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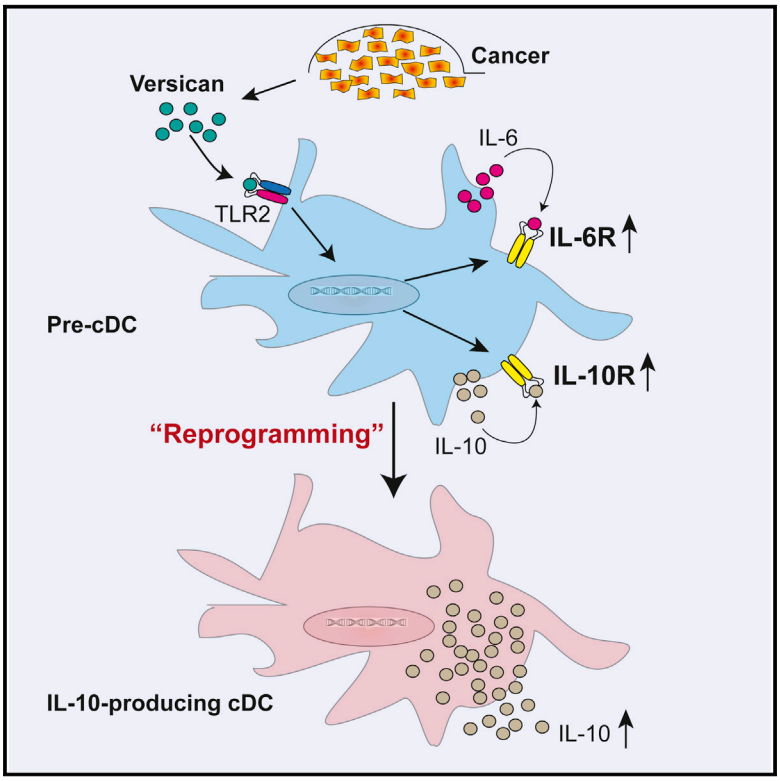
Toll-like Receptor 2 Activation Promotes Tumor Dendritic Cell Dysfunction by Regulating IL-6 and IL-10 Receptor Signaling

Graphical Abstract



Highlights

- Tumor-derived versican induces DC dysfunction through TLR2
- TLR2 ligation sensitizes DCs to IL-6/IL-10 by increasing IL-6R/IL-10R expression
- IL-10 and IL-6 reprograms sensitized DCs into immunosuppressive IL-10-producing DCs
- TLR2 blockade inhibits tumor DC dysfunction and improves immunotherapy

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In Brief

Although dendritic cell (DC) dysfunction is a well-recognized consequence of cancer-associated inflammation that contributes to immune evasion, the mechanisms that drive this process remain elusive. Here, Tang et al. show the critical importance of tumor-derived TLR2 ligands in the generation of immunosuppressive IL-10-producing human and mouse DCs.

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Toll-like Receptor 2 Activation Promotes Tumor Dendritic Cell Dysfunction by Regulating IL-6 and IL-10 Receptor Signaling

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SUMMARY

Although dendritic cell (DC) dysfunction in cancer is a well-recognized consequence of cancer-associated inflammation that contributes to immune evasion, the mechanisms that drive this process remain elusive. Here, we show the critical importance of tumor-derived TLR2 ligands in the generation of immunosuppressive IL-10-producing human and mouse DCs. TLR2 ligation induced two parallel synergistic processes that converged to activate STAT3: stimulation of autocrine IL-6 and IL-10 and upregulation of their respective cell surface receptors, which lowered the STAT3 activation threshold. We identified versican as a soluble tumor-derived factor that activates TLR2 in DCs. TLR2 blockade in vivo improved intra-tumor DC immunogenicity and enhanced the efficacy of immunotherapy. Our findings provide a basis for understanding the molecular mechanisms of DC dysfunction in cancer and identify TLR2 as a relevant therapeutic target to improve cancer immunotherapy.

INTRODUCTION

Inflammation is an integral feature of many cancers (Hanahan and Weinberg, 2011). Infiltrating leukocytes, mesenchymal stromal cells, extracellular matrix (ECM), and various cytokines and growth factors create a complex dynamic microenvironment that influences cancer growth, metastases, and prognosis (Coussens et al., 2013; Galon et al., 2006). The drivers of inflammation remain poorly understood, although evidence points to the importance of endogenous "danger signals" released from stressed and dying cancer cells and components of the ECM that activate pattern recognition receptors—such as the Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors—and downstream signaling cascades (Chen and Nuñez, 2010; Iwasaki and Medzhitov, 2010). In addition, TLR agonists produced by commensal bacteria in the gut have been shown recently to enhance the cytotoxic activity of tumor-associated myeloid cells during chemotherapy (Iida et al., 2013).

Dendritic cells (DCs) specialize in antigen capture, processing, and presentation and provide a key link between innate and adaptive immunity (Steinman, 2012). DCs prime naive tumor-antigen-specific $CD8^+$ T cells in lymphoid tissues, which results in the generation of cytotoxic T lymphocytes (CTLs) that migrate via the circulation into the tumor. In addition, recent reports have shown that tumor DCs stimulate naive T cells and CTL in situ, which appeared essential for the control of tumor growth. Indeed, the cancer microenvironment has been equated to a lymphoid organ (Zitvogel and Kroemer, 2014). Cancer impairs the function of DCs, which contributes to ineffective anti-tumor CTL responses (Gabrilovich et al., 2012). DC dysfunction in cancer is strongly associated with elevated expression levels of cytokines including interleukin (IL)-6 and IL-10, reduced expression of IL-12, and activation of signal transducer and activator of transcription 3 (STAT3) (Gabrilovich, 2004; Yu et al., 2007). Cancers may also fail to support the generation or recruitment of DC subpopulations that are known to have superior antigen cross-presentation activity, such as $CD103^+$ DCs (Broz et al., 2014). Efforts to further define these mechanisms and their relationship to cancer-associated inflammation have been hampered, however, by the poor understanding of the origin of tumor DCs and their evolution within the tumor microenvironment.

Common DC precursors (CDP), a defined bone marrow progenitor dedicated to the DC lineage (Guilliams et al., 2014; Merad et al., 2013; Naik et al., 2007; Onai et al., 2007, 2013), produce circulating pre-conventional DCs (pre-cDCs) and plasmacytoid DCs (pDCs) that are committed to become DCs in lymphoid and non-lymphoid tissues in the mouse (Diao et al., 2004, 2006; Liu et al., 2009; Meredith et al., 2012; Naik et al., 2006; Satpathy et al., 2012; Schlitzer et al., 2015). A parallel pathway exists in the human (Breton et al., 2015; Lee et al., 2015). We reported that tumors recruit circulating pre-cDCs through a CCL3-dependent mechanism where they generate normal immunostimulatory cDCs that undergo several rounds of cell division (Diao et al., 2010). Under the influence of the cancer milieu, some cells differentiated into immunosuppressive DCs and macrophages (Diao et al., 2011, 2012). IL-6 appeared to regulate this process, although the proximal signals remain unknown. Here, we report the fundamental importance

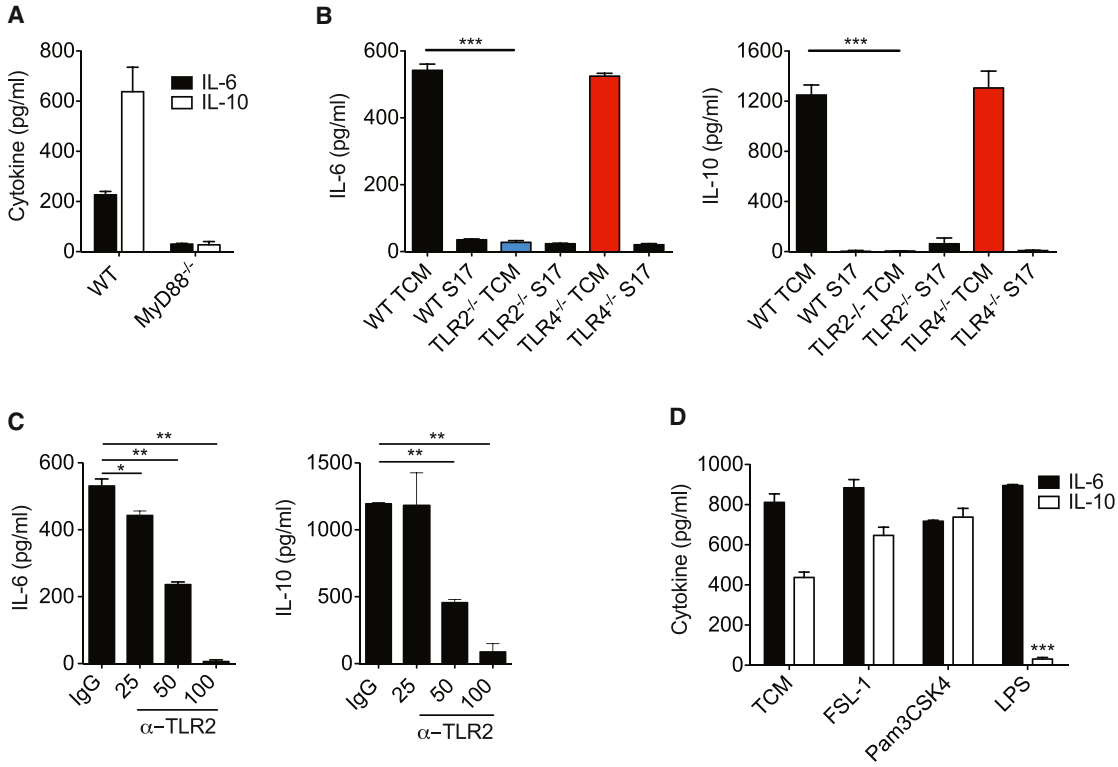

Figure 1. Tumors Stimulate Autocrine IL-6 and IL-10 in DCs through TLR2

(A) IL-6 and IL-10 production, as measured by ELISA, from WT and Myd88$^{-/-}$ mice pre-cDCs stimulated with TCM for 24 hr.

