Toll-Like Receptors on Tumor Cells Facilitate Evasion of Immune Surveillance

Bo Huang,[1] Jie Zhao,[3] Hongxing Li,[3] Kai-Li He,[4] Yibang Chen,[2] Lloyd Mayer,[3] Jay C. Unkeless,[3] and Huabao Xiong[3,5]

Departments of [1]Gene and Cell Medicine and [2]Biochemistry and Pharmacology, [3]Immunobiology Center, Mount Sinai School of Medicine; [4]Department of Cell and Developmental Biology, Weill Medical College of Cornell University, New York, New York; and [5]Department of Immunology, Shanghai Medical College, Fudan University, Shanghai

Abstract

The signal pathways that trigger tumor cell escape from immune surveillance are incompletely understood. Toll-like receptors (TLRs), which activate innate and adaptive immune responses, are thought to be restricted to immune cells. We show here that TLRs, including TLR4, are expressed on tumor cells from a wide variety of tissues, suggesting that TLR activation may be an important event in tumor cell immune evasion. Activation of TLR4 signaling in tumor cells by lipopolysaccharide induces the synthesis of various soluble factors and proteins including interleukin-6, inducible nitric oxide synthase, interleukin-12, B7-H1, and B7-H2, and results in resistance of tumor cells to CTL attack. In addition, lipopolysaccharide-stimulated tumor cell supernatants inhibit both T cell proliferation and natural killer cell activity. Blockade of the TLR4 pathway by either TLR4 short interfering RNA or a cell-permeable TLR4 inhibitory peptide reverses tumor-mediated suppression of T cell proliferation and natural killer cell activity *in vitro*, and *in vivo*, delays tumor growth and thus prolongs the survival of tumor-bearing mice. These findings indicate that TLR signaling results in a cascade leading to tumor evasion from immune surveillance. These novel functions of TLRs in tumor biology suggest a new class of therapeutic targets for cancer therapy. (Cancer Res 2005; 65(12): 5009-14)

Introduction

Tumors evade immune surveillance by multiple mechanisms, including the production of factors such as transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF), which inhibit dendritic cell activation and impair tumor-specific T cell immunity (1). To escape attack from natural killer (NK) cells and CTL, tumor cells up-regulate certain surface molecules (B7-H1 and HLA-G), down-regulate others (MHC class I and Fas), and shed surface molecules in soluble form (such as MIC, the ligand for NKG2D; refs. 2–5). However, the mechanisms regulating expression by tumor cells of factors/molecules that are important for tumor cell immune evasion are not well understood. Toll-like receptors (TLRs), critical players in both innate and adaptive immunity, recognize distinct pathogen-associated molecular patterns. The subsequent signal cascade triggers antimicrobial host defenses and the up-regulation of costimulatory molecules for DC maturation, resulting in effective antigen presentation to T and B cells. The TLR signaling pathway activates several different signaling elements, including nuclear factor κB (NF-κB) and extracellular signal-regulated kinase (ERK)/c-*Jun*-NH_2-kinase (JNK)/p38, which regulate many immunologically relevant proteins (6). Coincidentally, many of these same signaling elements are also involved in tumorigenesis and tumor growth, suggesting that TLRs may affect tumor growth. We hypothesize that TLR signaling, usurped by tumor cells, triggers tumor self-protection mechanisms leading to immune evasion.

Materials and Methods

Mice and cell lines. BALB/c (H-2^d) mice and transgenic DO11.10 (H-2^d) mice were purchased from the National Cancer Institute and Jackson Laboratory, respectively. TLR4-deficient mice were maintained in the animal facility of Mount Sinai School of Medicine. The mouse tumor cell lines MC26 (colon cancer), 4T1 (breast cancer), RM1 (prostate cancer), B16 (melanoma), and LLC1 (lung cancer) were grown in DMEM with 10% fetal bovine serum and 4 mmol/L glutamine. The NK cell–sensitive mouse lymphoma cell line yeast artificial chromosome-1 (YAC-1) was maintained in RPMI 1640 with 10% fetal bovine serum.

Flow cytometry. Cultured MC26 tumor cells were stained with phycoerythrin anti-mouse TLR4 antibody (eBioscience, San Diego, CA) or phycoerythrin-labeled mouse IgG_1 (BD Biosciences, isotype control). Tumor cells from tumor tissue were isolated and the purified tumor cells were stained for flow cytometry.

