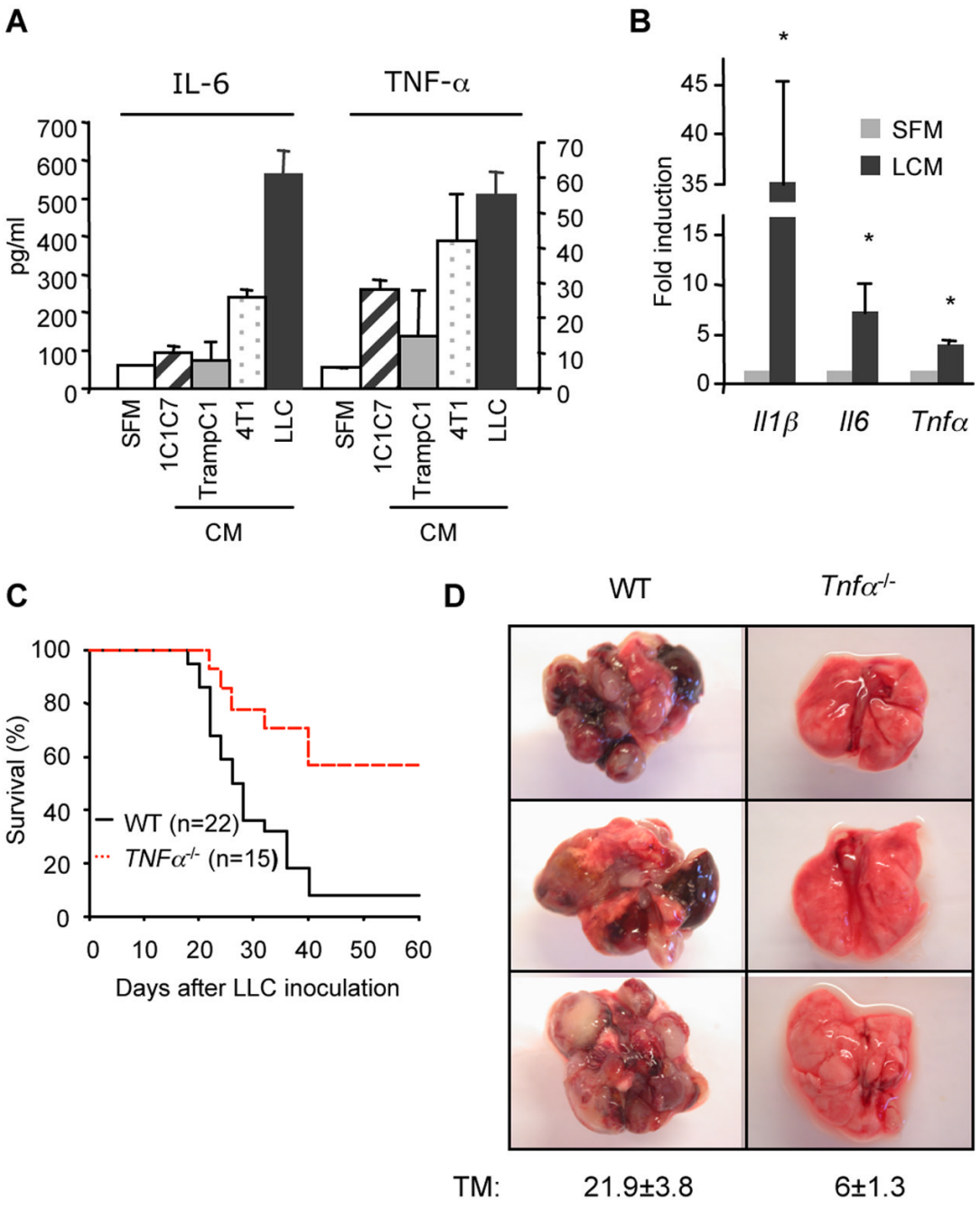

Figure 1. Metastatic carcinomas secrete factors that induce macrophage production of TNF-α needed for lung metastasis

A. BMDM were cultured with serum-free medium (SFM) or SFM conditioned by mouse carcinoma cells (CM) and cytokine production was measured (averages ± s.d., n=3). **B.** BMDM were cultured with SFM or LCM and cytokine mRNAs were measured by quantitative (Q)-RT-PCR. Fold-induction above SFM-treated cells was determined (averages ± s.d., n=3; *, $p<0.001$ by Student's *t* test). **C.** Survival of WT (n=22) and *Tnfα*$^{-/-}$ (n=15) mice inoculated with LLC (1×10^6 cells via the tail vein ($p<0.001$; Log-rank test for significance). **D.** Lungs of WT and *Tnfα*$^{-/-}$ mice 47 days after LLC inoculation (2×10^5 cells). Tumor multiplicities (TM) are shown underneath (averages ± s.d., n=11, $p<0.001$ by Student's *t* test).