(B) IL-6 and IL-10 production by WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice pre-cDCs incubated in TCM and S17 conditioned medium (S17) for 24 hr.

(C) IL-6 and IL-10 production by WT pre-cDCs cultured in TCM with neutralizing TLR2 mAb or immunoglobulin G1 (IgG1) control (25–100 ng/ml) for 24 hr.

(D) IL-6 and IL-10 production by WT pre-cDCs cultured in TCM, FSL-1 (100 ng/ml), Pam3CSK4 (100 ng/ml), and LPS (1 μg/ml) for 24 hr.

Results are displayed as mean ± SEM and are representative of more than three independent experiments. *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$, determined by Student's t test.

of TLR2 signaling in driving the differentiation of immunosuppressive DCs in cancer.

RESULTS

Tumors Stimulate Autocrine Production of IL-6 and IL-10 in Pre-cDCs through TLR2

Cancer has no apparent adverse effect on circulating pre-cDCs (Diao et al., 2010), at least for tumors <1 cm in diameter, suggesting that tumor DC dysfunction is initiated by direct exposure to the tumor microenvironment. We hypothesized that the immediate cytokine response of pre-cDCs to tumor-conditioned medium (TCM) might serve as a screening tool to investigate whether TLR signaling regulates cDC differentiation in cancer. All TLRs, except TLR3, signal through the adaptor molecule myeloid-differentiation factor 88 (MyD88) (Yamamoto et al., 2003). We first cultured wild-type (WT) and MyD88-deficient spleen pre-cDCs in TCM generated from Lewis lung carcinoma (LLC), and measured IL-6 and IL-10 concentrations in culture supernatants 24 hr later. WT pre-cDCs, but not MyD88$^{-/-}$ pre-cDCs, produced both IL-6 and IL-10 (Figure 1A); neither cytokine was detectable in TCM alone (data not shown). We obtained similar results using TCM generated from B16 melanoma.

TLR2 and TLR4 are well-recognized receptors for danger-associated molecular patterns (DAMPS) in the tumor microenvironment (Apetoh et al., 2007). Therefore, we investigated the response of spleen pre-cDCs from TLR2$^{-/-}$ and TLR4$^{-/-}$ mice to TCM. TCM failed to stimulate IL-6 and IL-10 production in TLR2$^{-/-}$ pre-cDCs, whereas pre-cDCs from TLR4$^{-/-}$ mice produced both cytokines at levels similar to that of WT control (Figure 1B). As expected, control S17 fibroblast-conditioned medium (S17) stimulated low levels of IL-6 and IL-10 in WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ pre-cDCs. We considered whether a cell-intrinsic defect accounted for the inability of TCM to stimulate IL-6 and IL-10 in TLR2$^{-/-}$ pre-cDCs; however, adding neutralizing anti-TLR2 antibodies to TCM also inhibited cytokine production in WT pre-cDCs in a dose-dependent manner (Figure 1C).

TLR2 forms heterodimers between TLR1 or TLR6 to recognize diacylated (e.g., FSL-1) or triacylated (Pam3CSK4) lipoproteins, respectively. Both FSL-1 and Pam3CSK4 mimicked TCM in their ability to stimulate IL-6 and IL-10 by WT pre-cDCs (Figure 1D), whereas lipopolysaccharide (LPS) only stimulated IL-6. Collectively, these findings indicated that tumor-derived factors stimulate production of autocrine IL-6 and IL-10 in pre-cDCs through a TLR2/MyD88 signaling pathway.

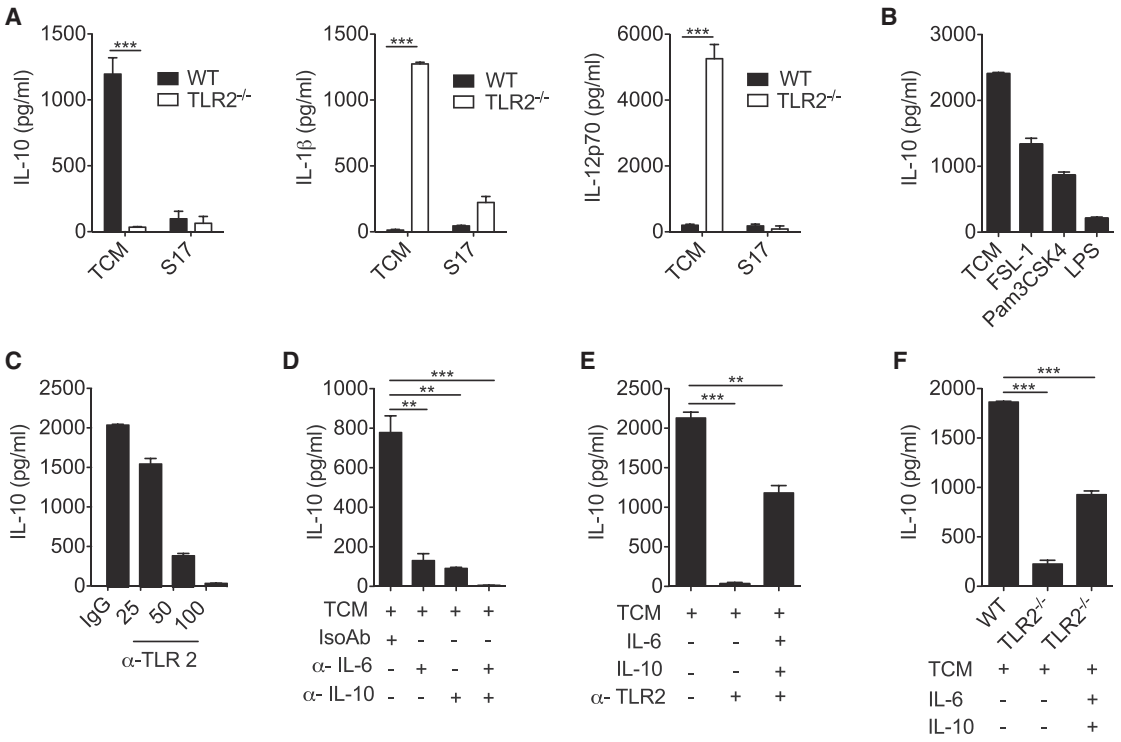

Figure 2. Tumors Promote Differentiation of IL-10-Producing DCs through TLR2

(A) Production of IL-10, IL-1β, and IL-12p70 by WT and TLR2$^{-/-}$ pre-cDCs pretreated with TCM or S17 for 72 hr, then recovered, washed, and recultured for 18 hr in fresh complete medium containing LPS (10 ng/ml).

(B) IL-10 production by WT pre-cDCs pretreated with TCM, FSL-1, Pam3CSK4, and LPS for 72 hr.

(C) IL-10 production by WT pre-cDCs pretreated with TCM with or without added anti-TLR2 antibodies (25–100 ng/ml) for 72 hr.

(D) IL-10 production by WT pre-cDCs pretreated with TCM in the presence or absence of blocking antibodies to IL-6 (10 μg/ml) and IL-10 (10 μg/ml) for 72 hr.

(E) IL-10 production by WT pre-cDCs pretreated with TCM in the presence or absence of blocking antibodies to TLR2 (100 ng/ml) and exogenous IL-6 and IL-10 (both 1 μg/ml) in the primary culture for 72 hr.

(F) IL-10 production by WT and TLR2$^{-/-}$ pre-cDCs pretreated with TCM in the presence or absence of exogenous IL-6 and IL-10 for 72 hr.

Results are displayed as mean ± SEM. **p < 0.005; ***p < 0.001. See also Figure S1.