RT-PCR. Total RNA was isolated from tumor cells using TRIzol reagent (Invitrogen, Carlsbad, CA). An RT-PCR protocol was used to determine relative quantities of mRNA after 28 PCR cycles (One-Step RT-PCR kit, Qiagen, Valencia, CA), and quantified relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The primers were synthesized by Gene Link, including: GAPDH 5′-GTGGAGATTGTTGCCATCAACG-3′ (sense), 5′-CAGTGGATGCAGGGATGATGTTCTG-3′ (antisense); TLR1 5′-CCCTCATCTTCTACTGTATC-3′ (sense), 5′-TCACCTTTAGCTCATTGTGG-3′ (antisense); TLR2 5′-GTCTTTCACCTCTATTCCCTC-3′ (sense), 5′-GTCTCTACATTTCCTATCCTG-3′ (antisense); TLR3 5′-GGATTCTTCTGGTGTCTTCC-3′ (sense), 5′-TCGAGTTCTGTCAGGTTCGTG-3′ (antisense); TLR4 5′-GAAACTCAGCAAAGTCCCT-G-3′ (sense), 5′-GAAAGGCTTGGTCTTGAATG-3′ (antisense); TLR5 5′-CTTCGGCTGTTTTCCTGTGG-3′ (sense), 5′-CTTCCCTGGATGATGTTGCTG-3′ (antisense); TLR6 5′-CCAAAGACCTGCCACCAAGAAC-3′ (sense), 5′-CACTAAGTCCAGAAGAATGC-3′ (antisense); TLR9 5′-CTCTCTCCATACACTGAACTC-3′ (sense), 5′-TGCTCTGCATCATCTGCCTC-3′ (antisense); interleukin-6 (IL-6) 5′-GAGAGGAGACTTCACAGAGGATAC-3′ (sense), 5′-GTACTCCAGAAGACCAGAGG-3′ (antisense); IL-12 p40 5′-GAGAAACAGTGAACCTCACC-3′ (sense), 5′-CAGACAGAGACGCCATTCCAC-3′ (antisense); tumor necrosis factor-α (TNF-α) 5′-GACACCATGAGCACAGAAAG-3′ (sense), 5′-GAGTAGACAAGGTACAACCC-3′ (antisense); VEGF 5′-CTTTCTGCTCTCTTGGGTGC-3′ (sense), 5′-CATGGTGATGTTGCTCTCTG-3′ (antisense); TGF-β1 5′-GTGGTATACTGAGACACCTTGG-3′ (sense), 5′-CCTTAGTTTGGACAGGATCTGG-3′ (antisense); inducible nitric oxide synthase (iNOS) 5′-GAGATTGGAGTTCGAGACTTCTGTG-3′ (sense), 5′-TGGCTAGTGCTTCAGACTTC-3′ (antisense); Fas 5′-GGAAACAAACTGCACCCTG AC-3′ (sense), 5′-AGCAGCTGGACTTTCTGCTC-3′ (antisense); B7-H1 5′-GGAAGATGAGAGTGATCAG-3′ (sense), 5′-CAATGAGGAACAACAGGATGG-3′

Requests for reprints: Huabao Xiong, Immunobiology Center, Box 1630, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029-6574. Phone: 212-659-9413; Fax: 212-849-2525; E-mail: Huabao.Xiong@mssm.edu.

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(antisense); B7-H2 5′-CTTGGTCTGTTCTTGCTGCTG-3′ (sense), 5′-GGCTATTGTCCGTTGTGTTG-3′ (antisense); CD40 5′-CAAACAGTACCTC-CACGATG-3′ (sense), 5′-CTCCATAACTCCA AAGCCAG-3′ (antisense).

Peptides. We have synthesized the $TLR4_{663-686}$ peptide with an NH_2-terminal Tat sequence: TLR4 peptide, RKKRRQRRRGKKYSRGESIYDAFVI-YSSQNEDWV; TLR4 mutant peptide, RKKRRQRRRGEEYSEG-ESIYDAF-VIYSSQNEDWV. The Tat sequence is underlined.

Cytokine release and nitric oxide production. Cytokine production in the tumor cell culture was quantified by ELISA (R&D Systems, Minneapolis, MN) and nitric oxide production was measured using Greiss reagent (Sigma, St. Louis, MO).

Construction of a TLR4 short interfering RNA expressing MC26 tumor cell line. TLR4 short interfering RNA (siRNA) sequence (GTCCCTGATGACATTCCTT) was inserted into RNAi-Ready pSIREN-RetroQ expressing vector with U6 promoter (BD Biosciences, Clontech, Palo Alto, CA). The recombinant siRNA and control plasmids were transfected into MC26 tumor cell using FuGENE 6 transfection reagent (Roche) for stable expression after selection.

Western blot. Western blot was done as described (7). All antibodies were purchased from Cell Signaling (Beverly, MA). The T cell proliferation assay procedures are described in the figure legends.

Natural killer and CTL cytotoxicity. $DX5^+$ NK cells were isolated from mouse splenocytes by immunoselection with biotin-labeled DX5 and streptavidin-magnetic beads for the cytotoxicity assay. For cytolysis of MC26 cell by CTL, splenocytes from MC26 tumor-bearing mice (10 days inoculation) were cocultured in 24-well plates with IL-2 (100 units/mL) and irradiated MC26 tumor cells (150 Gy) at a ratio of 10:1. The suspension cells were also stimulated under the same conditions. Viable lymphocytes were harvested 10 days later as CTLs for the cytotoxicity assay, as described in the figure legends.

