

## LETTERS

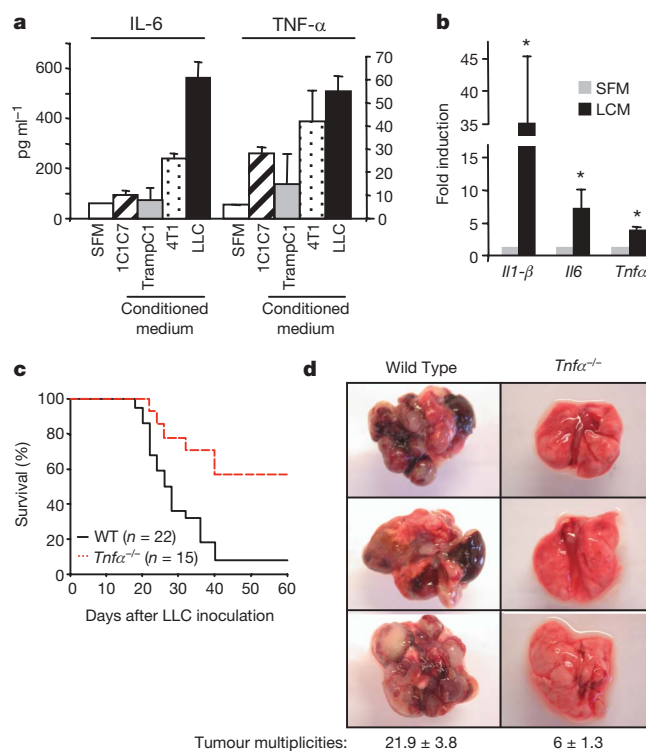
# Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis

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Metastatic progression depends on genetic alterations intrinsic to cancer cells as well as the inflammatory microenvironment of advanced tumours<sup>1,2</sup>. To understand how cancer cells affect the inflammatory microenvironment, we conducted a biochemical screen for macrophage-activating factors secreted by metastatic carcinomas. Here we show that, among the cell lines screened, Lewis lung carcinoma (LLC)<sup>3</sup> were the most potent macrophage activators leading to production of interleukin-6 (IL-6) and tumour-necrosis factor- $\alpha$  (TNF- $\alpha$ ) through activation of the Toll-like receptor (TLR) family members<sup>4</sup> TLR2 and TLR6. Both TNF- $\alpha$  and TLR2 were found to be required for LLC metastasis. Biochemical purification of LLC-conditioned medium (LCM) led to identification of the extracellular matrix proteoglycan versican, which is upregulated in many human tumours including lung cancer<sup>5,6</sup>, as a macrophage activator that acts through TLR2 and its co-receptors TLR6 and CD14. By activating TLR2:TLR6 complexes and inducing TNF- $\alpha$  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<sup>7</sup>, to generate an inflammatory microenvironment hospitable for metastatic growth.

Distant-site metastases are the leading cause of cancer-associated mortality. They depend on genetic and/or epigenetic alterations that are intrinsic to cancer cells, or extrinsic factors provided by the tumour microenvironment<sup>1</sup>. For instance, cytokines produced by inflammatory cells can enhance metastatogenesis by repressing the metastasis suppressor maspin within primary prostate carcinoma cells<sup>8</sup>. Furthermore, tumour progression and metastasis positively correlate with presence of infiltrates containing myeloid and lymphoid cells<sup>9</sup>. It has been shown that certain carcinomas secrete factors that upregulate fibronectin and recruit vascular endothelial growth factor receptor 1 (VEGFR1)-positive haematopoietic progenitors to sites of future metastatic growth, termed the pre-metastatic niche<sup>7</sup>. To examine whether cancer cells secrete factors that directly activate myeloid cells to produce tumour-promoting cytokines<sup>10</sup>, we collected serum-free conditioned medium from different cancer cell lines, derived mainly from C57BL6 mice, and applied it to bone-marrow-derived macrophages (BMDM), which were assayed for production of interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6 and TNF- $\alpha$ . 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. Conditioned medium from metastatic cells, especially LLC, induced higher amounts of IL-6 and TNF- $\alpha$  secretion than conditioned medium from non-metastatic cells (Fig. 1a). IL-1 $\beta$  secretion was

undetectable and the conditioned medium did not contain IL-6 or TNF- $\alpha$  (data not shown). LCM also induced expression of *Il1 $\beta$* , *Il6* and *Tnfx* messenger RNAs (mRNAs), whereas serum-free medium (SFM) and NIH3T3-conditioned medium 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 *Tnfx* and *Il6* knockout mice and wild-type (WT) controls. *Tnfx*<sup>-/-</sup> mice exhibited markedly ( $P < 0.001$ ) reduced



**Figure 1 | Metastatic carcinomas secrete factors that induce macrophage production of TNF- $\alpha$ , needed for lung metastasis.** **a**, BMDM were cultured with SFM or SFM conditioned by mouse carcinoma cells (conditioned medium), and cytokine production was measured (averages  $\pm$  s.d.,  $n = 3$ ). **b**, BMDM were cultured with SFM or LCM, and cytokine mRNAs were measured by quantitative PCR with reverse transcription. Fold-induction above SFM-treated cells was determined (averages  $\pm$  s.d.,  $n = 3$ ; \*,  $P < 0.001$  by Student's  $t$  test). **c**, Survival of WT ( $n = 22$ ) and *Tnfx*<sup>-/-</sup> ( $n = 15$ ) mice inoculated with LLC ( $1 \times 10^6$  cells through the tail vein ( $P < 0.001$ ; log-rank test for significance). **d**, Lungs of WT and *Tnfx*<sup>-/-</sup> mice 47 days after LLC inoculation ( $2 \times 10^5$  cells). Tumour multiplicities are shown underneath (averages  $\pm$  s.d.,  $n = 11$ ,  $P < 0.001$  by Student's  $t$ -test).