Autocrine IL-6 and IL-10 Promote Differentiation of IL-10-Producing DCs

Previous studies have shown that, in contrast with spleen DCs, tumor DCs preferentially produce IL-10 rather than IL-1β and IL-12 when stimulated with LPS. To determine whether TCM induces this aberrant response in pre-cDCs, we extended the culture period to 72 hr, recovered and washed the cells extensively, and stimulated them with LPS for 18 hr. WT pre-cDCs pretreated with TCM, but not S17, produced large quantities of IL-10 (Figure 2A). TLR2 deficiency blocked this response and increased their capacity to produce both IL-1β and IL-12p70. Pre-cDCs pretreated with FSL-1 and Pam3CSK4 also produced high amounts of IL-10, as compared to those pre-treated with LPS, whereas adding anti-TLR2 antibodies to TCM blocked IL-10 production (Figures 2B and 2C).

Next, we investigated the role of TCM-induced autocrine IL-10 and IL-6 in this process. Neutralizing anti-IL-6 or anti-IL-10 antibodies decreased the capacity of TCM to induce IL-10-producing cDCs from WT pre-cDCs, and blocking both cytokines was synergistic (Figure 2D). These findings suggested that paracrine stimulation with IL-10 and IL-6 was sufficient; however, exogenous IL-6 and IL-10 failed to restore IL-10 production completely when TLR2 was blocked with neutralizing antibodies or was genetically deficient (Figures 2E and 2F), even though the concentration of these cytokines was 2- to 3-fold higher than those produced by WT pre-cDCs in TCM. Furthermore, adding IL-6 and IL-10 to WT pre-cDCs cultured in Flt3 ligand or S17 medium for 72 hr failed to generate IL-10-producing DCs (Figures S1A and S1B). Collectively, these findings suggested that TLR2 signaling engages mechanisms beyond stimulating autocrine IL-6 and IL-10 to promote DC dysfunction.

TLR2 Ligation Sensitizes DCs to IL-6 and IL-10 Stimulation

We hypothesized that TLR2 signaling might increase the sensitivity of pre-cDCs to IL-6 and IL-10. To explore this idea, we first measured the expression levels of IL-6Rα and IL-10Rα, the cytokine-binding component of these receptors (Kishimoto, 2005; Moore et al., 2001). As compared to unstimulated control, stimulation of WT pre-cDCs with TCM and FSL-1 increased cell-surface expression of both IL-6Rα and IL-10Rα, whereas stimulation with S17 and LPS reduced expression levels slightly (Figures 3A and 3B). Induction of IL-6Rα and IL-10Rα mRNA, as measured by quantitative real-time PCR, accompanied IL-6Rα

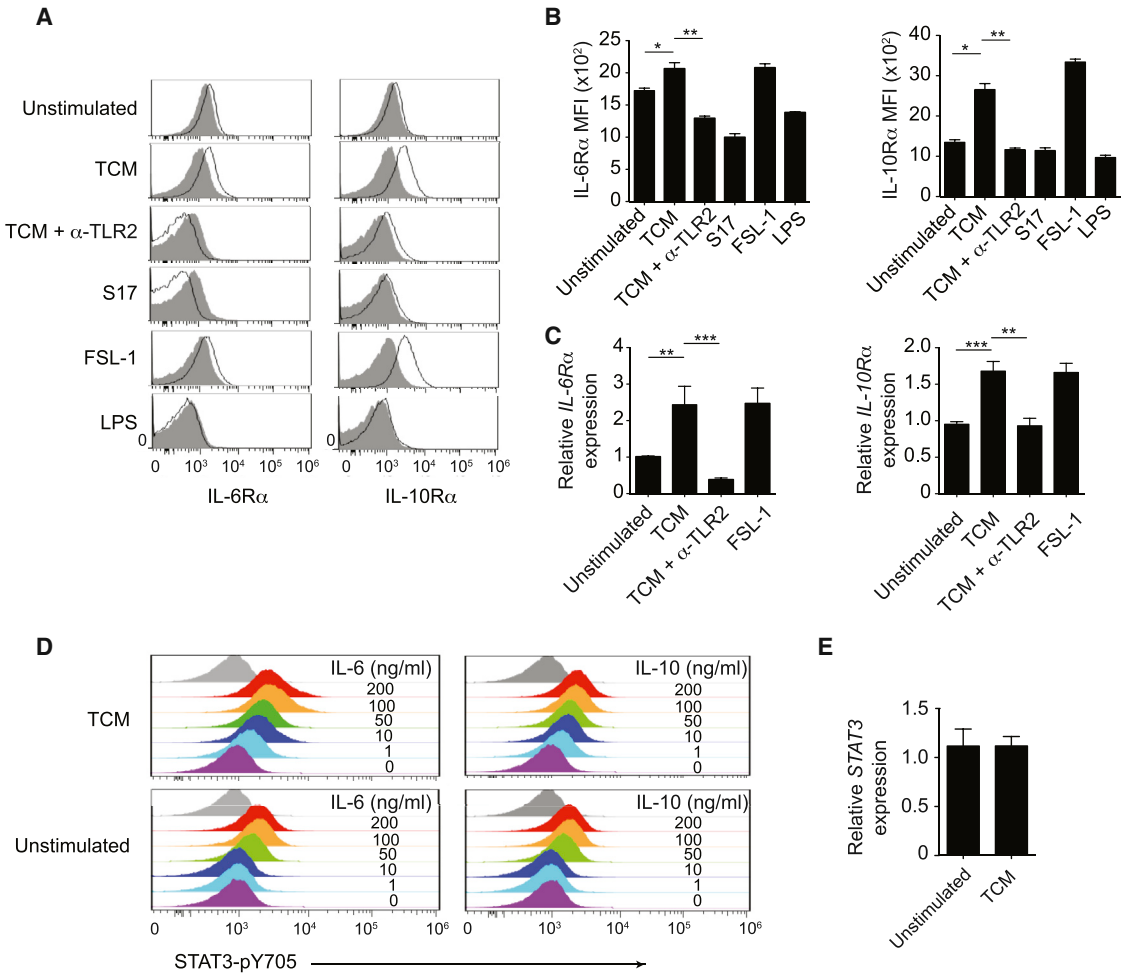

Figure 3. TLR2 Ligation Sensitizes DCs to IL-6 and IL-10 Stimulation

(A and B) Mean fluorescence intensity (MFI) of surface IL-6Rα or IL-10Rα expression by unstimulated DCs or DCs cultured in TCM, with or without neutralizing anti-TLR2 antibodies (100 ng/ml), S17, FSL-1, and LPS for 18 hr. Results are displayed as mean ± SEM. *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$ by Student's t test.

(C) Real-time PCR analysis of IL-6Rα (*il6ra*) and IL-10Rα (*il10ra*) mRNA in DCs. All values were normalized to *gapdh* expression, and fold changes are relative to unstimulated control. Results are displayed as mean ± SEM. **$p < 0.005$; ***$p < 0.001$ by Student's t test.

(D) Intracellular expression of phosphorylated STAT3 (STAT3-pY705) by DCs that were pretreated with TCM or left untreated for 18 hr then stimulated with the indicated concentrations of IL-6 and IL-10 for 30 min.

(E) STAT3 mRNA expression determined by real-time PCR of DCs cultured for 18 hr with or without TCM. Results are displayed as mean ± SEM.

See also Figures S2 and S3.

and IL-10Rα upregulation (Figure 3C). Adding anti-TLR2 antibodies to TCM blocked these responses completely.

Since TCM stimulated secretion of IL-6 and IL-10, we speculated that IL-6Rα and IL-10Rα expression levels were being self-regulated by their respective cytokine. We found, however, that TCM continued to induce IL-6Rα and IL-10Rα mRNA and surface receptor expression in the presence of neutralizing antibodies to IL-6 and IL-10 (Figure S2), suggesting that TLR2 signaling increases IL-6Rα and IL-10Rα expression independently of its effect on autocrine IL-6 and IL-10 production and signaling.

STAT3 is the main downstream molecular target for IL-6R and IL-10R signaling and promotes IL-10 while inhibiting IL-12 production in DCs (Kortylewski et al., 2009; Moore et al., 2001). To directly test the functional relevance of increased IL-6Rα and IL-10Rα expression, we assessed phosphorylated STAT3 (pSTAT3) levels in response to graded concentrations of IL-6 and IL-10. Spleen pre-cDCs from WT mice were cultured in TCM or control medium for 24 hr, recovered and rested for 4 hr, and then stimulated with IL-6 and IL-10 for 30 min. Before cytokine stimulation, pre-cDCs cultured in TCM and control medium exhibited similar basal levels of pSTAT3 (Figure 3D). The threshold IL-6 and IL-10 concentrations that induced pSTAT3 were about 50-fold lower for pre-cDCs cultured in TCM as compared to control (1 ng/ml versus 50 ng/ml). pSTAT3 expression peaked at IL-10 and IL-6 concentrations of 100 ng/ml for both unstimulated and TCM-pretreated DCs; however, at peak cytokine concentrations, TCM pre-treated DCs achieved higher