Isolation of T cells and natural killer cells from tumor. Large tumors from various treatment groups (different inoculation doses were used to obtain the same size tumors) were digested with collagenase and hyaluronidase for 1 hour at 37°C, and homogenized with semifrosted slides. After lysis of RBC, the dissociated cells were underlaid with 5 mL of Lymphocyte-M solution, centrifuged (2,200 rpm for 20 minutes). Tumor-infiltrating lymphocytes were harvested from the interface. T cells were isolated on a T cell enrichment column (R&D Systems), and NK cells were purified by magnetic sorting with biotinylated-DX5 antibody.

Results

Toll-like receptors are expressed on tumor cells. In this study, we first screened various murine tumor cell lines from different tissues, including colon cancer MC26, breast cancer 4T1, prostate cancer RM1, melanoma B16, and lung cancer LLC1, for the expression of major TLRs (including TLR1-6 and 9) by RT-PCR. Surprisingly, all the tumor cell lines expressed multiple TLRs (Fig. 1*A*). Experiments on human tumor cell lines showed a similar expression pattern of TLRs (data not shown). We focused our attention on TLR4, which had the most strongly expressed mRNA. Consistent with the RT-PCR result, fluorescence-activated cell sorting (FACS) analysis revealed that TLR4 protein was also present on the surface of tumor cell lines and absent from $TLR4^{-/-}$ PBMC (Fig. 1*B*). Confirming the results on cell lines, we found expression of TLR4 protein on isolated tumor cells from MC26 tumors (Fig. 1*C*). The broad expression of TLRs in tumors suggests that TLRs may have a previously unrecognized function in tumor biology.

Tumor cells respond to lipopolysaccharide through TLR4. We next asked if TLRs were functional in tumor cells. To activate TLR4 in MC26 cells, we incubated the cells with lipopolysaccharide, the natural ligand for TLR4. After incubation of MC26 tumor cells with lipopolysaccharide, we extracted total RNA for RT-PCR analysis. Interestingly, we found that tumor cells constitutively express VEGF, TGF-β1, and TNF-α. However, upon lipopolysaccharide stimulation, there was also a striking induction of iNOS, IL-6, and IL-12 p40 mRNA (Fig. 2*A*). Consistent with mRNA expression, lipopolysaccharide-stimulated tumor cell supernatant contains significant titers of IL-6, IL-12, and nitric oxide (Fig. 2*A*). To verify that the expression of these factors following lipopolysaccharide incubation was due to TLR4 activation, we constructed an MC26 tumor cell line stably expressing TLR4 siRNA (Fig. 2*B*). After knockdown of TLR4 expression, the effect of lipopolysaccharide was almost abolished, with essentially no nitric oxide, IL-6, or IL-12 produced in response to lipopolysaccharide (Fig. 2*A*). Furthermore, we confirmed the activation of the TLR4 signal pathway by analyzing the phosphorylation of downstream signaling molecules. The kinetics of phosphorylation of IκB, ERK, and JNK in tumor cells after lipopolysaccharide stimulation was very similar to that seen in macrophages (Fig. 2*C*).