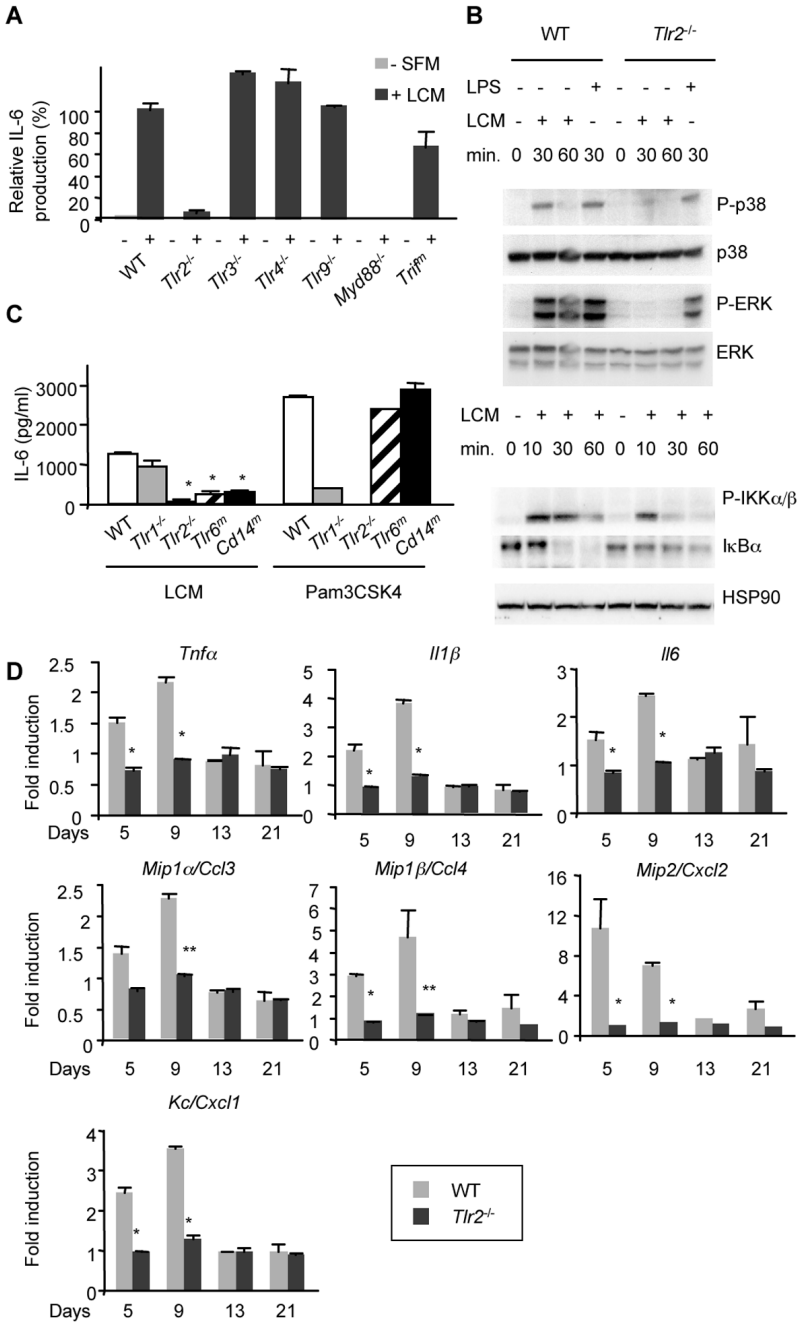

Figure 2. LLC-secreted factor activates TLR2 to induce lung inflammation

A. BMDM from indicated mouse strains were cultured with SFM or LCM and IL-6 production was measured (*m*: mutant allele; averages ± s.d., n=3, presented as % of WT LCM-stimulated value). **B.** BMDM were treated with LCM or LPS (100 ng ml^{-1}). Cell lysates were examined for kinase phosphorylation (P) and IκBα degradation by immunoblotting. Total ERK and HSP90 are loading controls. **C.** BMDM from indicated mouse strains were cultured with LCM or Pam3CSK4 (1 ng ml^{-1}), and IL-6 production was measured (averages ± s.d., n=4). **D.** RNA was extracted from lungs of WT or *Tlr2*$^{-/-}$ mice at indicated times after LLC inoculation (2×10^5 cells). mRNAs were quantitated as above and the amounts in non-inoculated WT or

Tlr2$^{-/-}$ lungs were given a value of 1.0 [averages ± s.e.m., n=3; *, $p<0.05$; **, $p<0.005$ (compared to WT) by Student's *t* test].