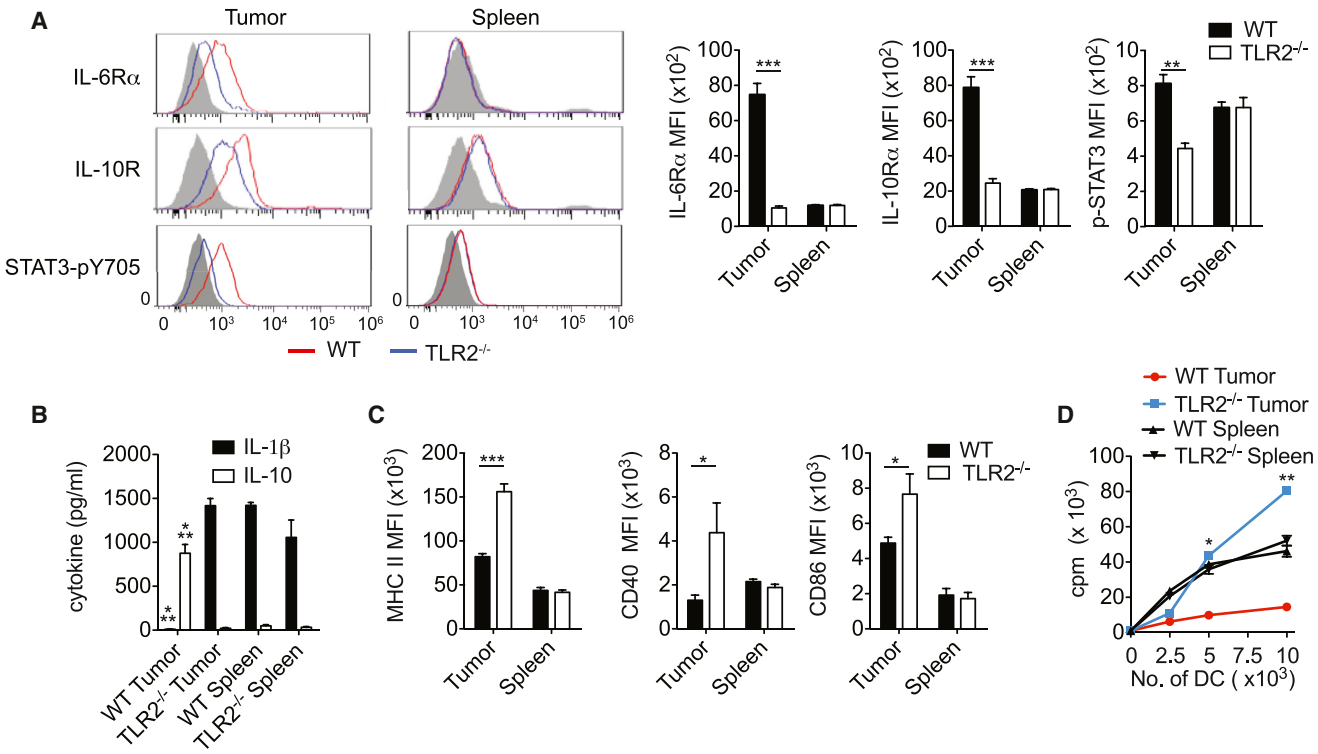

Figure 4. Superior Function of Tumor cDCs from TLR2$^{-/-}$ Mice

(A) Representative flow cytometric plots showing expression of IL-6Rα, IL-10Rα, and STAT3-pY705 by DCs sorted from B16 tumors and spleens of WT (red line) and TLR2$^{-/-}$ (blue line) mice. Bar graphs show MFI of IL-6Rα, IL-10Rα, and STAT3-pY705 by tumor DCs (n = 3–5 mice per group).

(B) Production of IL-10 and IL-1β by sorted tumor and spleen DCs after overnight culture in LPS (1 μg/ml).

(C) Flow cytometry for the indicated cell-surface molecules.

(D) Stimulation capacity of sorted tumor and spleen DCs from WT and TLR2$^{-/-}$ mice in mixed allogeneic lymphocyte reactions.

Results are expressed as mean counts per minute (cpm) × 10^3 ± SEM. *p < 0.05; **p < 0.005; ***p < 0.001, determined by Student's t test.

levels of pSTAT3 than control. STAT3 transcription levels in pre-cDCs pre-treated with TCM remained similar to that of control (Figure 3E), further supporting the hypothesis that the increased level of pSTAT3 resulted from increased IL-6 and IL-10 receptor signaling. Consistent with these findings, the increased expression levels of IL-6R and IL-10R in pre-cDCs pre-treated with FSL-1 reduced the concentrations of IL-6 and IL-10 needed to activate STAT3 (Figure S3). Collectively, these data indicate that TLR2 activation by tumor-derived factors promotes differentiation of IL-10-producing DCs by increasing the sensitivity of tumor DCs to IL-6 and IL-10 stimulation.

TLR2 Signaling Drives Tumor DC Dysfunction In Vivo

Next, we examined the influence of TLR2 on the expression levels of IL-6Rα, IL-10Rα, and pSTAT3 in cDCs isolated from spleen and tumors of mice bearing subcutaneous B16 melanoma. WT tumor cDCs displayed increased expression levels of both cytokine receptors and pSTAT3, as compared to tumor DCs from TLR2$^{-/-}$ mice (Figure 4A). Spleen DCs from tumor-bearing WT and TLR2$^{-/-}$ mice expressed similar low levels of IL-6Rα, IL-10Rα, and pSTAT3.

To determine whether these altered cytokine receptor and pSTAT3 expression levels correlate with DC function, we sorted cDCs from tumors and the spleen of tumor-bearing WT and TLR2$^{-/-}$ mice and compared their ability to produce IL-10 and IL-1β after LPS stimulation (Figure 4B). As expected, spleen DCs from both WT and TLR2$^{-/-}$ mice produced high and low amounts of IL-1β and IL-10, respectively (He et al., 2013). WT tumor DCs produced high amounts of IL-10 and no IL-1β, whereas tumor cDCs from TLR2$^{-/-}$ mice produced IL-10 and IL-1β at levels similar to that of spleen cDCs.

Tumor cDCs from TLR2$^{-/-}$ mice also displayed higher expression levels of cell-surface MHC II, CD40, and CD86 (Figure 4C) and stimulated proliferation of allogeneic lymphocytes more effectively than those from WT mice, consistent with a higher level of maturation or activation (Figure 4D). Collectively, these findings indicated that TLR2 deficiency inhibits the ability of the tumor milieu to upregulate IL-6Rα and IL-10Rα, activate STAT3, and impair the function of tumor cDCs. By contrast, spleen DCs in TLR2$^{-/-}$ mice remain similar to those in WT mice, which supports the view that DC dysfunction in B16-bearing mice occurs predominately in the cancer.

TLR2 Deficiency Improves T Cell Responses to Anti-cancer Vaccine

To test whether the improved function of tumor cDCs would enhance anti-tumor immunity, we compared the efficacy of an irradiated B16 melanoma vaccine engineered to express

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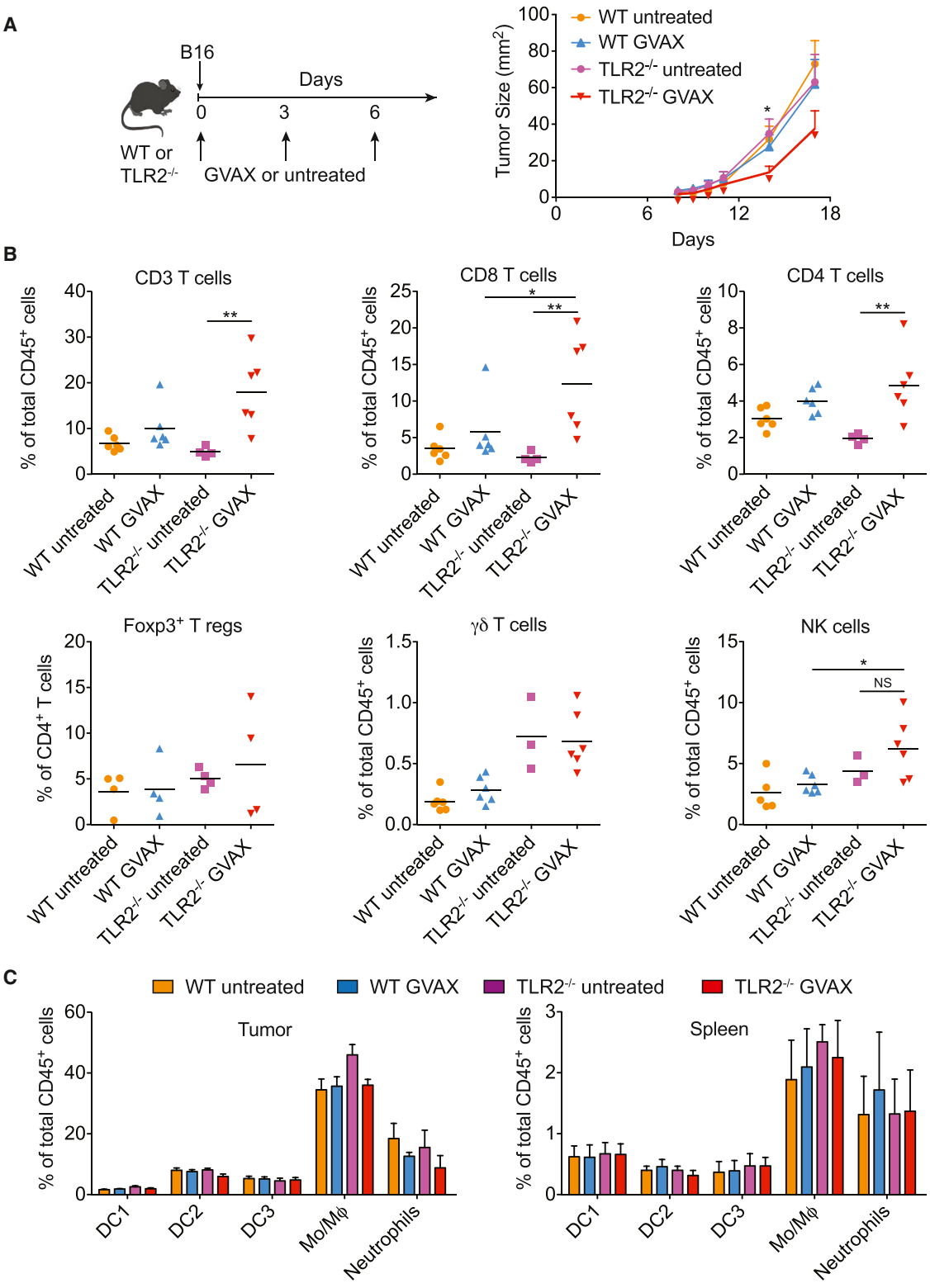