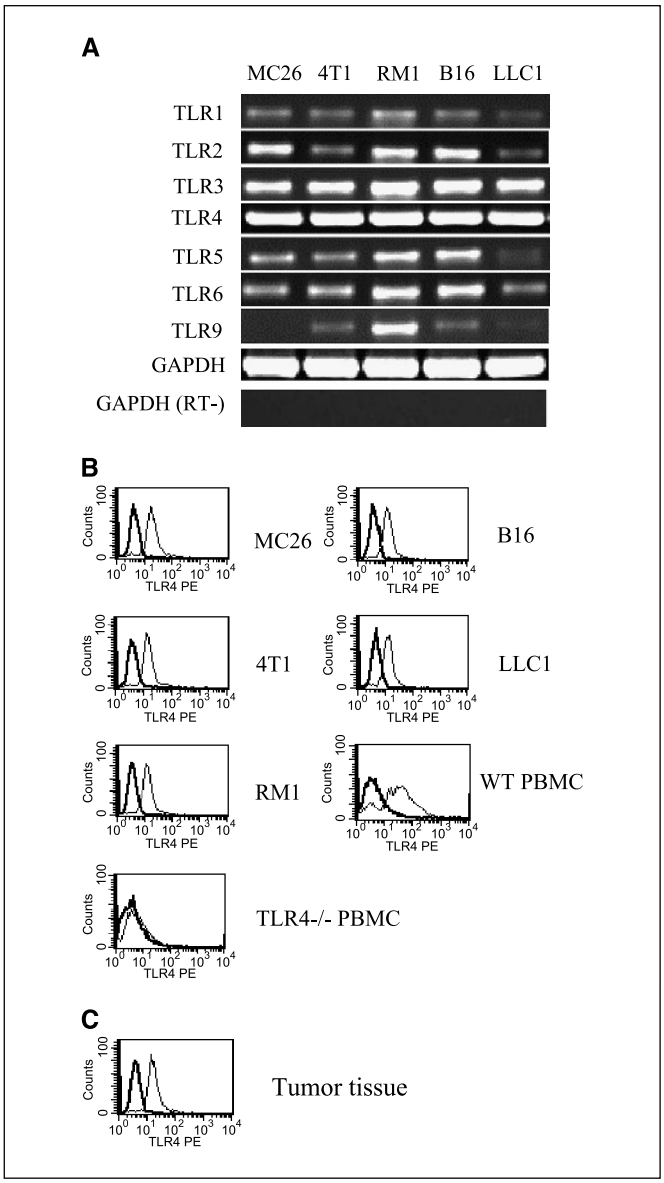

Figure 1. Expression of TLRs in tumor cells. *A,* expression of TLRs (1–6, 9) in different mouse tumor cell lines was analyzed by RT-PCR. One representative experiment (out of three) is depicted. *B* and *C,* FACS analysis of TLR4 expression in tumor cell lines, $TLR4^{-/-}$ PBMC, or isolated MC26 cells from tumor tissue. Heavy line, isotype controls.

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To confirm further that TLR4 was the target of lipopolysaccharide in MC26 cells, we synthesized a 34-residue peptide from the TLR4 juxtamembrane cytoplasmic domain (residues 663-686) with an NH_2-terminal Tat sequence, to facilitate cytoplasmic penetration. This TLR4 peptide, and a control in which the basic residues were mutated to Glu, were labeled with NH_2-terminal FITC. The TLR4 peptide efficiently inhibited the expression of IL-6, IL-12, iNOS, and the phosphorylation of IκB in MC26 cells induced by lipopolysaccharide (Fig. 2*D*). These data clearly indicate that TLR4 stimulation of tumor cells resulted in synthesis of nitric oxide and proinflammatory cytokines.

TLR4 activation in tumor cells enhances immune suppression *in vitro*. We first designed *in vitro* assays to analyze the consequences of TLR4 activation in MC26 cells. We incubated DO.10.11 OVA-specific splenocytes or naïve BALB/c splenocytes with tumor cell supernatants, and found that lipopolysaccharide-stimulated tumor cell supernatants strongly inhibited T cell proliferation in response to peptide (DO.10.11 splenocytes) or stimulation by anti-CD3 and CD8 antibodies (BALB/c splenocytes) relative to controls (Fig. 3*A*). Addition of lipopolysaccharide to the control medium had no effect on T cell proliferation, confirming that the lipopolysaccharide effect was due to stimulation of the tumor cells (data not shown). To confirm if the suppression of T cell proliferation was due to TLR4 activation, we blocked TLR4 signaling by either TLR4 siRNA or TLR4 peptide and found significant reversal of the suppressed T cell proliferation by the lipopolysaccharide-activated tumor cell supernatants (Fig. 3*A*). We used a comparable approach to examine the role of tumor TLR4 signaling in evasion of NK cell cytotoxicity. $DX5^+$ NK cells, isolated from naïve BALB/c spleen, were cultured with IL-2 and tumor cell supernatants. The cytotoxicity of NK cells towards carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled YAC-1 cells was determined by flow cytometry (8). NK cells efficiently killed YAC-1 cells in the control group (79.9% cytotoxicity; Fig. 3*B*). However, the lipopolysaccharide-stimulated MC26 supernatant significantly inhibited NK cell cytotoxicity compared with unstimulated tumor cell supernatant group (25.8% versus 43.3% cytotoxicity, respectively; $P < 0.05$). In addition, the inhibitory effect was reversed by both TLR4 siRNA and TLR4 peptide (40.4% and 43.9% cytotoxicity, respectively); irrelevant siRNA and mutated TLR4 peptide were without effect. Thus, the escape of tumor cells from NK cell attack is also TLR4-dependent.