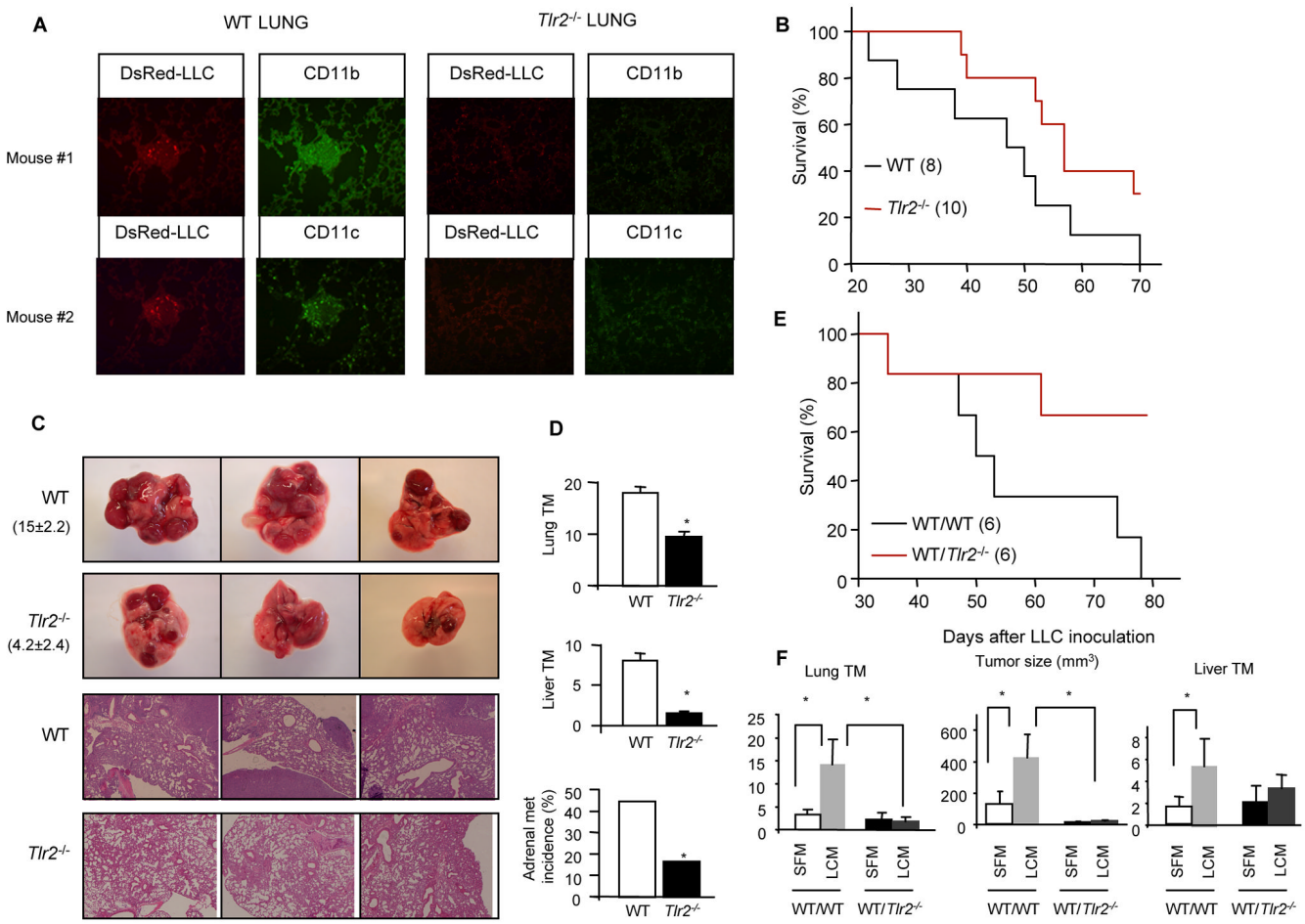

Figure 3. TLR2 is required for metastatic growth

A. WT and *Tlr2*$^{-/-}$ lungs were analyzed 9 days after inoculation of DsRed-LLC (2×10^5 cells) for DsRed and myeloid cells markers (CD11b, CD11c) using fluorescence microscopy (magnification: 200x). **B.** Survival of LLC inoculated (2×10^5 cells) WT (n=8) and *Tlr2*$^{-/-}$ (n=10) mice ($p<0.02$; Log-rank test for significance). **C.** Lungs and H&E-stained lung sections (magnification: 25×) 20 days after LLC inoculation. Tumor multiplicity is shown on the left (averages ± s.e.m., n=8, $p<0.001$ by Student's *t* test). **D.** Tumor multiplicities of lung and liver metastatic nodules and incidence of adrenal metastasis (met) 17 days after primary tumor removal (averages ± s.e.m., WT, n=9 and *Tlr2*$^{-/-}$, n=6; *, $p<0.05$ by Student's *t* test). **E.** Survival of WT/WT or WT/*Tlr2*$^{-/-}$ chimeric mice inoculated with LLC (2×10^5 cells) 6–7 weeks after BM reconstitution ($p<0.04$; Log-rank test for significance; n=6). **F.** Lung tumor multiplicity (left); size (middle); and liver tumor multiplicity (right) in chimeric mice, 27 (lung) or 48 (liver) days after LLC injection (2×10^5 cells) together with SFM or LCM (averages ± s.e.m., n=8; *, $p<0.05$ by Student's *t* test).

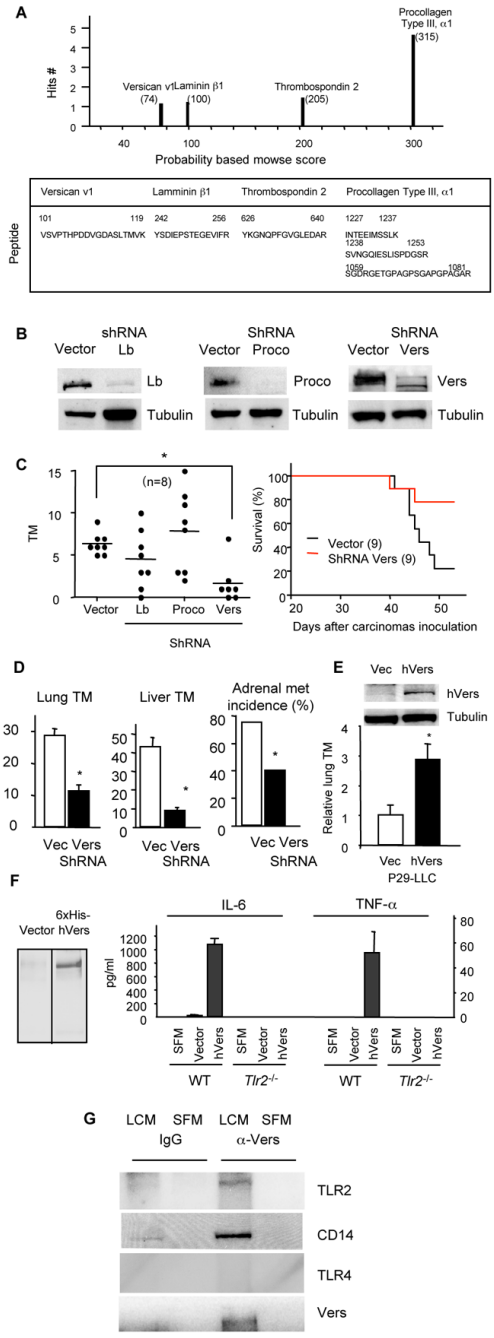

Figure 4. Versican is a TLR2 agonist and metastasis enhancing factor

