Toll-like Receptor 9 Activation of Signal Transducer and Activator of Transcription 3 Constrains Its Agonist-Based Immunotherapy

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Abstract

Although toll-like receptor (TLR) agonists, such as CpG, are used as immunotherapeutic agents in clinical trials for cancer and infectious diseases, their effects are limited and the underlying mechanism(s) that restrains CpG efficacy remains obscure. Here, we show that signal transducer and activator of transcription 3 (Stat3) plays a key role in down-modulating immunostimulatory effects of CpG. In the absence of interleukin-6 (IL-6) and IL-10 induction, CpG directly activates Stat3 within minutes through TLR9. Ablating Stat3 in hematopoietic cells results in rapid activation of innate immunity by CpG, with enhanced production of IFN-γ, tumor necrosis factor-α, IL-12, and activation of macrophages, neutrophils, and natural killer cells marked with Stat1 activation. Innate immune responses induced by CpG in mice with a Stat3-ablated hematopoietic system cause potent antitumor effects, leading to eradication of large (>1 cm) B16 melanoma tumors within 72 h. Moreover, ablating Stat3 in myeloid cells increases CpG-induced dendritic cell maturation, T-cell activation, generation of tumor antigen–specific T cells, and long-lasting antitumor immunity. A critical role of Stat3 in mediating immunosuppression by certain cytokines and growth factors in the tumor microenvironment has been recently documented. By demonstrating direct and rapid activation of Stat3 by TLR agonists, we identify a second level of Stat3-mediated immunosuppression. Our results further suggest that targeting Stat3 can drastically improve CpG-based immunotherapeutic approaches.

Introduction

Toll-like receptor (TLR) activation, originally studied in the context of microbial infections, is an attractive strategy for boosting prophylactic vaccines and anticancer therapies (1). The simplicity of the natural ligands for TLR9, fragments of unmethylated double-stranded DNA, allows for a large-scale synthesis of TLR9 agonists optimized for stability and cell type–specific and species-specific activation (2). Based on numerous studies in mice demonstrating their efficacy in breaking immune tolerance (3, 4) and promoting Th1-dependent immunity (2), several TLR9 agonists are currently used to treat solid and hematologic malignancies (1). Whereas TLR9 agonist–based therapies have no major toxicity and show some promise, the efficacy of the TLR9-mediated antitumor immune response is limited, which is largely attributed to the immunosuppression observed in cancer patients (1, 5).

It has been reported that antibody-mediated blockade of interleukin-10 (IL-10) restores TLR9-induced activation of tumor-infiltrating dendritic cells (DC), IL-12 production, and generation of antitumor immune memory (6). IL-10 is frequently overly produced in tumors and is a key activator of signal transducer and activator of transcription 3 (Stat3), which is activated in numerous cancers, promoting expression of diverse molecules important for tumor cell proliferation, survival, invasion, and metastasis (7). Recently, Stat3 was identified as an important mediator of tumor-induced immunosuppression at many levels (8–10). Several immunosuppressive factors commonly found in the tumor microenvironment, such as IL-10, IL-6, and vascular endothelial growth factor (VEGF; refs. 9, 11), are known to activate Stat3, leading to propagation of Stat3 activity from tumor cells to diverse immune cells infiltrating the tumor and affecting innate and adaptive antitumor immunity (9, 12, 13). Elevated Stat3 activity in tumor stromal immune cells further promotes expression of cytokines and growth factors, including but not limited to IL-10, IL-6, and VEGF, which in turn further activate Stat3 in tumor cells (9, 12, 13). This multidirectional Stat3 propagation facilitates the crosstalk between tumor cells and diverse immune cells in the tumor milieu, forming an immunosuppressive network (13). DCs are among the immune cells affected by Stat3 activity, which not only inhibits expression of Th1 cytokines, such as IL-12 and IFN-γ, but also reduces the levels of MHC class II, CD86 and CD80, contributing to the accumulation of tolerogenic DCs and regulatory T cells in the tumor (9, 12, 13). Whereas these observations document an important role for Stat3 in mediating immunosuppression in the tumor microenvironment, it remains to be further explored whether or not Stat3 constrains TLR9 activation.