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mortality compared with WT mice after inoculation with  $1 \times 10^6$  LLC cells (Fig. 1c) and showed an even greater survival advantage when given a smaller LLC inoculum (Supplementary Fig. 1a). Similar differences were seen in lung tumour multiplicity (Fig. 1d). By contrast, there was little difference in survival of *Il6*<sup>-/-</sup> and WT mice inoculated with  $1 \times 10^6$  LLC cells (Supplementary Fig. 1b). Thus, TNF- $\alpha$  but not IL-6 is important for LLC metastasis.

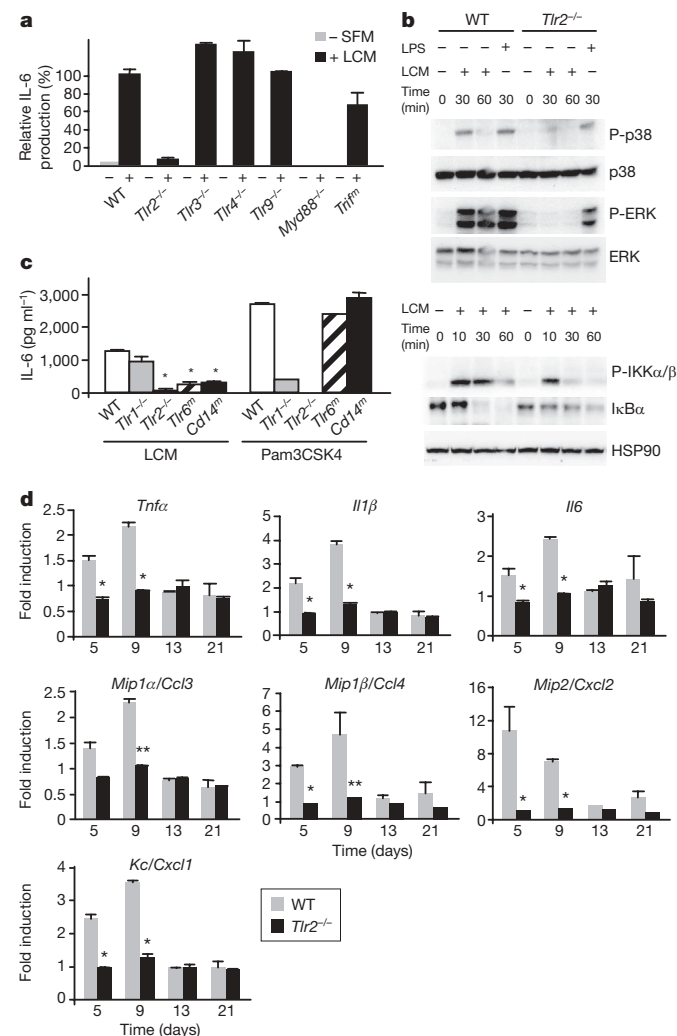
We explored the involvement of TLR family members in sensing LCM components. BMDM from mice deficient in TLR2, TLR3, TLR4 or TLR9 or their adaptor proteins, Myd88 and TRIF (which is inactivated by the *Lps2* mutation: *Trif*<sup>tm</sup>)<sup>4</sup>, were examined for production of IL-6, a convenient 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*<sup>-/-</sup> BMDM were also defective in LCM-induced *Il1 $\beta$*  and *Il6* mRNA expression and LCM did not induce anti-tumorigenic type I interferon genes

(Supplementary Fig. 2a), which are readily induced after TLR3 or TLR4 engagement<sup>11</sup>. TLR2 was also required for LCM-induced IL-6 and TNF- $\alpha$  secretion by alveolar macrophages, which produced tenfold more TNF- $\alpha$  than BMDMs (Supplementary Fig. 2b). TLR2 was required for optimal LCM-induced activation of mitogen-activated protein kinases and I $\kappa$ B kinase or I $\kappa$ B $\alpha$  degradation (Fig. 2b). TLR2 uses TLR1, TLR6 or CD14 as co-receptors<sup>12</sup>. 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 analogue<sup>4</sup>, 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).