A. Identification of candidate LCM macrophage activators by mass spectrometry. Probability based Mowse scores (upper) and tryptic peptides corresponding to the indicated proteins (lower). **B.** LLC were transduced with indicated shRNA lentiviruses. After selection, expression of the indicated proteins was analyzed. **C.** (left) LLC transduced with indicated lentiviruses were injected (2×10^5 cells) into mice (n=8). Lung tumor multiplicity was enumerated at 20 days (averages ± s.e.m., $p<0.001$ by Student's *t* test). (right) Survival of mice inoculated with LLC transduced with control or Vers shRNAs (n=9; *, $p<0.05$; Log-rank test for significance). **D.** Lung and liver tumor multiplicities and incidence of adrenal metastasis 17 days after primary tumor removal (averages ± s.e.m., n=8 and n=5 for mice injected with

control or Vers shRNA transduced cells, respectively; *, $p<0.05$ by Student's *t* test). **E.** (upper) Non-transfected and human Versican (hVers) transfected P29-LLC cells were analyzed for versican expression. (lower) hVers non-expressing and expressing P29-LLC cells were tail vein injected (n=7). After 27 days, lung metastatic nodules were enumerated (*, $p<0.05$ by Student's *t* test). **F.** (left) Silver staining of purified 6xHis-hVers. (right) BMDM were incubated without or with His-hVers for 20–24 hrs, and cytokine secretion was measured (averages ± s.d., n=3). **G.** Lysates of Raw264.7 cells incubated with LCM or SFM for 1 hr were immunoprecipitated with versican-specific or control antibody and analyzed by immunoblotting with the indicated antibodies.