What has become evident is that TLR agonists, such as CpG oligodeoxynucleotides (ODN), can down-modulate their own activity, which likely represents a physiologic mechanism for limiting collateral damage during infection (14). However, the mechanisms by which TLR agonists restrain TLR activity remain poorly defined. The current study tests the hypothesis that Stat3 constrains TLR activity and that targeting Stat3 can improve the efficacy of TLR9-based immunotherapy. We show that both TLR9 and TLR4 agonists activate Stat3 rapidly and continuously, which can occur in the absence of IL-6 and IL-10 induction. Furthermore, activation of Stat3 by CpG is abrogated in TLR9−/− immune cells. Removing Stat3 in hematopoietic cells in vivo allows much greater induction of several key Th1 cytokines, such as IFN-γ and IL-12, by CpG, activating components of innate immunity. Stat3 ablation, combined with local CpG treatment, activates innate and adaptive...
antitumor immune responses, leading to the rapid and long-term regression of large B16 melanoma tumors. To show the feasibility of future clinical applications, we show that systemic Stat3 targeting with a small molecule drug synergizes peritumoral CpG treatment. These results indicate that Stat3 restrains CpG-induced immune responses not only via tumor-induced immunosuppression but also by TLR9 activation itself. As such, targeting Stat3 is a potential viable strategy to improve various TLR9-based immunotherapeutic approaches.

**Materials and Methods**

**Cells.** Mouse B16 melanoma cells were purchased from American Type Culture Collection. Mouse C4 melanoma cells were generous gifts from Dr. J. Fidler (M. D. Anderson Cancer Center).

**In vivo experiments.** Mouse care and experimental procedures were performed under pathogen-free conditions in accordance with institutional guidance from Research Animal Care Committees of City of Hope. Mx1-Cre mice were purchased from the Jackson Laboratory. Stat3<sup>flox/flox</sup> mice were kindly provided by Dr. S. Akira and Dr. K. Takeda (University of Osaka). Generation of mice with Stat3<sup>−/−</sup> hematopoietic cells by inducible Mx1-Cre recombinase system has been reported (12, 15). For s.c. tumor challenge, B16 tumor cells (1 × 10<sup>6</sup>) were injected into 7-wk-old to 8-wk-old Mx1-Cre/Stat3<sup>flox/flox</sup> mice and Stat3<sup>flox/flox</sup> littermates 4 d after poly(I:C) treatment to induce Stat3 ablation. At 7 to 10 d later, mice were injected peritonally with 5 μg of phosphothioated CpG (CpG1668, TCCATGACGTTCCTGATGCT; GATGCT) or control GpC (GpC1668, TCCATGACGTTCCTGATGCT) ODN, and tumor growth was monitored trice weekly. For studies on CpG effects, mice were sacrificed at 1, 2, or 3 d after CpG treatment and spleens, lymph nodes, and tumor specimens were harvested. For immune cell depletion, mice were pretreated with anti-CD8 plus anti-CD4 antibodies (clones 2.43 and GK1.5, respectively) before tumor inoculation and then injected weekly. For treatment using Stat3 inhibitor CPA7 (12, 16), B16 and C4 tumors were implanted into male C57BL/6 or C3H mice, respectively, and allowed to grow until 5 to 7 mm in diameter. Mice were given i.v. injections of vehicle (10% DMSO/PBS) or CPA7 (5 mg/kg) twice weekly and treated with 5 μg CpG injected peritonally on the following days.

**Nitric oxide measurements.** CD11b-positive myeloid cells were magnetically separated from spleenocyte suspensions derived from tumor-bearing mice using EasySep-positive selection kits (StemCell Technologies) and incubated (48 h) with or without CpG (5 μg/mL). Supernatants collected from cultured cells were treated with 1% sulfanilamide and 0.1% N-1-naphthylethenediamine dihydrochloride. The absorbance was measured within 30 min on a microplate reader (520–550 nm band pass filter), normalizing for the basal nitrate content in the RPMI 1640. Nitrate concentration in each sample was calculated in relation to a standard curve.