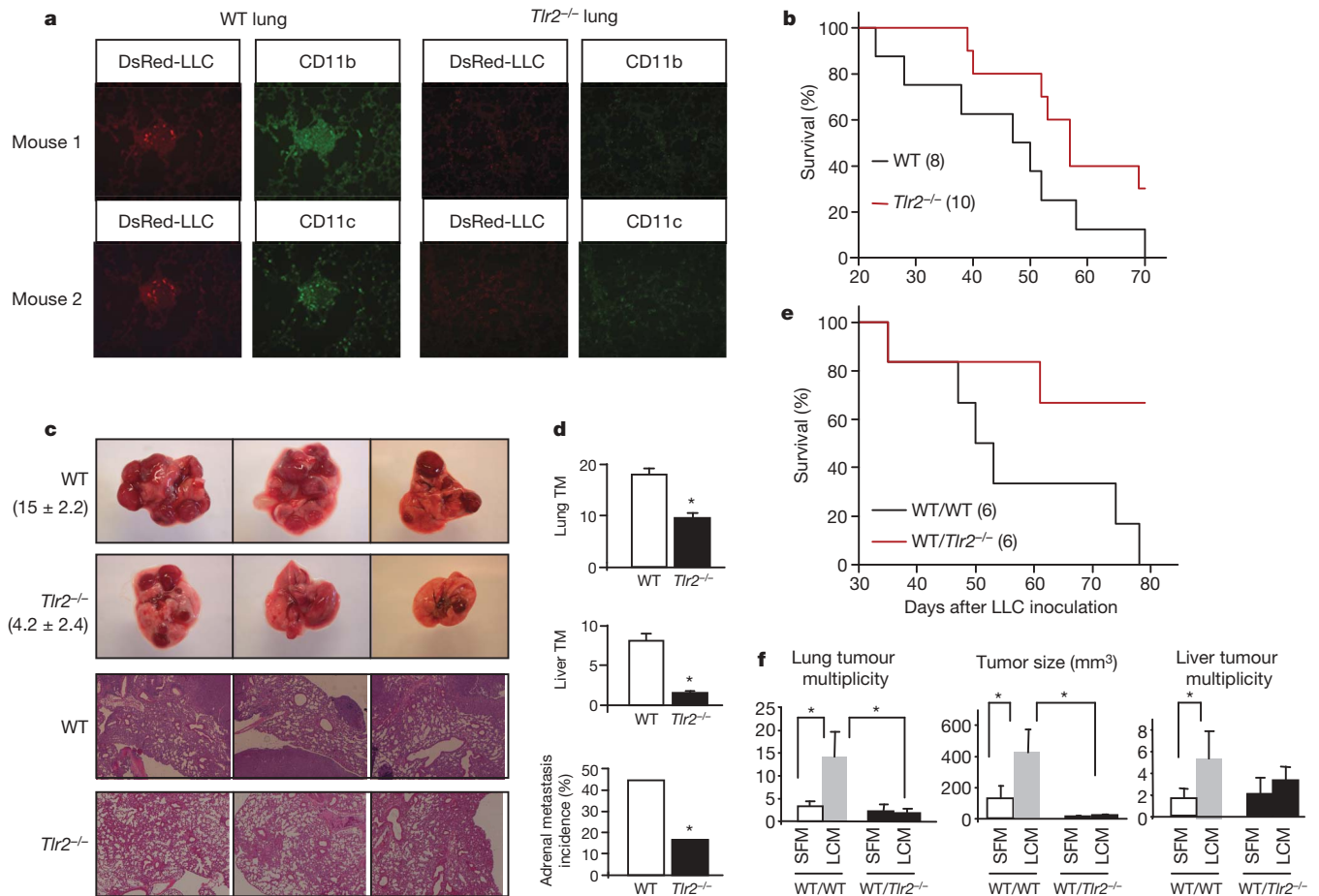
To examine the *in vivo* role of TLR2, we inoculated sex- and age-matched *Tlr2*<sup>-/-</sup> and WT mice with  $2 \times 10^5$  LLC cells through the tail vein and measured mRNAs encoding cytokines and chemokines in their lungs. LLC-induced lung inflammation has been previously described<sup>13</sup>, but its mechanism was unknown. *Tnfa*, *Il1 $\beta$* , *Il6* and inflammatory chemokine mRNAs were induced 5 days after LLC inoculation in WT lungs, peaking at 9 days after inoculation (Fig. 2d). None of these mRNAs was induced in lungs of *Tlr2*<sup>-/-</sup> mice, whose basal content of *Mip1 $\beta$ /Ccl4*, *Mip2/Cxcl2* and *Kc/Cxcl1* mRNAs was higher than WT lungs (Supplementary Fig. 3). In addition, *Tlr2*<sup>-/-</sup> and WT mice were subcutaneously inoculated with LLC cells and examined for lung macrophage infiltration and inflammatory cytokine gene expression days later. Although no difference was observed in primary subcutaneous tumour growth, macrophage infiltration and inflammatory cytokine gene expression were greatly reduced in *Tlr2*<sup>-/-</sup> mice compared with WT mice (Supplementary Fig. 4a-c).

To investigate whether TLR2 signalling contributes to LLC metastatogenesis, we inoculated age- and sex-matched *Tlr2*<sup>-/-</sup> and WT mice with dsRed-labelled LLC cells through the tail vein and examined their lungs for micrometastases. WT but not *Tlr2*<sup>-/-</sup> lungs showed small clusters of DsRed-LLC cells with adjacent CD11b<sup>+</sup> and CD11c<sup>+</sup> myeloid cells (Fig. 3a). We also detected a few cells that carried the CD3 antigen (T cells) in micro-metastases of WT lungs (data not shown). Importantly, *Tlr2*<sup>-/-</sup> mice exhibited significantly greater ( $P < 0.02$ ) survival than WT mice after LLC inoculation and their lungs contained fewer and smaller tumour nodules (Fig. 3b, c). Tumour nodules in WT mice contained more CD11b<sup>+</sup>/Gr1<sup>+</sup> inflammatory monocytes/myeloid suppressors and IL-10<sup>high</sup>/F4/80<sup>+</sup> M2 macrophages (Supplementary Fig. 5). *Tlr2*<sup>-/-</sup> mice exhibited significantly fewer lung and liver tumour nodules than WT mice and a lower incidence of adrenal gland metastasis after subcutaneous implantation of LLC cells (Fig. 3d). To investigate whether TLR2 acts in bone-marrow-derived cells, we examined survival of LLC-inoculated chimaeric mice. Mice reconstituted with *Tlr2*<sup>-/-</sup> bone marrow (WT/*Tlr2*<sup>-/-</sup>) exhibited markedly improved ( $P < 0.04$ ) survival compared with mice reconstituted with WT bone marrow (WT/WT) (Fig. 3e). WT/WT and WT/*Tlr2*<sup>-/-</sup> mice were also inoculated with  $2 \times 10^5$  LLC cells followed by intraperitoneal injections of LCM or SFM. Lung and liver tumour loads were significantly higher ( $P < 0.05$ ) in WT/WT mice receiving LCM than in 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*<sup>-/-</sup> 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, 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 (Supplementary Fig. 6). Most of the IL-6-inducing activity eluted in a few high molecular mass (greater than 400 kDa) fractions that contained several polypeptides larger than 200 kDa. These fractions were pooled, deglycosylated and