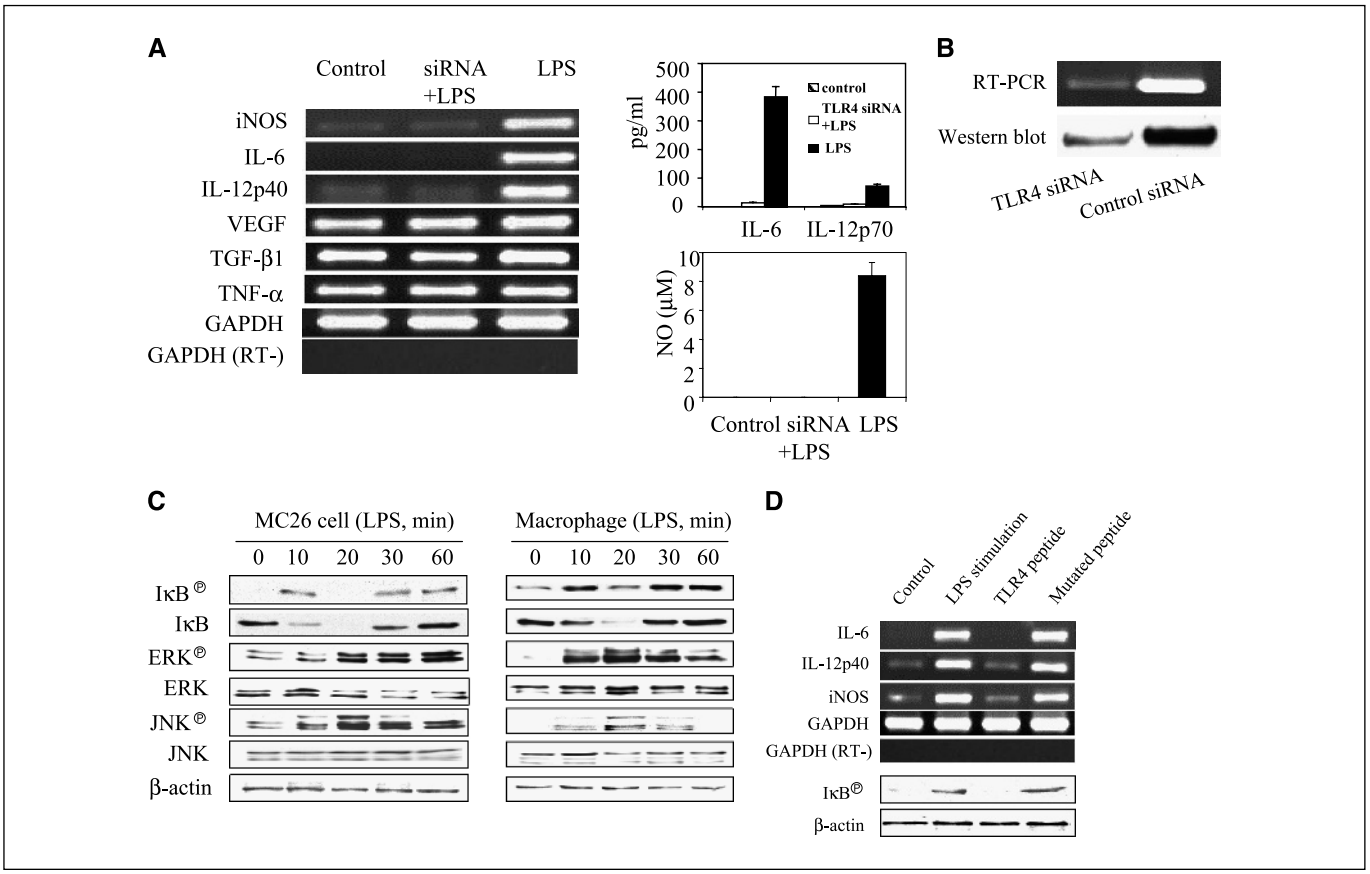

Figure 2. TLR4 signaling in tumor cells. *A,* expression of cytokine and growth factor in MC26 cells after lipopolysaccharide stimulation. MC26 cells were activated with lipopolysaccharide (1 μg/mL) for 6 hours for the expression of cytokine and growth factor mRNAs analyzed by RT-PCR, or 24 hours for the measurement of the production of nitric oxide, IL-6, and IL-12p70 by Greiss reagent and ELISA, respectively. *B,* siRNA silences TLR4 expression. After stable transfection of TLR4 siRNA vector into the MC26 tumor cell line, TLR4 mRNA and protein expression was examined by RT-PCR and Western blot. *C,* activation of NF-κB and mitogen-activated protein kinases in MC26 cells and peritoneal macrophages. MC26 cells or peritoneal macrophages were activated with lipopolysaccharide (1 μg/mL) for 10, 20, 30, and 60 minutes, and phosphorylation of IκB, ERK, and JNK was analyzed by immunoblotting with phosphorylation-specific and control antibodies. *D,* TLR4 peptide blocks TLR4 signaling in MC26 cells. MC26 cells were pretreated with TLR4 peptide or control peptide for 1 hour, followed by activation with lipopolysaccharide (1 μg/mL) for 6 hours. IL-6, iNOS, and IL-12 mRNAs were analyzed by RT-PCR. IκB phosphorylation was analyzed 10 minutes after lipopolysaccharide activation by immunoblotting.