**Flow cytometry.** Single-cell suspensions of spleen, lymph node, or tumor tissues were prepared by mechanic disruption followed by collagenase D/DNase I treatment as described (12). For extracellular staining, freshly prepared cells suspended in PBS/2% FCS/0.1% w/v sodium azide were stained with fluorochrome-coupled antibodies to CD11c, I-A<sup>+</sup> (MHCI), CD40, CD80, CD86, CD11b, CD49b, CD3, CD8, CD4, or CD69 (BD Biosciences). Before intracellular staining with antibodies to phosphotyrosine Stat3 and Stat1 (BD Biosciences), cells were fixed in paraformaldehyde and permeated in methanol. For intracellular cytokine staining using antibodies specific for IL-6 and IL-10 (BD Pharmingen), cells were incubated (4 h) with Leukocyte Activation Cocktail (BD Pharmingen) and stained according to the manufacturer’s protocol. Fluorescence data were collected on a FACSAria (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**ELISPOT assays.** Lymph nodes were harvested from Stat3<sup>−/−</sup> and Stat3<sup>−/−</sup> mice 2 wk after B16 tumor challenge and 3 d after CpG treatment. Lymph node cells were seeded into 96-well filtration plate (5 × 10<sup>5</sup> per well) in the presence or absence of p15E peptide (10 μg/mL) and incubated (37°C, 24 h). Peptide-specific IFNγ-positive spots were detected following the manufacturer’s protocol (Cell Sciences), scanned, and quantified using Immunospot Analyzer (Cellular Technology Ltd.).

**Immunohistochemistry, immunofluorescence, and intravitral two-photon microscopy.** Immunohistochemical staining with H&E (DAKO) was performed on formalin-fixed and paraffin-embedded tissue sections (5 μm). Flash-frozen tumor specimens were fixed in 2% paraformaldehyde, permeabilized with methanol, and stained with antibodies specific to neutrophils (7/4, Cedarlane), macrophages (Mac3), and inducible nitric oxide (NO) synthase (iNOS; BD Biosciences), and detected with fluorochrome-coupled secondary antibodies (Invitrogen). After staining the nuclei (Hoechst 33342, Invitrogen), slides were mounted and analyzed by fluorescence microscopy. For intravitral two-photon imaging, CpG-treated Stat3<sup>−/−</sup> and Stat3<sup>−/−</sup> B16 tumor-bearing mice received a single retroorbital injection of dextran-rhodamine (Invitrogen) 1 h before imaging. Mice were anesthetized, and intravitral two-photon microscopy was carried out using the equipment and software from Ultima Multiphoton Microscopy Systems.

**Electromobility shift assay, Western blotting, and antibody arrays.** Electromobility shift assay to detect Stat3 DNA-binding and Western blot analyses were performed as described (12). Cytokine antibody arrays for detecting cultured DC protein secretion were developed according to the manufacturer’s protocol (Panomics).

**Statistical analysis.** Unpaired t test was used to test differences between two treatment groups. Statistically significant P values were labeled as follows: ***, P < 0.001; **, P < 0.01; *, P < 0.05. Data were analyzed using the Prism software (GraphPad).

**Results**

Direct and rapid induction of Stat3 activity in DCs by TLR agonists. Whereas it has recently become evident that TLR agonists can induce their own inhibition (17–19), the role played by TLRs in mediating this feedback mechanism remains poorly understood. Because Stat3 is known to inhibit the expression of multiple Th1 immunostimulatory cytokines (9, 13), we tested the possibility that certain TLR agonists might directly activate Stat3, thereby leading to the down-modulation of TLR activity. We first tested Stat3 activation by TLR agonists in freshly prepared splenic CD11c<sup>+</sup> DCs. At 3 hours after treatment, lipopolysaccharide (LPS), agonist of both CpG and TLR4, induced tyrosine phosphorylation of Stat3, whereas poly(I:C), a TLR3 agonist shown to have potent antitumor immunostimulatory effects (20), did not (Fig. 1A). We further assessed the effects of CpG on the activation of Stat3, as well as Stat1 and Stat5 (Fig. 1B). Like Stat3, both Stat1 and Stat5 were phosphorylated on tyrosine residues critical for their dimerization within the first 2 hours after CpG treatment. However, their activation was transient compared with phosphorylated Stat3 levels, which still remained elevated after 24 hours. Both the brief Stat1 and prolonged Stat3 activation induced by CpG within the first 24 hours was confirmed by testing DNA binding activity in primary splenocytes (Supplementary Fig. S1).