**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  $\pm$  s.d.,  $n = 3$ , presented as percentage of WT LCM-stimulated value). **b**, BMDM were treated with LCM or LPS (100 ng ml<sup>-1</sup>). Cell lysates were examined for kinase phosphorylation (P) and I $\kappa$ B $\alpha$  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<sup>-1</sup>), and IL-6 production was measured (averages  $\pm$  s.d.,  $n = 4$ ). **d**, RNA was extracted from lungs of WT or *Tlr2*<sup>-/-</sup> mice at indicated times after LLC inoculation ( $2 \times 10^5$  cells). mRNAs were quantified as above and the amounts in non-inoculated WT or *Tlr2*<sup>-/-</sup> lungs were given a value of 1.0 (averages  $\pm$  s.e.m.,  $n = 3$ ; \* $P < 0.05$ , \*\* $P < 0.005$  (compared with WT) by Student's *t*-test).



**Figure 3** | **TLR2 is required for metastatic growth.** **a**, WT and *Tlr2*<sup>-/-</sup> lungs were analysed 9 days after inoculation of DsRed-LLC ( $2 \times 10^5$  cells) for DsRed and myeloid cells markers (CD11b, CD11c) using fluorescence microscopy (magnification  $\times 200$ ). **b**, Survival of LLC inoculated ( $2 \times 10^5$  cells) WT ( $n = 8$ ) and *Tlr2*<sup>-/-</sup> ( $n = 10$ ) mice ( $P < 0.02$ ; log-rank test for significance). **c**, Lungs and haematoxylin-and-eosin-stained lung sections (magnification  $\times 25$ ) 20 days after LLC inoculation. Tumour multiplicity is shown on the left (averages  $\pm$  s.e.m.,  $n = 8$ ,  $P < 0.001$  by Student's *t*-test). **d**, Tumour multiplicities of lung and liver metastatic nodules and incidence

of adrenal metastasis 17 days after primary tumour removal (averages  $\pm$  s.e.m., WT  $n = 9$ , *Tlr2*<sup>-/-</sup>  $n = 6$ ;  $*P < 0.05$  by Student's *t*-test). **e**, Survival of WT/WT or WT/*Tlr2*<sup>-/-</sup> chimaeric mice inoculated with LLC ( $2 \times 10^5$  cells) 6–7 weeks after bone-marrow reconstitution ( $P < 0.04$ ; log-rank test for significance;  $n = 6$ ). **f**, Lung tumour multiplicity (left), size (middle) and liver tumour multiplicity (right) in chimaeric mice, 27 (lung) or 48 (liver) days after LLC injection ( $2 \times 10^5$  cells) together with SFM or LCM (averages  $\pm$  s.e.m.,  $n = 8$ ;  $*P < 0.05$  by Student's *t*-test).