Figure 5. TLR2 Deficiency Improves Tumor Vaccination

(A) Relative growth of subcutaneous B16 melanoma in untreated and GVAX-treated WT and TLR2$^{-/-}$ mice. GVAX treatment schedule is indicated in the schematic. Results are displayed as mean ± SEM with 13 mice per group. *$p < 0.05$, by unpaired Mann-Whitney test.

(legend continued on next page)

granulocyte/macrophage colony-stimulating factor (GM-CSF) (GVAX) in WT and TLR2$^{-/-}$ mice bearing established B16 melanoma (Figure 5A). Tumor growth rates were similar in unvaccinated WT and TLR2$^{-/-}$ mice, consistent with the poor immunogenicity of B16 melanoma (Dranoff, 2012). In agreement with previous reports, GVAX alone had no effect on tumor growth in WT mice (van Elsas et al., 1999). By contrast, GVAX suppressed tumor growth significantly in TLR2$^{-/-}$ mice.

Flow cytometric analysis of the tumors revealed that GVAX immunization in TLR2$^{-/-}$ mice was associated with a significant increase in the frequency CD3$^+$ T cells, CD8$^+$ T cells, and CD4$^+$ T cells, whereas the frequencies of these populations were similar in vaccinated and unvaccinated WT mice. (Figure 5B). We found no difference in the frequencies of Foxp3$^+$ T regulatory cells (Tregs) and $\gamma\delta$ T cells after GVAX in WT or TLR2$^{-/-}$ mice. The frequency of natural killer (NK) cells was higher in vaccinated TLR2$^{-/-}$ mice than in WT mice. Analysis of the corresponding lymphoid populations in spleen showed no differences in the four groups of mice (data not shown), indicating that the absence of TLR2 signaling did not have a systemic effect in the lymphoid compartment.

We also analyzed tumor DC subsets and other myeloid populations. The frequency of intra-tumor CD103$^+$ cDCs, which have a proclivity for cross-presentation of tumor-derived antigens to T cells, can influence the efficacy of anti-tumor immune responses and tumor growth (Broz et al., 2014; Ruffell et al., 2014). Assessment of the frequency of CD11c^{hi}MHC II^{hi} cDC subpopulations in WT and TLR2$^{-/-}$ mice according to the gating schema—DC1 (CD103$^+$CD11b$^-$), DC2 (CD103$^-$CD11b$^+$CD64$^-$F4/80$^-$), and DC3 (CD103$^-$CD11b$^+$CD64$^+$F4/80$^+$)—showed no differences in tumors and spleen (spleen DC1s are CD8$^+$CD11b$^-$) (Figure 5C; Figure S4). pDCs exist in trace numbers in B16 melanoma and were not compared. Tumor-infiltrating macrophages and neutrophils can both support and suppress anti-tumor T cell responses (Gabrilovich et al., 2012). We detected no difference in the frequency of macrophages/monocytes (F4/80$^+$CD11b$^+$MHCII$^-$) and neutrophils (Ly6G$^+$). Notably, the frequency of myeloid-derived suppressor cells (MDSC), as defined by Lin$^-$CD11b$^+$Gr-1$^+$, was also similar in WT and TLR2$^{-/-}$ mice (Figure S5).

Thus, the anti-tumor effects of GVAX in TLR2$^{-/-}$ mice correlated with the intra-tumor frequency of T cells and, to a lesser extent, with NK cells but not with the frequency of Tregs, DC subsets, or MDSC.

TLR2 Deficiency Enhances Anti-tumor CTL Responses in Tumors

Both T cell priming in lymphoid tissues and cognate interactions with intra-tumor DCs contribute to T cell expansion in tumors (Broz et al., 2014; Diao et al., 2011; Kerkar et al., 2011; Ma et al., 2013). Since the function and frequencies of spleen DCs were similar in WT and TLR2$^{-/-}$ mice (Figure 4), it seemed unlikely that differences in T cell priming would account for the increased frequency of intra-tumor T cells. Further examination of lymphoid DCs in GVAX-treated mice revealed no difference in the frequencies of resident and migrated lymph node DCs; surface expression levels of CD40, CD80, and CD86; or T cell stimulation capacity (Figure S6). Consistent with these findings, we found that the frequency of primed T cells in lymphoid tissues using an in vitro antigen-recall assay was indistinguishable in WT and TLR2$^{-/-}$ mice (data not shown).

To eliminate the possibility that TLR2 signaling might affect T cell survival/proliferation (Quigley et al., 2009), and to facilitate the study of tumor-specific CTL responses in situ, we injected antigen-activated CD45.1 OT-I CTLs intravenously into CD45.2 WT and TLR2$^{-/-}$ mice bearing established B16 (control) or B16 tumors expressing OVA (B16-OVA) and recovered the tumors 3 days later, when they were ~1 cm in diameter, for analysis. As expected, B16 tumors contained few OT-I CTLs. B16-OVA tumors from TLR2$^{-/-}$ mice contained a 5- to 10-fold higher frequency of OT-I CTLs than those from WT mice (Figures 6A and 6B). In addition, almost 50% of these cells expressed interferon (IFN)-γ after re-stimulation with SIINFEKL peptides as compared to <10% in WT mice (Figure 6C), consistent with more robust effector activity. The spleen and non-draining lymph nodes of tumor-bearing TLR2$^{-/-}$ and WT mice contained a low frequency of OT-I CTL, regardless of tumor OVA expression (data not shown).

We and others reported previously that tumor DCs possess the unique ability to stimulate proliferation of tumor-antigen-specific CTLs, even in the presence of other tumor-infiltrating cells, including MDSC, monocytes, and macrophages (Broz et al., 2014; Diao et al., 2011). Since the frequency of CTLs in lymphoid tissues was similar to that in WT and TLR2$^{-/-}$ mice, we hypothesized that the higher intra-tumor CTL frequency in TLR2$^{-/-}$ mice was due to increased CTL proliferation within the tumor (Thompson et al., 2010). To monitor cell division, we injected CFSE-labeled OT-I CTL into tumor-bearing mice and assessed tumors and lymphoid tissues 5 days later (Figure 6D). OT-I CTL divided more extensively in B16-OVA tumors from TLR2$^{-/-}$ mice than from WT mice, as measured by CFSE dilution. In both WT and TLR2$^{-/-}$ mice, low levels of OT-I CTL proliferation occurred in B16-OVA tumor-draining lymph nodes, whereas non-draining lymph nodes and spleen contained no divided OT-I CTL. In the absence of OVA, OT-I CTL did not divide in any of the tissues examined. These findings support the view that CTL proliferation and acquisition of effector functions within the tumor regulate anti-tumor T cell responses in TLR2$^{-/-}$ mice.

To evaluate whether the increased intra-tumor frequency of CTL in TLR2$^{-/-}$ mice affects tumor growth, we transferred OT-I CTL into TLR2$^{-/-}$ and WT mice 3 days after B16-OVA or B16 inoculation. Analysis of tumor growth rates revealed enhanced efficacy of OT-I CTL therapy in TLR2$^{-/-}$ mice compared to WT mice bearing B16-OVA tumors (Figure 6E). Without CTL therapy, tumors grew at similar rates in WT and TLR2$^{-/-}$

(B) Flow cytometric quantitation of lymphoid cell populations in B16 melanoma. Results are displayed as mean ± SEM. *$p < 0.05$; **$p < 0.005$, by unpaired Mann-Whitney test; NS, not significant.