It has been suggested that CpG can activate IL-10, a known Stat3 activator, through a negative feedback mechanism (18). Because the kinetics of Stat3 activation by CpG occurs rapidly, it is highly unlikely that Stat3 activation involves induced expression of IL-6 or IL-10. To rule out the participation of these Stat3-activating cytokines in mediating CpG-induced rapid Stat3 activation, we measured IL-6 and IL-10 levels in splenic CD11c<sup>+</sup> DCs at 4 and 24 hours after CpG stimulation. Our data indicated that, although CpG treatment induced higher levels of IL-6 at 24 hours, there was no detectable induction within 4 hours (Fig. 1C) in contrast to CpG-induced rapid Stat3 activation (Fig. 1B). The induction of IL-10 within the tested time points was negligible. Finally, we also observed induction of Stat3 activity in response to CpG treatment
in splenic DCs isolated from IL-10--deficient mice, but not in TLR9--deficient DCs (Fig. 1D). These results show direct TLR9-mediated Stat3 activation triggered by CpG ODN.

Stat3 signaling in DCs restrains TLR9 agonist--induced expression of Th1 type cytokines and chemokines. To directly test the role of Stat3 in restraining TLR9 agonist--induced Th1 type immune responses, we examined the effects of Stat3 ablation on CpG-induced Th1 cytokines and chemokine expression in Stat3<sup>−/−</sup> and Stat3<sup>−/−</sup> DCs. Wild-type (WT) splenic DCs stimulated in vitro with CpG ODN secreted several proinflammatory mediators, including IL-12, MIG (CXCL9), macrophage inflammatory protein 1α (CCL3), RANTES (CCL5), and IL-6, as well as low levels of IFNγ and tumor necrosis factor α (TNFα; Fig. 2A). Both IFNγ and TNFα expression levels stimulated by CpG were drastically increased upon Stat3 ablation by 8-fold and 26-fold, respectively (Fig. 2B). In addition, after TLR9 activation, Stat3<sup>−/−</sup> DCs secreted immunosuppressive IP-10 (CXCL10). Results from real-time PCR confirmed very strong induction of innate immunity activators, such as IFNβ, IFNγ, TNFα, and IL-6, in splenic Stat3<sup>−/−</sup> DCs after 4 hours of CpG stimulation ex vivo compared with WT DCs (Supplementary Fig. S2A). Similarly, the mRNA expression of IL-12, RANTES, and IL-6 was up-regulated in vivo within 18 hours after peritumoral injection of CpG, as determined in DCs freshly isolated from
tumors (Supplementary Fig. S2B) or mouse tumor-draining lymph nodes (Supplementary Fig. S2C).

**Targeted Stat3 ablation allows potent antitumor innate immune responses by TLR9 triggering.** To assess whether Stat3 restrained CpG-induced innate immunity against tumors, we induced Stat3 allele truncation in hematopoietic cells of adult mice using the Mx1-Cre-loxP system (12). In the Cre-loxP system, in addition to hematopoietic cells, some organs also undergo partial Stat3 truncation upon poly(I/C) treatment (21). To avoid interference from poly(I/C) treatment, s.c. B16(F10) tumor challenge was performed 4 days after the last poly(I/C) administration. Established B16 tumors (>1 cm diameter) were treated 10 days later with a single peritumoral injection of CpG1668 oligonucleotide (Fig. 3A). Our previous study showed that Stat3 ablation in myeloid cells leads to activation of tumor-infiltrating immune cells and antitumor immune response (12). To assess the combined effects of Stat3 targeting and CpG treatment on established tumors, the experimental conditions were setup to allow tumor-induced immunosuppression to mask the effects of Stat3 ablation. Relative to our previous study, we doubled the number of tumor cells for the initial challenge and allowed B16 tumors to reach a larger size before treatment (diameter exceeding 1 cm versus 3–5 mm). Under these conditions, no significant difference in tumor size in Stat3+/+ and Stat3−/− mice was noted (Fig. 3B). Although CpG treatment did not show significant antitumor activity in control littermates (Stat3+/+) with heavy tumor load, the same treatment resulted in the eradication of large B16 tumors (some reaching 1.5 cm in diameter) within 3 days after injection in mice lacking intact Stat3 alleles (Fig. 3A and B). In contrast, in WT mice with smaller initial B16 tumors (4–6 mm diameter at day 7), single treatment with CpG only reduced tumor growth similar to the effect of Stat3 truncation alone (Fig. 3C). However, combination of Stat3 ablation with local TLR9 stimulation led to complete regression of rapidly growing B16 tumors (Fig. 3C). To gain a glimpse of the tumor in vivo after CpG treatment/Stat3 ablation, we examined tumor vasculature by intravital two-photon imaging. Whereas tumors treated only with CpG displayed relatively intact vasculature, peritumoral injection of