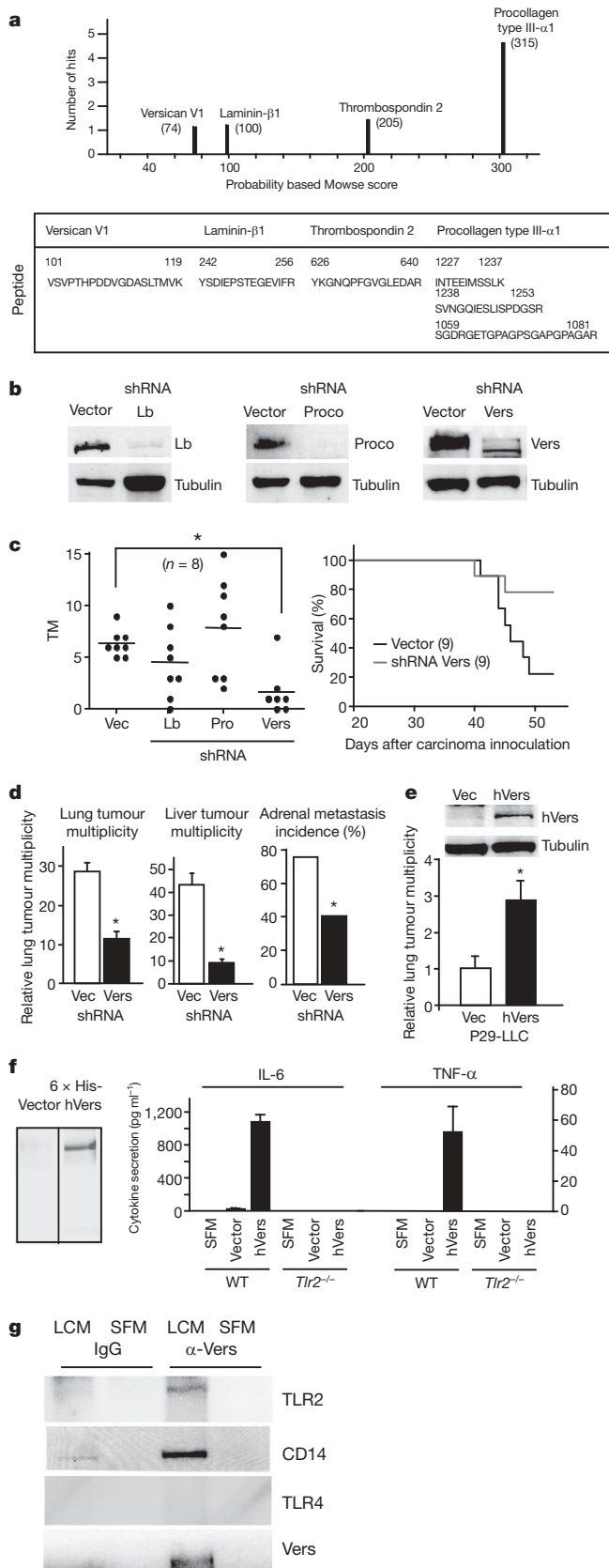
subjected to mass spectrometry, resulting in identification of several peptides derived from the extracellular matrix proteins: versican V1, laminin- $\beta$ 1, thrombospondin 2 and procollagen type III- $\alpha$ 1 (Fig. 4a). To examine which protein accounts for induction of inflammatory cytokines, we incubated LCM with individual neutralizing antibodies before BMDM stimulation and measurement of cytokine production. Incubation of LCM with antibodies to versican, laminin- $\beta$ 1 or procollagen type III- $\alpha$ 1, but not thrombospondin 2, reduced IL-6 and TNF- $\alpha$  production (Supplementary Fig. 7). A control antibody to HMGB1, an inflammatory mediator released by necrotic cells<sup>14</sup>, did not inhibit cytokine induction.

To investigate the role of these proteins in LCM-enhanced metastatogenesis, we generated stable LLC cell lines containing short hairpin RNA (shRNAs) specific to versican V1, laminin- $\beta$ 1 or procollagen III- $\alpha$ 1. 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 tumour nodules and their survival monitored. Silencing of versican V1 significantly reduced ( $P < 0.001$ ) tumour multiplicity, but silencing of laminin- $\beta$ 1 resulted only in a modest inhibition, whereas procollagen III- $\alpha$ 1 silencing slightly enhanced tumour multiplicity (Fig. 4c). Furthermore, silencing of versican V1 reduced lung nodule multiplicity fourfold (Supplementary Fig. 8a). Tumours isolated from mice inoculated with versican-silenced LLC cells displayed very low versican expression

(Supplementary Fig. 8b), whereas lung tumours from LLC-inoculated mice expressed more versican than normal lung (Supplementary 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 subcutaneous implantation model (Fig. 4d). To ascertain the proinflammatory and premetastatic functions of versican, we used the low metastatic LLC variant P29-LLC<sup>15</sup>. Conditioned medium from P29-LLC did not induce IL-6 in BMDM and contained very little versican (Supplementary Fig. 9). Ectopic expression of human versican in P29-LLC cells increased lung tumour multiplicity after tail vein injection (Fig. 4e).

To examine how versican activates macrophages, we produced His-tagged human versican V1 in LLC cells and purified it on an Ni-chelate column. The purified protein induced IL-6 and TNF- $\alpha$  production in WT but not *Tlr2*<sup>-/-</sup> 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



**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 analysed. **c**, Left: LLC transduced with indicated lentiviruses were injected ( $2 \times 10^5$  cells) into mice ( $n = 8$ ). Lung tumour multiplicity was enumerated at 20 days (averages  $\pm$  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 tumour multiplicities and incidence of adrenal metastasis 17 days after primary tumour removal (averages  $\pm$  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 analysed for versican expression. Lower: hVers non-expressing and expressing P29-LLC cells were injected through the tail vein ( $n = 7$ ). After 27 days, lung metastatic nodules were enumerated ( $*P < 0.05$  by Student's *t*-test). **f**, Left: silver staining of purified  $6 \times$  His-hVers. Right: BMDM were incubated without or with His-hVers for 20–24 h, and cytokine secretion was measured (averages  $\pm$  s.d.,  $n = 3$ ). **g**, Lysates of Raw264.7 cells incubated with LCM or SFM for 1 h were immunoprecipitated with versican-specific or control antibody and analysed by immunoblotting with the indicated antibodies.

depends on TLR2-mediated myeloid cell activation and TNF- $\alpha$  production. Versican is an aggregating chondroitin sulphate proteoglycan that accumulates both in tumour stroma and cancer cells<sup>5,6</sup>. It can bind hyaluronan, and both versican and hyaluronan are highly expressed in non-small-cell lung cancer (NSCLC), especially in advanced disease with high recurrence rate, whereas versican in normal lung is rather low<sup>6</sup>. Versican or fragments thereof enhance tumour cell migration, growth and angiogenesis, processes that are of direct relevance to metastasis<sup>17</sup>. Versican also binds to several adhesion molecules expressed by inflammatory cells and has pro-inflammatory activity<sup>18</sup>. A related extracellular matrix proteoglycan, biglycan, has been reported to activate both TLR2 and TLR4<sup>19</sup>, 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<sup>4</sup>. This activity mainly depends on TLR2:TLR1 dimers<sup>20</sup>, 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 hyaluronan. Indeed, hyaluronan fragments can activate macrophages through TLR2 (ref. 21), and hyaluronan accumulation and the enzyme that converts large hyaluronan polymers to smaller fragments, hyaluronidase, have been linked to metastasis<sup>22</sup>.