(C) Flow cytometric quantitation of DCs, neutrophils and monocyte/macrophages in B16 melanoma and spleen. DCs were separated into three subpopulations: DC1 (CD103$^+$CD11b$^-$), DC2 (CD103$^-$CD11b$^+$CD64$^-$F4/80$^-$), and DC3 (CD103$^-$CD11b$^+$CD64$^+$F4/80$^+$). DC1s in spleen are CD8$^+$ rather than CD103$^+$. Results are displayed as mean ± SEM. See also Figures S4–S6.

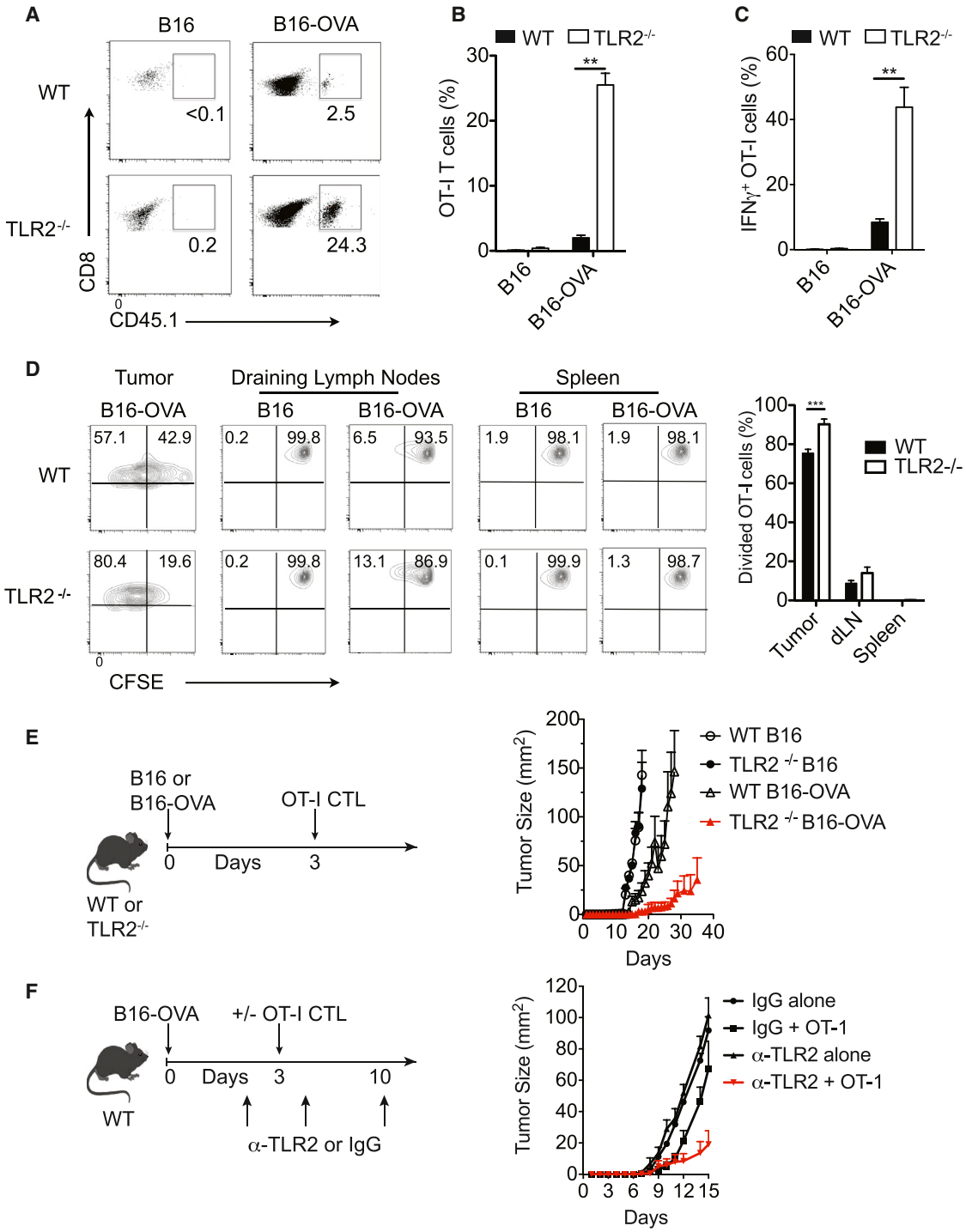

Figure 6. TLR2 Deficiency Enhances the Expansion and Function of Transferred Tumor-Antigen-Specific CTLs

(A and B) Frequency of OT-I CTLs in B16 and B16-OVA tumors 3 days after adoptive transfer of CD45.1 OT-I CTL into WT and TLR2$^{-/-}$ mice. Numbers in the dot plots indicate percentage of CD45.1^{+} CD8^{+} cells in B16 and B16-OVA tumors.

(C) Frequency of CD45.1^{+} IFNγ^{+} CD8^{+} T cells in B16 and B16-OVA tumors of WT or TLR2$^{-/-}$ mice.

(D) CFSE dilution of CD45.1^{+} OT-I CTL analyzed 5 days after transfer into WT and TLR2$^{-/-}$ mice bearing B16 or B16-OVA tumors. dLN, draining lymph nodes.

(E) Adoptive CTL therapy in WT versus TLR2$^{-/-}$ mice inoculated with 0.5 × 10^{6} B16 or B16-OVA cells. Graph shows mean tumor growth.

(F) Adoptive CTL therapy in WT mice inoculated with 1 × 10^{6} B16-OVA cells and treated with α-TLR2 (1 mg/kg) or control IgG antibodies, as indicated.

Results in (B) and (C) are displayed as mean ± SEM. **p < 0.005; ***p < 0.001. See also Figure S7.

mice. Collectively, these data suggest that the improved function of tumor cDCs in TLR2$^{-/-}$ mice increased the expansion and functional activity of adoptively transferred CTL.

We also determined whether TLR2 could be targeted in vivo with neutralizing antibodies as an adjunct for CTL immunotherapy. We confirmed that a single intravenous injection of anti-TLR2 antibodies (1 mg/kg) inhibited Pam3CSK4-induced cytokine secretion in recovered splenocytes for at least 2 days (data not shown) (Meng et al., 2004). We found that anti-TLR2 antibody therapy boosted the efficacy of adoptive CTLs significantly (Figure 6F).

Published reports indicate that B16 melanoma express TLR2 by qPCR (Oldford et al., 2010; Yang et al., 2009), which raised the possibility that anti-TLR2 antibodies might influence their behavior directly. However, we found that, in contrast with splenocytes, B16 melanoma cells neither express TLR2 on their cell surface nor produce IL-6 and IL-10 when stimulated with Pam3CSK4 or FSL-1 (Figure S7). Consistent with these findings, a short course of anti-TLR2 therapy alone had no effect on tumor growth (Figure 6F). Thus, the benefit of blocking TLR2 signaling on CTL therapy does not depend on TLR2 expression in B16 melanoma.

Human Cancers Induce Human DC Dysfunction through TLR2

Next, we addressed whether TLR2 affects the response of human DCs to human cancers. We measured IL-6 and IL-10 production by monocyte-derived human DCs incubated for 24 hr in conditioned medium generated from lung (H-125) and liver (HepG2) cancer cell lines, primary colon cancer cells (Colon 328), and control human spleen fibroblasts (FCM). As compared to unstimulated control, TCM from all three cancers stimulated autocrine production of IL-6 and IL-10 (Figure 7A), which was inhibited by the addition of anti-TLR2 antibodies to the culture medium (Figure 7B). FCM stimulated no IL-10 and little IL-6, which was blocked by anti-TLR2 antibodies. Further analysis of the effects of H-125-TCM on human DCs showed that it increased the surface expression of IL-6Rα and IL-10Rα, as well as the mRNA levels for these receptors (Figures 7C and 7D).

We also found that H-125 downregulated cell-surface expression of major histocompatibility complex class II (MHC II) and CD86 (Figure 7E) and induced the differentiation of IL-10-producing DCs, as detected by LPS stimulation in secondary cultures (Figures 7F). Anti-TLR2 antibodies blocked these changes and restored the production of IL-1β and IL-12p70 to levels similar to those produced by DCs that were maintained in GM-CSF. Furthermore, anti-TLR2 antibodies improved their stimulatory capacity in mixed allogeneic lymphocyte reactions (Figure 7G). Collectively, these data indicate that the mechanisms mediating DC dysfunction in mice also operate in human DCs.

Tumor-Derived Versican Induces cDC Dysfunction

Versican, an ECM proteoglycan that is upregulated in many cancers, activates macrophages through TLR2 to secrete tumor necrosis factor (TNF) and IL-6 (Kim et al., 2009). Accordingly, we speculated that versican may trigger DC dysfunction. Consistent with this possibility, the addition of anti-versican antibodies to TCM decreased IL-6 and IL-10 secretion by pre-cDCs at 24 hr, whereas the addition of antibodies to high mobility group box 1 (HMGB1), a pro-inflammatory TLR4 ligand released by dying cancer cells (Apetoh et al., 2007), inhibited neither IL-6 nor IL-10 secretion (Figure S8A). To further investigate the role of versican, we generated TCM from LLC cells transduced with versican and control short hairpin RNA (shRNA) (Figures S9A and S9B). Versican knockdown decreased the capacity of TCM to stimulate IL-6 and IL-10 production in pre-cDCs at 24 hr (Figure S8B); and cells recovered and re-stimulated with LPS at 72 hr showed a lower and higher capacity to produce IL-10 and IL-12p70, respectively. These results mirrored those obtained with the addition of anti-TLR2 antibodies to control shRNA TCM (Figure S8C), supporting the view that versican is a key TLR2 ligand in LLC TCM.