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To further understand the factors involved in immune suppression (Fig. 2), we inhibited nitric oxide production by the nitric oxide synthase inhibitor *N*-nitro-L-arginine methyl ester (L-NAME), or neutralized the function of IL-6 or IL-12 in the supernatants with neutralizing antibodies. L-NAME or IL-6 antibody significantly restored T cell proliferation and NK cell activity, but IL-12 antibody had no effect (data not shown), suggesting that nitric oxide and IL-6 induced by lipopolysaccharide contributes to immune suppression. Furthermore, we also tested whether the activation of tumor cell TLR4 alters their

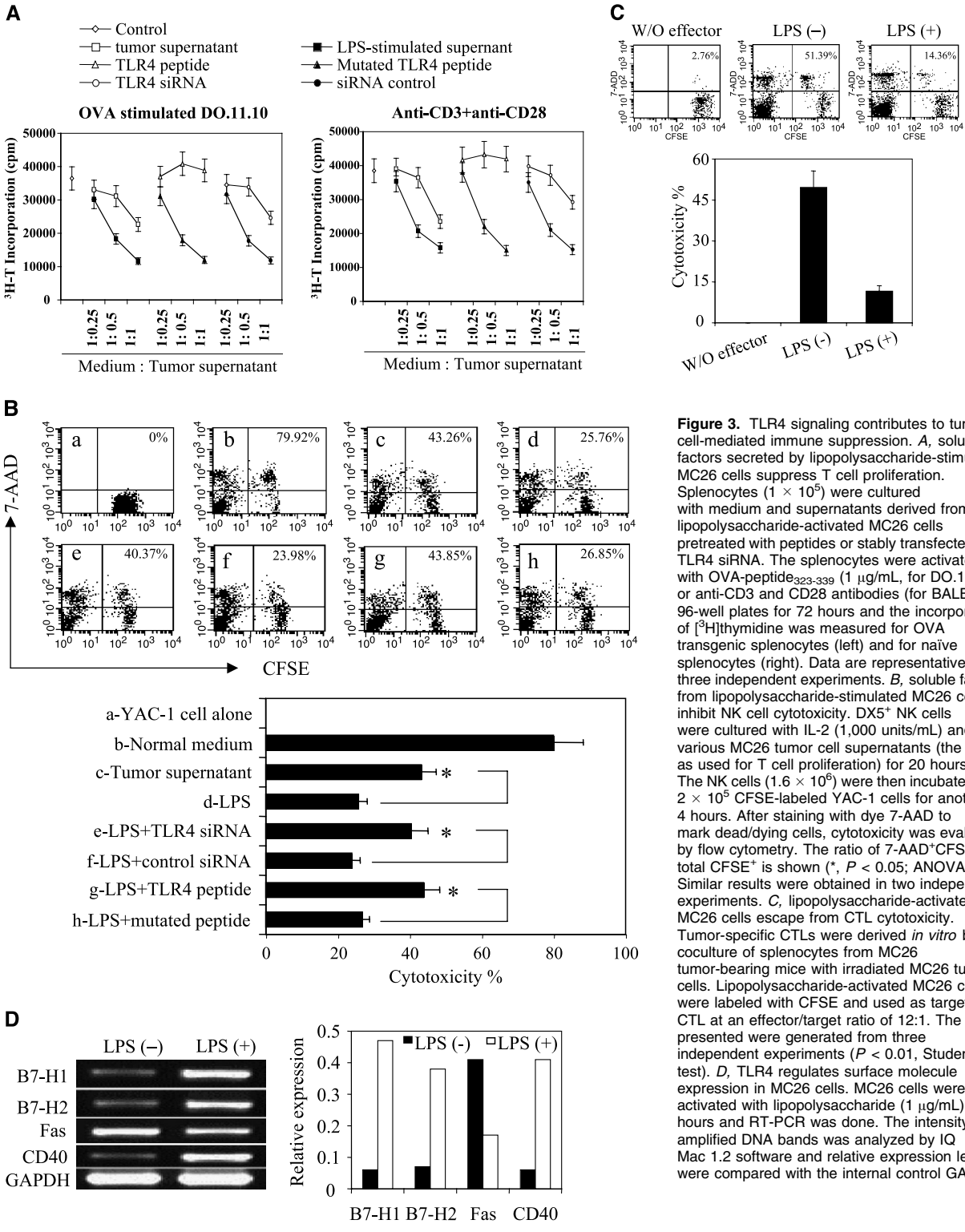

Figure 3. TLR4 signaling contributes to tumor cell-mediated immune suppression. *A,* soluble factors secreted by lipopolysaccharide-stimulated MC26 cells suppress T cell proliferation. Splenocytes (1×10^5) were cultured with medium and supernatants derived from lipopolysaccharide-activated MC26 cells pretreated with peptides or stably transfected with TLR4 siRNA. The splenocytes were activated with OVA-peptide$_{323\text{-}339}$ (1 μg/mL, for DO.11.10) or anti-CD3 and CD28 antibodies (for BALB/c) in 96-well plates for 72 hours and the incorporation of [^{3}H]thymidine was measured for OVA transgenic splenocytes (left) and for naïve splenocytes (right). Data are representative of three independent experiments. *B,* soluble factors from lipopolysaccharide-stimulated MC26 cells inhibit NK cell cytotoxicity. DX5$^+$ NK cells were cultured with IL-2 (1,000 units/mL) and various MC26 tumor cell supernatants (the same as used for T cell proliferation) for 20 hours. The NK cells (1.6×10^6) were then incubated with 2×10^5 CFSE-labeled YAC-1 cells for another 4 hours. After staining with dye 7-AAD to mark dead/dying cells, cytotoxicity was evaluated by flow cytometry. The ratio of 7-AAD$^+$CFSE$^+$/total CFSE$^+$ is shown (*, $P < 0.05$; ANOVA). Similar results were obtained in two independent experiments. *C,* lipopolysaccharide-activated MC26 cells escape from CTL cytotoxicity. Tumor-specific CTLs were derived *in vitro* by coculture of splenocytes from MC26 tumor-bearing mice with irradiated MC26 tumor cells. Lipopolysaccharide-activated MC26 cells were labeled with CFSE and used as targets for CTL at an effector/target ratio of 12:1. The data presented were generated from three independent experiments ($P < 0.01$, Student's *t* test). *D,* TLR4 regulates surface molecule expression in MC26 cells. MC26 cells were activated with lipopolysaccharide (1 μg/mL) for 4 hours and RT-PCR was done. The intensity of amplified DNA bands was analyzed by IQ Mac 1.2 software and relative expression levels were compared with the internal control GAPDH.