TLR2 on host myeloid cells and their product TNF- $\alpha$  are important positive modulators of LLC metastatic behaviour, although neither protein influences primary tumour growth of subcutaneously implanted LLC. It appears that TNF- $\alpha$  is one of the major pro-metastatic factors produced by host myeloid cells. TNF- $\alpha$  can suppress the apoptosis of cancer cells and stimulate their proliferation through NF- $\kappa$ B activation<sup>23</sup>. In addition, by increasing vascular permeability<sup>24</sup>, TNF- $\alpha$  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- $\alpha$  by activated myeloid cells provide potential points for anti-metastatic intervention.

**METHODS SUMMARY**

Detailed methods are given in Supplementary Information. Briefly, LLC cells were injected through the tail vein or subcutaneously implanted into 6- to 7-week-old mice at  $2 \times 10^5$  to  $2 \times 10^6$  cells per mouse to measure metastases to lung, liver or adrenal gland. Metastasis-enhancing factors were purified from LCM by column chromatography, and identified on a QSTAR XL qTOF mass spectrometer. Factor activity was determined by the ability to induce IL-6 production by BMDM. Gene and protein expression were monitored by quantitative PCR and immunoblot analysis, respectively. Tumours and their composition were analysed by immunohistochemistry and indirect immunofluorescence.

distant site, extravasation, growth, survival and neangiogenesis<sup>16</sup>. Bone-marrow-derived cells, such as macrophages<sup>2</sup> and haematopoietic progenitors<sup>7</sup>, are important participants in this process; however, 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

**Full Methods** and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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- Kopfstein, L. & Christofori, G. Metastasis: cell-autonomous mechanisms versus contributions by the tumor microenvironment. *Cell. Mol. Life Sci.* **63**, 449–468 (2006).
- Pollard, J. W. Tumour-educated macrophages promote tumour progression and metastasis. *Nature Rev. Cancer* **4**, 71–78 (2004).
- Weiss, L. & Ward, P. M. Lymphogenous and hematogenous metastasis of Lewis lung carcinoma in the mouse. *Int. J. Cancer* **40**, 570–574 (1987).
- Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124**, 783–801 (2006).
- Isogai, Z. *et al.* 2B1 antigen characteristically expressed on extracellular matrices of human malignant tumors is a large chondroitin sulfate proteoglycan, PG-M/versican. *Cancer Res.* **56**, 3902–3908 (1996).
- Pirinen, R. *et al.* Versican in nonsmall cell lung cancer: relation to hyaluronan, clinicopathologic factors, and prognosis. *Hum. Pathol.* **36**, 44–50 (2005).
- Kaplan, R. N. *et al.* VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* **438**, 820–827 (2005).
- Luo, J. L. *et al.* Nuclear cytokine-activated IKK $\alpha$  controls prostate cancer metastasis by repressing Maspin. *Nature* **446**, 690–694 (2007).
- DeNardo, D. G., Johansson, M. & Coussens, L. M. Immune cells as mediators of solid tumor metastasis. *Cancer Metastasis Rev.* **27**, 11–18 (2008).
- Lin, W. W. & Karin, M. A cytokine-mediated link between innate immunity, inflammation, and cancer. *J. Clin. Invest.* **117**, 1175–1183 (2007).
- Hsu, L. C. *et al.* The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4. *Nature* **428**, 341–345 (2004).
- Bas, S. *et al.* The proinflammatory cytokine response to *Chlamydia trachomatis* elementary bodies in human macrophages is partly mediated by a lipoprotein, the macrophage infectivity potentiator, through TLR2/TLR1/TLR6 and CD14. *J. Immunol.* **180**, 1158–1168 (2008).
- Hiratsuka, S. *et al.* MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. *Cancer Cell* **2**, 289–300 (2002).
- Scaffidi, P., Misteli, T. & Bianchi, M. E. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* **418**, 191–195 (2002).
- Nakanishi, H. *et al.* Structural differences between heparan sulphates of proteoglycan involved in the formation of basement membranes *in vivo* by Lewis-lung-carcinoma-derived cloned cells with different metastatic potentials. *Biochem. J.* **288**, 215–224 (1992).
- Fidler, I. J. The pathogenesis of cancer metastasis: the ‘seed and soil’ hypothesis revisited. *Nature Rev. Cancer* **3**, 453–458 (2003).
- Zheng, P. S. *et al.* Versican/Pg-M G3 domain promotes tumor growth and angiogenesis. *FASEB J.* **18**, 754–756 (2004).
- Wight, T. N. Versican: a versatile extracellular matrix proteoglycan in cell biology. *Curr. Opin. Cell Biol.* **14**, 617–623 (2002).
- Schaefer, L. *et al.* The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. *J. Clin. Invest.* **115**, 2223–2233 (2005).
- Buwitt-Beckmann, U. *et al.* Toll-like receptor 6-independent signaling by diacylated lipopeptides. *Eur. J. Immunol.* **35**, 282–289 (2005).
- Scheibner, K. A. *et al.* Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J. Immunol.* **177**, 1272–1281 (2006).
- Lokeshwar, V. B., Cerwinka, W. H., Isoyama, T. & Lokeshwar, B. L. HYAL1 hyaluronidase in prostate cancer: a tumor promoter and suppressor. *Cancer Res.* **65**, 7782–7789 (2005).
- Luo, J. L., Maeda, S., Hsu, L. C., Yagita, H. & Karin, M. Inhibition of NF-kappaB in cancer cells converts inflammation-induced tumor growth mediated by TNF $\alpha$  to TRAIL-mediated tumor regression. *Cancer Cell* **6**, 297–305 (2004).
- Tracey, K. J. *et al.* Shock and tissue injury induced by recombinant human cachectin. *Science* **234**, 470–474 (1986).