![Figure 3](https://example.com/figure3.png)

**Figure 3.** CpG ODN triggers rapid eradication of large B16 tumors in mice with Stat3 ablation. Mice with Stat3+/+ or Stat3−/− hematopoietic cells were challenged with B16 melanoma cells (1 × 10⁶ s.c.). Mice with established tumors were treated with a single peritumoral injection of CpG ODN (5 μg). A, tumor size before and after CpG treatment. B and C, changes in tumor volume within 3 d after CpG treatment; two independent experiments (n = 4 mice per group), with either larger, −10 mm (B), or smaller, −5 mm (C), average tumor diameter at the time of CpG ODN injection. Columns, mean; bars, SE. D, two-photon intravital imaging of vasculature stained using dextran-rhodamine within tumor or tumor-draining lymph node in Stat3+/+ or Stat3−/− mice 18 h after CpG ODN treatment. Images composed of multiple scans from 52 μm z-dimensional stack (two independent experiments). Scale bars, 100 μm.
CpG into mice with Stat3 truncation in hematopoietic cells led to a complete disruption of tumor vasculature within 18 hours (Fig. 3D). A closely located tumor-draining lymph node served as a positive control and showed a normal vasculature pattern. These in vivo data further implicated rapid antitumor effects by CpG in the absence of Stat3.

**Cellular mechanisms for CpG/Stat3 ablation–induced antitumor innate immune responses.** Because CpG treatment in mice with conditional Stat3 knockout led to rapid tumor regression, we assessed the underlying cellular mechanism(s) responsible for the antitumor innate immunity. We observed that within the first 18 hours after CpG injection, the inflammatory cell populations migrating into tumor tissues of Stat3-deficient mice were predominantly neutrophils (Fig. 4A and Supplementary Fig. S3) and, to a lesser extent, macrophages (Fig. 4B). Immunohistochemical staining of B16 tumor specimen using apoptosis marker (active caspase-3) revealed induction of cell death in tumor areas strongly infiltrated by neutrophils within 24 hours after CpG injection and necroses by 48 hours (Supplementary Fig. S4). Because increased NO synthesis by activated macrophages is known to induce antitumor innate immune response, we also assessed the level of iNOS expression in tumor-infiltrating Mac3+ cells within 24 hours after CpG treatment (Fig. 4B). iNOS expression was detectable only in Stat3+/−/− mice treated with CpG and mostly overlapped with the staining for macrophage-specific (Fig. 4B), but not neutrophil-specific, markers.3 In vitro experiments confirmed that Stat3 ablation led

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3 Unpublished data.
to increased nitrate levels secreted by CD11b+ myeloid cells in response to TLR9 stimulation (Fig. 4C). These results showed that innate immunity caused by Stat3 ablation contributed to the observed rapid tumor regression after CpG ODN injection.

In addition to macrophages and neutrophils, natural killer (NK) cells can also play an important role in antitumor innate immunity. To determine if ablating Stat3 promotes CpG-induced NK cell activity, we used flow cytometry to assess the presence of NK cells in tumor tissues 24 hours after CpG injection. In vivo treatment with CpG enhanced the number of tumor-infiltrating NK cells in mice with a Stat3-negative hematopoietic system but failed to stimulate NK activity in WT tumor-bearing controls (Fig. 4D, left). Stat1 activation has been shown to be critical for NK antitumor activity (22). Flow cytometric analysis of phosphorylated Stat1 in NK cells prepared from tumor-draining lymph nodes indicated that Stat3 ablation allowed strong Stat1 activation by CpG ODN (Fig. 4D, right).

Ablating Stat3 sensitizes DCs to TLR9-mediated priming and augments tumor antigen–specific immunity. We next assessed if Stat3 inhibition affects CpG-induced DC activation in tumor-bearing mice, as DCs are critical for the regulation of T-cell antitumor immunity (20, 23). Flow cytometric analysis of CD11c+ DCs isolated from tumor-draining lymph nodes of mice lacking Stat3 in hematopoietic cells showed enhanced DC activation, as measured by increased expression of MHC class II, together with costimulatory molecules CD86 (Fig. 5A, two top rows) or CD80 and CD40 (Fig. 5A, two bottom rows) 2 days after peritumoral injection of CpG but not control GpC ODN. Control in vitro experiments on CpG-treated splenic DCs (with low endogenous Stat3 activity) further confirmed that TLR9-induced Stat3 limits DC activation (Supplementary Fig. S5). Furthermore, whereas tumor-isolated WT CD11c+ DCs displayed little Stat1 activation after CpG, their Stat3−/− counterparts showed high levels of phosphorylated Stat1 (Fig. 5B).