Next, we analyzed the in vivo effect of tumor-derived versican on tumor DCs. LLC transduced with versican-shRNA and control shRNA were inoculated into B6 mice subcutaneously; both tumors grew at a similar rate, as reported previously (Kim et al., 2009). We detected 4-fold higher expression levels of intracellular IL-12 p40 in DCs isolated from versican knockdown tumors as compared to those from control tumors (Figure S8D). The frequency of tumor DC subsets was similar in control- and versican-knockdown tumors (Figure S9). Collectively, these data suggest that tumor-derived versican promotes tumor DC dysfunction.

DISCUSSION

In this study, we show that TLR2 activation is a critical proximal signal leading to DC dysfunction in cancer. TLR2 ligation not only stimulated secretion of autocrine IL-10 and IL-6 but also, perhaps more importantly, led to sustained elevation of the cell-surface receptors for these cytokines, which decreased the threshold concentration required to activate STAT3. This feed-forward amplification loop reprogramed DCs to produce high amounts of IL-10 rather than IL-12 and IL-1β when stimulated with LPS, a classic pro-inflammatory stimulus. We found that tumor-derived versican can trigger this pathway. TLR2 deficiency improved the immunogenicity of intra-tumor DCs and enhanced expansion and proliferation and CTLs in tumors, acquisition of CTL effector functions, and anti-tumor responses.

Earlier studies established a linkage between STAT3, tumorigenesis, and impairment of anti-tumor immunity (Yu et al., 2007). Elevated STAT3 activity in tumor DCs is ascribed mostly to paracrine stimulation by tumor-derived cytokines such as IL-6, IL-10, and VEGF (Lee et al., 2010; Nefedova et al., 2004; Wang et al., 2004), although macrophages and other tumor-infiltrating cell populations can also produce these cytokines (Ruffell et al., 2014). Our study reveals the previously unrecognized role of autocrine cytokines induced by TCM in causing DC dysfunction. We found that both IL-6 and IL-10 were key to this process and behaved synergistically. Although typically considered a pro-inflammatory cytokine, IL-6 dampens immune responses in some settings (Hunter and Jones, 2015; Mauer et al., 2014). TCM also stimulates the release of IL-1β from pre-cDCs; however, neutralizing anti-IL-1β fails to prevent the subsequent differentiation of IL-10-producing DCs (M.S.C. and

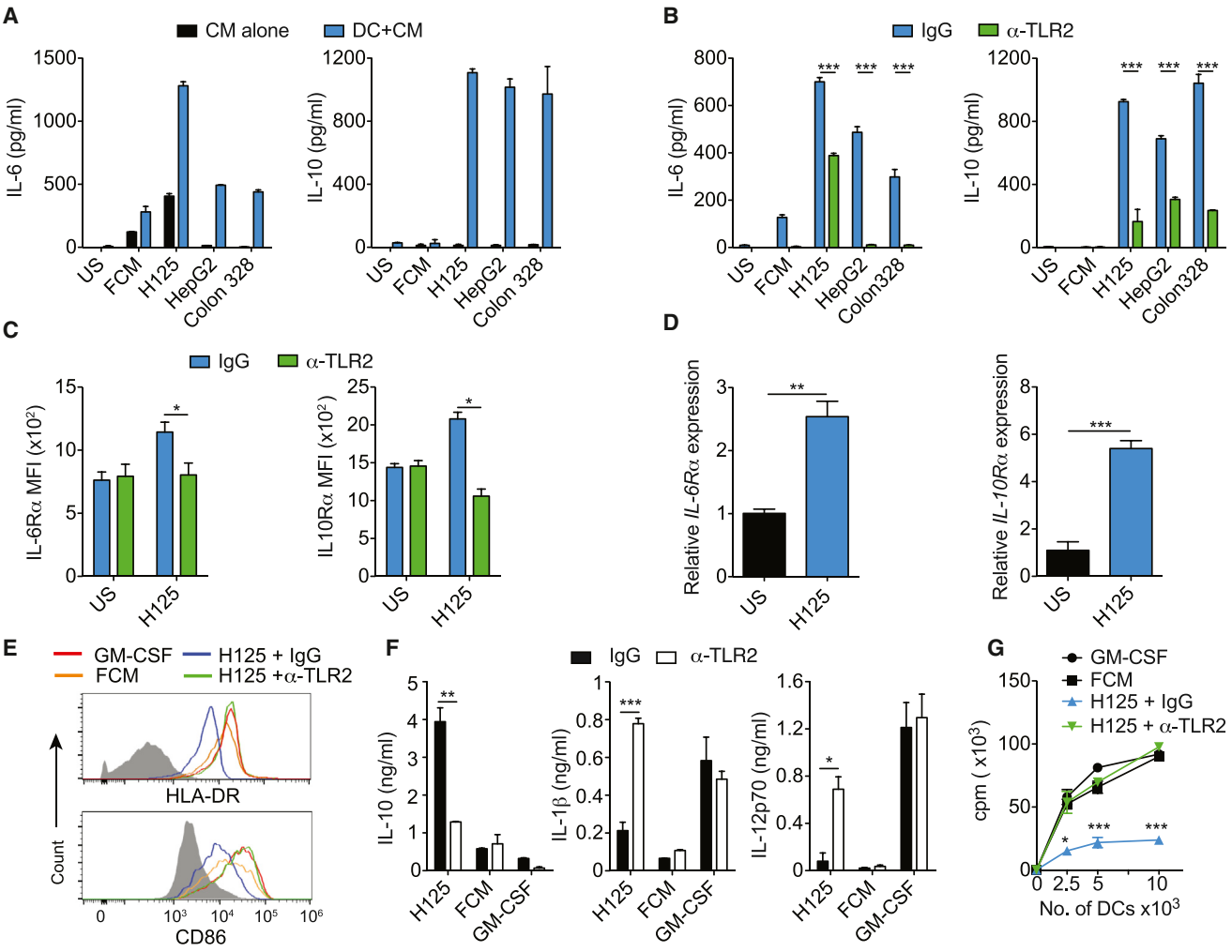

Figure 7. Human Cancers Induce Human DC Dysfunction through TLR2

(A) IL-6 and IL-10 production by human DCs unstimulated (US) or cultured for 24 hr in conditioned medium (CM) derived from FCM, NCI-H125, HepG2, and Colon328.

(B) IL-6 and IL-10 production by human DCs under the same conditions as in (A) in the presence of anti-TLR2 (10 μg/ml) blocking antibodies or control IgG2b antibodies.

(C) Surface expression of IL-6Rα and IL-10Rα at 24 hr human DCs unstimulated (US) or cultured for 24 hr in conditioned medium derived from NCI-H125.

(D) IL-6Rα and IL-10Rα mRNA expression at 24 hr determined by quantitative real-time PCR. Fold change is relative to unstimulated control.

(E) Flow cytometric analysis of HLA-DR and CD86 expression of human DCs cultured for 48 hr in GM-CSF, FCM, or NCI-H125 TCM with or without anti-TLR2 antibodies.

(F) IL-10, IL-1β, and IL-12p70 production by human DCs pretreated with NCI-HI25 TCM and FCM or left in GM-CSF for 72 hr and then washed and restimulated with LPS for 18 hr.

(G) Stimulation capacity of human DCs cultured as in (E) in mixed allogeneic lymphocyte reactions.

Results are displayed as mean ± SEM and are representative of three independent experiments. *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$, assessed by unpaired Student's t tests.

M.T., unpublished data). Although autocrine IL-6 and IL-10 mediated DC dysfunction in vitro, the relative importance of autocrine versus paracrine signaling in vivo remains unclear.

The intensity and duration of STAT3 signaling governs whether an anti-inflammatory signal is delivered to DCs (Braun et al., 2013; Yasukawa et al., 2003). We show that IL-10R and IL-6R expression levels calibrate the sensitivity of DCs to IL-10 and IL-6 based on their ability to activate STAT3. TLR2 ligation increased expression levels IL-6R and IL-10R independently of IL-6 and IL-10 signaling. In the absence of receptor upregulation, the capacity of paracrine IL-6 and IL-10 to induce STAT3 and DC dysfunction was diminished markedly. Thus, TLR2 ligation imprints the capacity to respond to lower concentrations of these cytokines, which helps explain the relative tumor specificity of DC dysfunction. These findings also suggest that cytokine receptor expression levels, rather than the source of the cytokines

per se, may be the ultimate arbiter of DC fate and function in vivo. In support of this theory, a recent study found that IL-10R expression was absolutely required for intestinal macrophages to prevent spontaneous colitis, whereas autocrine IL-10 was dispensable (Zigmond et al., 2014).