**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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**Author Contributions** S.K., H.T., W.-W.L. and M.K. conceived the project and planned experiments and analyses, which were performed by S.K., H.T. and W.-W.L. Y.K. and J.-L.L. helped with protein purification and tail-vein injection of cancer cells and tumour analysis, respectively. P.D. and S.G. analysed M2 macrophages and tissue versican content, and effect of TNF- $\alpha$  neutralization on lung metastasis. M.K. oversaw the entire project and wrote the manuscript with S.K.

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## METHODS

**Animal and cell culture.** *Tlr1*<sup>-/-</sup>, *Tlr2*<sup>-/-</sup>, *Tlr3*<sup>-/-</sup>, *Tlr4*<sup>-/-</sup>, *Tlr9*<sup>-/-</sup>, *Myd88*<sup>-/-</sup> and *Trif*<sup>m</sup>, *Tlr6*<sup>m</sup> and *Cd14*<sup>m</sup> (other names: *Lps*, *Insouciant* and *Heedless*, respectively) mutant mice were provided by S. Akira (Osaka University) and B. Beutler (The Scripps Research Institute). *Tnfr*<sup>-/-</sup> and *Il6*<sup>-/-</sup> mice were from the Jackson Laboratory. All strains used in metastasis studies were backcrossed to C57BL6 mice more than ten times. Mice were maintained under specific pathogen-free conditions, and experimental protocols were approved by the University of California, San Diego Animal Care Program, following National Institutes of Health guidelines. LLC, 1C1C7, TrampC1 and 4T1 cells were from the American Type Culture Collection. DsRed-LLC cells were provided by R. Hoffman (Anti-Cancer, Inc.). The cells were cultured under standard conditions, except for collection of conditioned media, and were confirmed to be mycoplasma free.

**Metastasis assays.** Formation of lung and liver metastases by cancer cells inoculated through the tail vein was examined as described<sup>23</sup>. Briefly, subconfluent LLC cells were harvested and passed through a 40- $\mu$ m cell strainer, washed three times in PBS, re-suspended in PBS and inoculated at  $2 \times 10^5$  or  $1 \times 10^6$  cells per 7- to 8-week-old mouse through the tail vein. At designated time points, mice were killed, and their lungs were removed, weighed and histologically examined. Some mice were kept until death and survival data were obtained. Lung tumour nodules were microdissected using an 18G needle under a microscope for protein and RNA analysis. Alternatively, the entire tumour-bearing lung was used. Subcutaneous cancer cell implantation was as described<sup>25</sup>. Briefly,  $2 \times 10^6$  control or versican-silenced LLC cells were subcutaneously injected into the right flank. When necessary, the primary tumour was surgically removed using aseptic techniques at 3–4 weeks after inoculation, and lung, liver and adrenal gland metastases were analysed 2–3 weeks after removal of the primary tumour.

**Bone marrow transplantation.** C57BL6 mice were lethally irradiated (1050 rad) and transplanted with  $1 \times 10^6$  bone marrow cells from WT or *Tlr2*<sup>-/-</sup> mice.

**DsRed visualization, immunohistochemistry, histological analysis and flow cytometry.** Tissues were immediately frozen in OCT compound (Tissue-Tek) and post-fixed with acetone. Sections were mounted with Vectashield containing DAPI (4,6-diamidino-2-phenylindole), and observed under a fluorescent microscope (Zeiss) to detect DsRed-labelled cells. The sections were also incubated with FITC-labelled antibodies to CD11b, CD11c and CD3 (all from Pharmingen) to detect inflammatory cells. Histological analysis of M2 macrophages was conducted as described<sup>26</sup>. Briefly, tumour-bearing lungs were fixed in 10% neutral buffered formalin. Paraffin-embedded lung sections (5  $\mu$ m) were kept at 60 °C for 1 h, and then incubated at 94 °C for 40 min in Dako's antigen retrieval solution S2368. Slices were stained with primary antibodies against IL-10 (Santa Cruz Biotechnology) and F4/80 (Caltag) for 1 h at room temperature. A specific signal was detected using the appropriate Dako EnVision System, HPR (DAB) kit. CD11b<sup>+</sup>/Gr1<sup>+</sup> myeloid suppressors or inflammatory monocytes present in lungs were analysed by flow cytometry using CD11b-Alexa 647 and Gr1-PE-Cy7 (eBioscience) antibodies and an ACCURI flow cytometer.

**Conditioned media.** Conditioned medium was collected from cells incubated in SFM for 20 h and filtered through a 0.22- $\mu$ m filter. Conditioned medium samples were added to BMDM for 16–20 h, after which inflammatory cytokine gene expression or secretion were assayed. Conditioned medium (300  $\mu$ l) were injected intraperitoneally three times, once every three days into WT mice that had received WT or *Tlr2*<sup>-/-</sup> bone marrow transplants 6–7 weeks before being inoculated with LLC cells. After 27 days, mice were killed and lungs were removed and histologically analysed by staining sections with Harris haematoxylin and eosin.