To evaluate the effect of Stat3 ablation on CpG-induced effector lymphocyte activity, we analyzed CD8+ T cells within tumor-draining lymph nodes of Stat3-positive and Stat3-negative mice after CpG ODN treatment. CD8+ lymphocytes in tumor-draining lymph nodes of Stat3-ablated mice showed high levels of CD69 activation marker 24 hours after peritumoral injection of CpG (Fig. 5C). Importantly, 10 days after CpG treatment, Stat3-deficient mice displayed enhanced ability to mount tumor antigen–specific T-cell immune response. ELISPOT assays, after ex vivo exposure to the B16 tumor–specific p15E peptide antigen, indicated significantly higher numbers of IFNγ-secreting T cells in tumor-draining lymph nodes derived from Stat3−/− mice treated with CpG, relative to their WT or untreated counterparts (Fig. 5D).

Ablating Stat3 in myeloid cells strongly promotes CpG-mediated antitumor immune responses. We next assessed whether Stat3 restrained the long-term effects of TLR9 triggering on tumor growth. CpG treatment prevented tumor outgrowth for over 3 weeks in mice with Stat3-deficient myeloid cells (Fig. 6A). Importantly, the prolonged antitumor immunity was abrogated after Stat3−/− mice were depleted of CD4+ and CD8+ T cells. In the absence of CD4+ and CD8+ T cells, tumors reoccurred within the primary tumor site ∼10 days later, suggesting the role of T cells in the development of antitumor memory responses. Lack of both lymphocyte populations did not prevent the initial robust tumor regression, confirming the critical role of innate immunity in eliminating established tumors. This is consistent with the fact that, regardless of Stat3 status, only a few CD8+ T cells were present in tumor specimens within 18 hours after CpG stimulation (Supplementary Fig. S6). In agreement with our previous study (12), Stat3 ablation alone reduced tumor growth rate but did not lead to complete tumor regression (Fig. 6A). Treatment with control CpG oligonucleotide did not significantly inhibit tumor progression.4 These results suggested that Stat3 restrained CpG-induced adaptive antitumor immune responses.

**Superior antitumor efficacy by combining small molecule Stat3 inhibitor with TLR9 agonist.** Our genetic studies showed that eliminating Stat3 signaling in myeloid cells allowed potent local and systemic antitumor responses induced by peritumoral injection of CpG ODN. We then used a small molecule inhibitor, CPA7 (12, 16), to confirm the therapeutic potential of such a strategy. The therapeutic effect of systemic Stat3 inhibition followed by local treatment with CpG was tested using two metastatic tumor models, B16F10 (Fig. 6B) and C4 melanoma (Fig. 6C). In both tumor models, CpG/Stat3 inhibitor combination yielded the strongest tumor growth retardation compared with the effects of each reagent alone. In addition, only cotreatment with the Stat3 inhibitor and CpG produced sufficient concomitant antitumor immunity to prevent B16F10 tumor outgrowth at the site of the secondary challenge (Fig. 6D). Similar to our genetic studies, the combinatorial therapy might have coactivated mechanisms of innate and adaptive immunity because CD8 depletion only partially affected the initial antitumor effect of CPA7/CpG treatment but clearly prevented the long-term mice survival (Supplementary Fig. S7).

**Discussion**

We and others have identified a critical role for Stat3 in inducing immunosuppression in the tumor microenvironment by inhibiting the expression of Th1 immunostimulatory molecules and promoting the expression of several immunosuppressive factors (9, 24). In the tumor setting, activation of Stat3 is induced by cytokines, such as IL-10, IL-6, and growth factors, including VEGF and basic fibroblast growth factor, among many other tumor-associated factors. Our current study showed that Stat3 was a key molecule limiting the efficacy of TLR agonists. We showed that both TLR9 and TLR4 agonists activated Stat3 rapidly and that CpG ODN activated Stat3 directly through TLR9. Moreover, targeted deletion or pharmacologic targeting of Stat3 allowed potent antitumor innate and adaptive immune responses after CpG treatment, leading to the regression of established tumors. These findings revealed a second mechanism by which Stat3 mediates immunosuppression: activation by TLRs without the induction of cytokines and growth factors. Our results also showed that targeting Stat3 can drastically improve CpG-based immunotherapies.