Tumor DCs from TLR2$^{-/-}$ mice exhibited several key attributes of effective antigen-presenting cells: (1) high expression levels of MHC II and co-stimulatory molecules; (2) expression of pro-inflammatory IL-1β rather than IL-10; and (3) potent stimulation of allogeneic lymphocytes. These functional properties correlated with better anti-tumor immune responses elicited by a GVAX vaccine and higher rates of proliferation and expression of IFN-γ in adoptively transferred OT-1 CTL. Since other tumor-infiltrating immune cells express TLR2, we cannot exclude the possibility that the absence of TLR2 signaling in these cells also influenced anti-tumor immune responses. It is noteworthy, however, that growing evidence points to a unique role for tumor DCs in regulating the proliferation, expansion, and function of tumor-infiltrating CTLs through cross-presentation of acquired tumor antigens (Broz et al., 2014; Broz and Krummel, 2015; Diao et al., 2011; Ma et al., 2013). This role for tumor DCs aligns well with the function of tissue DCs in restimulating effector and memory T cells in situ (McGill et al., 2008; Wakim et al., 2008).

TLR2 signaling can increase CD8$^+$ T cell effector activity and survival and reduce the suppressive function of Tregs (Geng et al., 2010). In our study, GVAX immunization in TLR2$^{-/-}$ mice resulted in higher frequencies of intra-tumor CD8$^+$ T cells and no difference in the frequency of Foxp3$^+$ Tregs, as compared to those in WT mice. We propose that the immunologic benefit of blocking TLR2 signaling in tumor DCs in B16 melanoma outweighs the loss of TLR2 signaling in T cells. This dynamic may vary in different tumors, which may explain why TLR2 agonists have been reported to both promote and inhibit tumor growth (Huang et al., 2007; Zhang et al., 2011). In some tumors, TLR2 signaling directly contributes to tumorigenesis and tumor cell proliferation and survival (Tye et al., 2012). In our study, we failed to detect cell-surface expression of TLR2 or a functional response to TLR2 agonists in B16 cells.

Recent reports suggest that particular DC subpopulations are particularly well equipped to promote anti-tumor immunity. Tumor CD103$^+$ DCs, which are linked ontogenetically to spleen CD8$^+$ DCs (Hildner et al., 2008), express higher levels of IL-12 and stimulate T cells more effectively than tumor CD11b$^+$ DCs (Broz et al., 2014). Indirect or direct inhibition of tumor macrophage-derived IL-10 (anti-CSF and anti-IL-10R antibodies, respectively) increased the density of tumor CD103$^+$ DCs, which correlated with a better response to paclitaxel in a spontaneous breast cancer model (Ruffell et al., 2014). In our study, TLR2 deficiency had no effect on the frequencies of cDC subpopulations; and all populations displayed a similar increase in the expression levels of MHC II and co-stimulatory molecules as compared to tumor DCs from WT mice. Although CD11b$^+$ DCs are less effective antigen-presenting cells than CD103$^+$ DCs on a per-cell basis, they have a substantial numerical advantage in most tumors and likely contribute to anti-tumor immunity. Indeed, depletion of tumor CD11b$^+$ DCs abolished the efficacy of chemotherapy-induced anti-tumor T cell responses (Ma et al., 2013).

Human and mouse tumors produce a variety of TLR2 ligands, including versican, laminin-β1, procollagen III-α1, Hsp60, and Hsp72 (Chalmin et al., 2010; Kim et al., 2009; Yang et al., 2009). The microbiome also generates TLR2 ligands that could potentially influence the tumor microenvironment and tumor progression (Schwabe and Jobin, 2013). This panoply of TLR2 ligands suggests that targeting the receptor rather than individual ligands may be a more effective strategy to modulate anti-tumor immunity. The tumor microenvironment also produces immunostimulatory molecules, such as HMGB1 (a TLR4 ligand) and ATP (Apetoh et al., 2007; Ghiringhelli et al., 2009). How tumor DCs respond to ostensibly conflicting signals merits further investigation. Our findings raise the possibility that treatments aimed to stimulate the release TLR4 ligands in tumors may augment IL-10 production from "re-programmed" DCs.

Immunotherapy offers great potential for long-term cure and survival for cancer patients (Rosenberg et al., 2008). Advances in understanding T cell biology has led to new approaches to increase the efficacy of immunotherapy, including cytokine therapy (e.g., IL-2, IL-7, and IL-15) and blockade of regulatory molecules (CTLA-4/CD28, PD1/PD-L1) and immunosuppressive cells (Tregs) (Mellman et al., 2011). Although the success rates of immunotherapy continue to improve and there are examples of spectacular responses, this therapy is not consistently effective, and many patients derive little or no benefit (Restifo et al., 2012). Our study suggests that targeting TLR2 to prevent tumor DC dysfunction may be a practical adjunct to improve the results of immunotherapy.

EXPERIMENTAL PROCEDURES

Mice

Male C57BL/6, BALB/c, TLR2$^{-/-}$, TLR4$^{-/-}$, and OT-I mice and C57BL/6.SJL (CD45.1) congenic mice were purchased from The Jackson Laboratory or Taconic Farms and bred in our animal facility. MyD88$^{-/-}$ mice were provided by Dr. Peter Liu (University of Toronto). Mice were maintained in pathogen-free conditions in accordance with University Health Network Animal Resource Center guidelines and used at 2–3 months of age. The Animal Research Committee of University Health Network reviewed and approved the studies.

Tumor Models

B16-F10 melanoma (B16), LLC, and human cancer cell lines were purchased from the American Type Culture Collection. B16-OVA was kindly provided by R.W. Dutton at the Trudeau Institute (Dobrzanski et al., 2004). Primary colon cancer cells were kindly provided by C. O'Brien (Ontario Cancer Institute).

To establish tumors, 0.5–1 × 10^6 B16 or B16-OVA tumor cells in 100 μl PBS were injected subcutaneously (s.c.) into the flank of C57BL/6 mice. Tumor dimensions were measured with calipers, and tumor size was calculated by multiplying 2/3 length and width.

Cell Isolation

Single-cell suspensions generated from tumors, spleen, and lymph nodes were prepared and analyzed as described in the Supplemental Experimental Procedures.

Flow Cytometry

Cell suspensions were preincubated with anti-CD16/32 to block Fc receptors and then were washed and incubated with the indicated monoclonal antibody (mAb) conjugates for 30 min at 4°C in a final volume of 100 ml PBS containing 0.5% BSA and 2 mM EDTA. In all experiments, appropriate control isotype matched mAbs were included to determine the level of background staining.

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For intracellular cytokine detection, surface antibody-labeled cells were fixed, permeabilized, and stained with anti-cytokine antibodies according to the instructions from the BD Cytofix/Cytoperm Kit (BD Biosciences). The antibodies and reagents are described in detail in the Supplemental Experimental Procedures.

Preparation of Conditioned Medium

Cancer cell lines, S17 cells (a kind gift from K. Dorshkind, David Geffen School of Medicine at UCLA), and human spleen-derived fibroblasts were cultured at 90% confluence in serum-free medium for 20–24 hr. Supernatants were filtered through a 0.22-μm filter, stored at −80°C, and thawed immediately before use as described in the Supplemental Experimental Procedures.

Cytokine Assays

IL-6, IL-1β, IL-10, and IL-12p70 concentrations were measured by ELISA with commercial kits (OptEIA; BD Biosciences). Notably, mouse pre-cDCs do not require pre-treatment with a caspase activator to produce IL-1β (He et al., 2013).

Gene Expression Analysis

Total cell RNA was prepared from DCs with the RNAeasy Kit (QIAGEN), followed by first-strand cDNA synthesis using M-MLV Reverse Transcriptase (Invitrogen). qPCR for IL-6R, IL-10R, and STAT3 gene expression was performed as described in the Supplemental Experimental Procedures.

Versican Knockdown with Lentiviral Transduction

Versican shRNA (SHCLNG-NM_004385) and control shRNA (SHC016) constructs were purchased from Sigma-Aldrich and packaged into lentivirus vectors in our laboratory. LLC and B16 cells were incubated with lentivirus culture supernatants containing polybrene (10 μg/ml) for 48 hr and selected in the presence of puromycin (1 mg/ml; Invivogen). Knockdown of versican expression was verified by quantitative real-time PCR and western blot.

Statistical Analysis

Results are expressed as means ± SEM and mean ± SD. Data were analyzed by Student's t test, Mann-Whitney test, two-way ANOVA, and the log-rank test. The analyses were performed with the GraphPad Prism statistical program. p values < 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and nine figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.11.053.

AUTHOR CONTRIBUTIONS

M.T. designed and performed research and wrote the manuscript. J.D. designed and performed research and wrote the manuscript. H.G., I.K., and J.Z. performed experiments. M.S.C. designed and supervised research and wrote the manuscript.

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