**Analysis of gene expression.** Total tissue RNA was prepared using RNeasy kit (Qiagen). Quantitative PCR was performed as described<sup>23</sup>. Primer sequences for *Tnfr*, *Il1 $\beta$* , *Il6* and *Cph* and RT-PCR conditions were as previously described<sup>27</sup>.

Primer pairs for *Mip1 $\alpha$ /Ccl3*, *Mip1 $\beta$ /Ccl4*, *Mip2/Cxcl2* and *Kc/Cxcl1* were 5'-TCTGCCGGTTTCTCTTAGTCA-3'/5'-ACCATGACACTCTGCAACCA-3', 5'-CTGCCTCTTTGGTCCAGGAA-3'/5'-TTCTGTGCTCCAGGGTTCTC-3', 5'-CTAGCTGCCTGCCTCATTCTAC-3'/5'-CAACAGTGTACYYACGCAGACG-3', 5'-CTTGGGGACACCTTTTAGCA-3'/5'-GCTGGGATTACCTCAA-GAA-3'.

Cells and tumour tissues were lysed and analysed by SDS-polyacrylamide gel electrophoresis and immunoblotting<sup>23</sup> with antibodies to p38, I $\kappa$ B $\alpha$ , procollagen III- $\alpha$ 1, laminin- $\beta$ 1, tubulin (all from Santa Cruz Biotechnology), ERK, P-ERK, P-p38, P-IKK $\alpha$ / $\beta$  (all from Cell Signalling, Inc.), versican V1 (Abcam), actin (Sigma) and hsp90 (Pharmingen). IL-6 and TNF- $\alpha$  were measured by enzyme-linked immunosorbent assay (R&D and Pharmingen).

**Lentiviral and retroviral transduction.** siRNAs to mouse versican V1, laminin- $\beta$ 1 and procollagen III- $\alpha$ 1 mRNAs were generated as described<sup>8</sup>, cloned into pLSLPw, provided by I. Verma (The Salk Institute), and transfected into 293T cells. siRNA sequences for indicated genes were as follows: versican V1, GTACACAGTTGATGAAATA and TATTTTCATCAACTGTGTAC; laminin- $\beta$ 1, ACAGATACTTCGCCTACGA and TCGTAGGCGGAAGTATCTGT; procollagen III- $\alpha$ 1, CCAGAACCATGTCAAATAT and ATATTTGACATGGTTCTGG. Human versican V1 vector was provided by D. Zimmermann (University of Zurich) and transfected into GP2-293T cells (Clontech). Lentivirus and retrovirus stocks were prepared as described<sup>8</sup>. Virus-containing supernatants were incubated with LLC cells for 2 days with polybrene, and infected cells were selected in 5  $\mu$ g ml<sup>-1</sup> puromycin (Invitrogen) for shRNA or 10  $\mu$ g ml<sup>-1</sup> Zeocin (Invitrogen) for versican expression.

**Protein purification.** Protein purification was conducted using a ÄKTA FPLC (GE Healthcare) system. Initially, samples were loaded onto a Mono-Q HR 5/5 column (Amersham Bioscience) that was developed with a gradient of 0–1 M NaCl in 20 mM Tris-HCl pH 8.0 buffer (flow rate 1 ml min<sup>-1</sup>). Fractions possessing IL-6 inducing activity were collected for further analysis and separation on a HiLoadTM 16/60 SuperdexTM 200 gel filtration column (GE Healthcare) eluted with 20 mM Tris-150 mM NaCl pH 8.0 buffer. Molecular masses were estimated by calibrating the column with a molecular mass marker kit (Sigma). Active fractions were pooled and analysed by SDS-polyacrylamide gel electrophoresis and mass spectrometry.

**Mass spectrometry.** Samples were separated on a 1D NuPage 4–12% Bis-Tris gradient gel (Invitrogen) in MOPS buffer (Invitrogen) and stained with GelCode Blue. Protein bands were excised and in-gel digested with trypsin. The resulting peptides were extracted and separated on an LC Packings liquid chromatographic system equipped with a 75- $\mu$ m C18 column at a flow rate of 300 nl min<sup>-1</sup>. The column was interfaced through a nanospray source directly into a QSTAR XL QTOF mass spectrometer. A 1-s survey scan was followed by 3-s product ion scans for the two most abundant parent ions. The resulting mass spectrometry data were analysed using MASCOT searching of the Swiss Prot database. The MASCOT search was performed allowing one miscleavage and common modifications of methionine oxidation and amino-terminal pyroglutamic acid.

**Statistical analyses.** Results are expressed as means  $\pm$  s.e.m. or s.d. Data were analysed by Student's *t*-test and two-way analysis of variance using the GraphPad Prism statistical program. *P* values < 0.05 were considered significant. Error bars depict s.e.m. or s.d.

- O'Reilly, M. S. et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* **79**, 315–328 (1994).
- Urosevic, M., Kamarashev, J., Burg, G. & Dummer, R. Primary cutaneous CD8<sup>+</sup> and CD56<sup>+</sup> T-cell lymphomas express HLA-G and killer-cell inhibitory ligand, ILT2. *Blood* **103**, 1796–1798 (2004).
- Eckmann, L., Fierer, J. & Kagnoff, M. F. Genetically resistant (Ityr) and susceptible (Itys) congenic mouse strains show similar cytokine responses following infection with *Salmonella dublin*. *J. Immunol.* **156**, 2894–2900 (1996).