Although signaling through TLR9 also involves two other Stat factors, Stat1 and Stat5, their early immediate activation is transient. The sustained Stat activity apparently requires the production of secondary mediators like IL-6 and/or IL-10 for Stat3 (Supplementary Fig. S2; ref. 18) or IFNγ and IFNγ for Stat1 (Supplementary Fig. S2; ref. 25). However, because Stat3 strongly inhibits IFN expression, it is likely that immunostimulation by the secondary effect of TLR9 activation is affected by Stat3 activity.

Currently, CpG-based immunotherapies involve both prophylactic vaccines and therapeutic antitumor approaches. Our results...
Figure 5. Ablating Stat3 promotes TLR9-induced DC maturation and antigen-specific T-cell responses. Stat3 deletion enhances CpG-induced tumor DC maturation in vivo. A, the phenotypic analysis of CD11c+ DCs accumulated in tumor-draining lymph nodes of Stat3+/+ and Stat3−/− mice 48 h after CpG injection. The maturation of CD11c+ DCs was increased by Stat3 ablation, as shown by a greater percentage of double-positive MHC class II+ and CD86+ DCs (top two rows), and higher expression of CD80 and CD40 on DCs (bottom two rows), as seen by FACS analyses (three independent experiments; n = 3–4 mice per group). B, Stat1 is activated in Stat3−/− DCs from tumor-draining lymph nodes following CpG but not control GpC ODN treatment. Intracellular flow cytometric analysis of DCs isolated from tumor-draining lymph nodes using an antibody to detect tyrosine-phosphorylated Stat1 (Phospho-Stat1; three mice per group). C, Stat3 deletion promotes tumor antigen–specific T-cell responses. Expression of the early lymphocyte activation marker CD69 was analyzed by flow cytometry on CD8+ T cells 24 h after injecting CpG or control GpC ODNs (three independent experiments using lymph node cell suspensions; n = 3–4 mice per group). D, Stat3−/− mice mount a stronger T-cell response against an endogenous B16 tumor antigen than their Stat3+/+ counterparts after treatment with CpG ODN. IFNγ production in T cells derived from tumor-draining lymph node was assessed by ELISPOT assay. Columns, mean of p15E-specific IFNγ-producing cells; bars, SE (two independent experiments; n = 4 mice per group).
suggest that injection of CpG ODN in individuals without chronic pathogen infection or cancer may also lead to Stat3 activation, which limits CpG Th1 immunostimulatory effects. For cancer therapies, previous studies in mice showed improved TLR9-mediated therapeutic activity when CpG treatment was combined with blocking the immunosuppressive effects of IL-10 (6) or regulatory T cells (26) using neutralizing antibodies. The role of Stat3 as a critical mediator of tumor-induced immunosuppression is supported by numerous studies demonstrating Stat3-mediated inhibition of antigen presentation (13). Additionally, IL-10 is a key factor responsible for activating Stat3 in immune cells in the tumor milieu. Because IL-10 activates Stat3 and Stat3 is required for IL-10 expression, the anti–IL-10 antibody–mediated enhanced antitumor efficacy induced by CpG is likely due to the reduction in Stat3 signaling. A role of TLR in inducing regulatory T cells in tumors has also been shown, and recent reports indicate a possible function for Stat3 in promoting regulatory T-cell function (27). Because both TLR4 and TLR9 agonists activate Stat3, it is possible that regulatory T cells induced by TLR are in part mediated by Stat3 activation. In addition, as recently shown by Cheng and colleagues (28) and our own studies (12, 29), Stat3 is critical for the accumulation and function of CD11b+Gr1+ myeloid-derived suppressor cells in tumor-bearing mice. Our results indicate that combination of Stat3 ablation and TLR9 activation may convert immunosuppressive/proangiogenic myeloid-derived suppressor cells into cytotoxic neutrophils and stimulate their massive recruitment into tumor site.