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sensitivity to CTL attack. MC26-specific CTLs were generated from MC26 tumor-bearing mice by coculture of spleen cells with irradiated MC26 cells. The cytolysis of tumor cells by CTL was measured by determining the ratio of 7-AAD$^+$CFSE$^+$/total CFSE$^+$ MC26 cells. As expected, CTL killing of lipopolysaccharide-stimulated MC26 cells was significantly lower than control (11.6% and 48.6%, $P < 0.01$, Fig. 3*C*). Then we examined the mRNA expression of B7 costimulatory family members and several members of TNF/TNFR superfamily in MC26 cells activated with lipopolysaccharide, because these factors are directly involved in cytotoxicity. Lipopolysaccharide stimulation up-regulated the expression of B7-H1 (8-fold increase), B7-H2 (5-fold increase), and CD40 (7-fold increase), but down-regulated the expression of Fas (2.5-fold decrease) compared with control (Fig. 3*D*). These results show that TLR4 activation renders MC26 cells resistant to CTL attack.

Blockade of tumor TLR4 signaling prolongs the survival of tumor-bearing mice. We used the MC26 s.c. tumor model to validate our *in vitro* results *in vivo*. BALB/c mice inoculated with TLR4 siRNA-expressing tumor cells survived much longer compared with the mice inoculated with MC26 cells expressing control siRNA (Fig. 4*A*), and the TLR4 siRNA tumors were significantly smaller (Fig. 4*A*). As an alternative approach, direct injection of the TLR4 peptide to local tumors retarded tumor growth and prolonged the survival of tumor-bearing mice relative to the mutated TLR4 peptide control (Fig. 4*B*). We hypothesized that T cells and NK cells within TLR siRNA or TLR4 peptide–treated tumors might differ from those in control tumors. We isolated T cells and NK cells from 10 to 12 mm diameter tumors and analyzed T cell proliferation driven by anti-CD3 and anti-CD28 antibodies or NK IFN-γ release. T cells and NK cells from tumors expressing TLR4 siRNA or treated with TLR4 peptide showed enhanced T cell proliferation and higher titers of NK cell derived IFN-γ than control groups ($P < 0.01$; Fig. 4*C* and *D*). Therefore, TLR4 stimulation both renders the tumor cells less sensitive to CTL attack, and also blunts the T cell and NK cell function.

Discussion

Our study shows that tumors from a variety of tissues express multiple TLRs, most dramatically TLR4. Furthermore, blockade of TLR4 signaling in MC26 cells retards tumor growth and prolongs the survival of tumor-bearing mice. Thus, the inhibition of TLR function in tumors may be beneficial to the host. However, other studies suggest that enhancement of TLR function is beneficial to drive DC maturation (9) and block CD4$^+$CD25$^+$ T cell regulatory function (10, 11). TLRs, thus may act as a double-edged sword, enhancing host immunity against the tumor by stimulating antigen presenting cells, and protecting the tumor from host surveillance.

Although the function of nitric oxide in tumor biology is still controversial, nitric oxide inhibits T cell activation and results in immune suppression (12). IL-6 inhibits dendritic cell maturation, NK T cell activation, and may also affect NK cell anergy (13–15). Both iNOS and IL-6 are induced in tumor cells by TLR4 activation, and we show that both nitric oxide and IL-6 inhibit T cell proliferation and NK cell activity. Thus, blocking TLR4 signaling may have a beneficial effect. In contrast, although TLR4 activation induces IL-12 expression, we found that neutralization of IL-12 had no effect. However, there are reports indicating that IL-12 also has suppressive effects on allogeneic or tumor-specific CTL generation (16, 17).

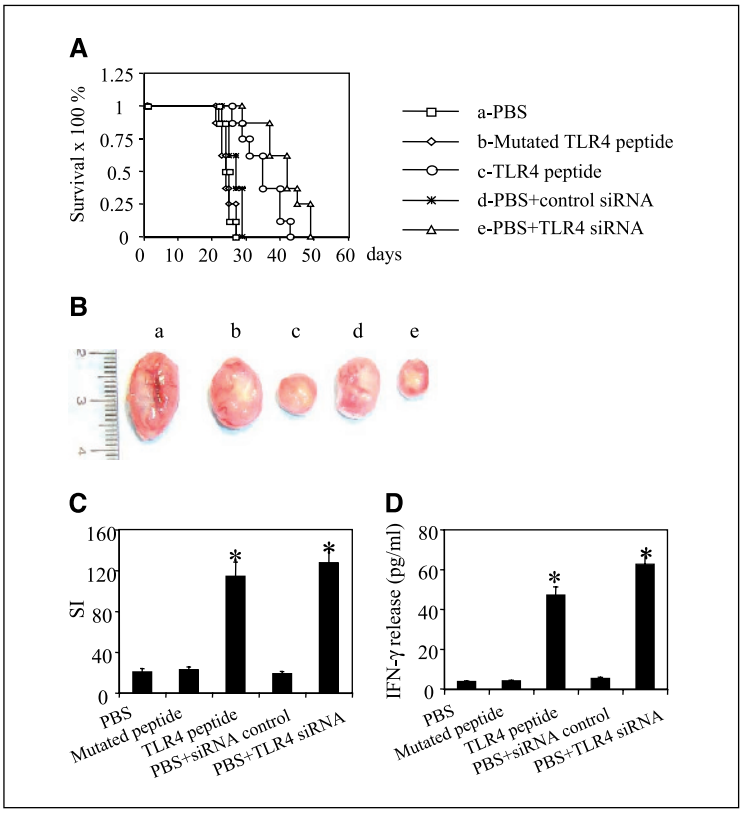

Figure 4. Blockade of TLR4 signaling prolongs survival of tumor-bearing mice. BALB/c mice were inoculated s.c. with 3×10^5 WT or TLR4 siRNA expressing MC26 tumor cells. Seven days after inoculation, TLR4 peptide was injected directly into the local tumor of WT tumor-bearing mice (100 μg/mouse, once every 3 days for 18 days). *A,* long-term survival of tumor-bearing mice. The survival period of the TLR4 siRNA-expressing tumor-bearing mice or peptide-treated mice was significantly prolonged compared with control groups ($n = 8$, $P < 0.002$; Kaplan-Meier analysis). *B,* TLR4 siRNA or TLR4 peptide inhibits tumor growth. The typical size of tumors on day 16 is shown. *C,* tumor-infiltrating T cell proliferation. T cells (10^5 cells per well) isolated from large tumors of each group were activated with anti-CD3 and CD28 (1 μg/mL). The stimulation index is the ratio of [^{3}H]thymidine incorporation in the presence of anti-CD3 and CD28/control without stimulation. Data are representative of two independent experiments. *, $P < 0.01$ (ANOVA). *D,* IFN-γ release from tumor-infiltrating NK cells. NK cells were isolated from large tumor tissues of various groups and after incubation for 24 hours in 96-well plates (3×10^4 per well) supernatants were assayed for IFN-γ by ELISA. *, $P < 0.01$ (ANOVA).

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In addition to soluble factors, surface molecules on tumor cells are also involved in tumor evasion from immune surveillance. B7-H1/PD1 interaction leads to T cell inactivation (2), and ligation of tumor cell CD40 results in resistance to apoptosis by tumor cells (18). Furthermore, down-regulation of Fas on tumor cells may facilitate tumor cell escape from CTL attack (4). Our data that lipopolysaccharide up-regulated the expression of B7-H1, B7-H2, and CD40, and down-regulated Fas expression is consistent with the decreased CTL cytotoxicity to lipopolysaccharide-activated tumor cells (Fig. 3*C*).

Because the endogenous ligand for TLR4 in tumors is not known, we have used lipopolysaccharide to activate the signal pathway. However, endogenous ligands for TLR4, such as Hsp70 and β-defensin2 have been identified (19, 20). Hsp70, which is abundantly expressed by tumor cells, may be a ligand for tumor TLR4. In addition, the tumor cell surface has a variety of altered glycan/lipid moieties that might activate TLRs. Identification of the endogenous ligands for tumor TLRs would help us understand the molecular mechanisms for tumor cell growth and escape from the host defense system.

Unregulated or inappropriate TLR activation resulting in excessive production of proinflammatory factors is involved in several inflammatory diseases. Our study indicates that tumor cells produce proinflammatory factors including nitric oxide, IL-6, and IL-12 upon TLR4 activation, mimicking some characteristics of inflammatory cells. The role of inflammation, thought to be mediated exclusively by immune cells, has to be reevaluated in light of these results. Finally, we suggest that these studies represent only a beginning in the analysis of TLR function in tumor biology, which may lead to discovery of new therapeutic targets in cancer therapy.

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