A role for TLRs in recognizing a variety of pathogen-associated patterns and thereby initiating innate and adaptive immunity has been well appreciated. However, recent evidence indicates that TLRs are also expressed by tumor cells that promote tumor cell proliferation and survival, as well as invasion and metastasis. A critical role of Stat3 in up-regulating a large number of genes critical for proliferation, survival, angiogenesis, and metastasis has been widely documented (7). Recent research in our laboratory further indicated that activated Stat3 in tumor-associated diverse immune cells, especially in myeloid cells, stimulated the expression of many factors involved in immunosuppression, tumor invasion, and metastasis in these cells (29). Because CpG and LPS directly activate Stat3, it is likely that the observed tumor-promoting effects of TLRs (and their agonists; ref. 14) are partially mediated by Stat3 activation. This is consistent with an emerging role for Stat3 in wound healing (30, 31), a physiologic process involving immunosuppression; cell proliferation, invasion, and angiogenesis; and

Figure 6. Stat3 targeting in vivo improves TLR9-mediated antitumor immunity. A, single peritumoral injection of CpG results in long-term complete rejection of tumors in Stat3−/− but not in Stat3+/+ mice through CD4-mediated and CD8-mediated immunity. Mice with established B16 tumors were treated with CpG ODNs. Depleting antibodies against CD4+ and CD8+ T cells or control rat IgG were given to the indicated group of mice (three independent experiments; n = 6 mice per group). Statistically significant differences between the CpG-treated Stat3−/− group of mice with or without CD4/CD8 T-cell depletion are indicated with asterisks: **, P < 0.01; *, P < 0.05. B-D, growth of B16F10 (B) and C4 (C) tumors is significantly inhibited when peritumoral CpG ODN treatment is combined with systemic inhibition of Stat3 activity by a Stat3 inhibitor, CPA7. Mice with established tumors (average diameter, 4–8 mm) were treated with CPA7, followed by peritumoral CpG injection a day later. The treatment was repeated twice weekly. Results of three (B) and two (C) independent experiments. D, combination of Stat3 targeting with CpG-treatment generates concomitant antitumor immunity. Surviving mice treated as in B were rechallenged with the same number of B16F10 cells injected in the opposite flank 2 wk after the first tumor challenge. Statistically significant differences between the group treated with combination of CPA7 with CpG compared with controls treated with CpG only are indicated with asterisks: **, P < 0.01; *, P < 0.05.
characteristic of cancer development. However, whereas wound healing is self-limiting, cancer progression is not. Taken together, blocking Stat3 not only lifts the “brake” on TLR-mediated immune responses but also reduces the oncogenic potential mediated by TLR activation. It is also interesting to note that, unlike LPS and CpG, polyI:C did not rapidly induce Stat3 activity. A recent publication supports its use for cancer immunotherapies (20).

Despite several challenges, TLR9 agonists remain attractive anticancer agents because they have shown some efficacy and are well tolerated in patients, with relatively minor adverse effects (1). Our study strongly supports the use of combination therapies in which TLR9 activation is preceded by or simultaneous with Stat3 targeting. Although the potential side effects resulting from Stat3 targeting remain to be fully determined, first insights into possible effects of Stat3 deletion in the human system were revealed in patients with hyper-IgE syndrome (32, 33). These patient studies suggested that long-term Stat3 inhibition could cause immune disorders and complications due to microbial infections, that is, manageable chronic diseases. Therefore, temporary Stat3 inhibition by small molecule drugs or small interfering RNA may not generate severe undesirable effects. Our studies support the use of Stat3 targeting to improve the outcome of immunotherapies based on TLR activation.

Disclosure of Potential Conflicts of Interest

The authors have no conflicting financial interests.

Acknowledgments

Received 8/5/2008; revised 11/21/2008; accepted 12/22/2008; published OnlineFirst 3/3/09.

Grant support: This work is funded by grants from Harry Lloyd Charitable Trust, Keck Foundation, NIH R01CA122976, and by the Board of Governors at City of Hope. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. H. Kay of Paradigm Biotech LLC for providing CPAT, Dr. S. Akira and Dr. K. Takeda of University of Osaka for Stat3flox mice, the members of the pathology core at City of Hope for tissue processing, the staff members at the animal facilities and flow cytometry cores at City of Hope for their dedication, and Dr. S. Da Costa for editing the manuscript.

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