Attenuating Regulatory T Cell Induction by TLR Agonists through Inhibition of p38 MAPK Signaling in Dendritic Cells Enhances Their Efficacy as Vaccine Adjuvants and Cancer Immunotherapeutics[1]

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TLR ligands are potent adjuvants and promote Th1 responses to coadministered Ags by inducing innate IL-12 production. We found that TLR ligands also promote the induction of IL-10-secreting regulatory T (Treg) cells through p38 MAPK-induced IL-10 production by dendritic cells (DC). Inhibition of p38 suppressed TLR-induced IL-10 and PGE_2 and enhanced IL-12 production in DC. Incubation of Ag-pulsed CpG-stimulated DC with a p38 inhibitor suppressed their ability to generate Treg cells, while enhancing induction of Th1 cells. In addition, inhibition of p38 enhanced the antitumor therapeutic efficacy of DC pulsed with Ag and CpG and this was associated with an enhanced frequency of IFN-γ-secreting T cells and a reduction of Foxp3$^+$ Treg cells infiltrating the tumors. Furthermore, addition of a p38 inhibitor to a pertussis vaccine formulated with CpG enhanced its protective efficacy in a murine respiratory challenge model. These data demonstrate that the adjuvant activity of TLR agonists is compromised by coinduction of Treg cells, but this can be overcome by inhibiting p38 signaling in DC. Our findings suggest that p38 is an important therapeutic target and provides a mechanism to enhance the efficacy of TLR agonists as vaccine adjuvants and cancer immunotherapeutics. *The Journal of Immunology,* 2008, 180: 3797–3806.

R egulatory T (Treg)[5] cells play a central role in immune homeostasis and in preventing autoimmune diseases (1). Natural Treg cells that express Foxp3 and Ag-specific Treg cells that secrete IL-10 and/or TGF-β, termed Tr1 or Th3 cells, play a protective role in immunity to infection by controlling infection-induced immunopathology (2). However, induction of Treg cells is also a potent immune subversion strategy developed by many pathogens to suppress protective immune responses and to prolong their survival in the host (2). Furthermore, tumor growth is associated with recruitment and activation of Treg cells (3). Depletion of Treg cells can enhance the development of protective T cell responses during chronic infection (4) and can enhance the induction of antitumor immunity (5, 6). Indeed, the potent immunosuppressive effects of Treg cells may in part explain the failure of many immunotherapeutic approaches to cancer (7, 8). Treatment with cyclophosphamide to reduce suppressor cells has been shown to enhance antitumor immunity during vaccination in melanoma patients (9), but it is now recognized that more specific strategies are required to eliminate Treg cells to improve the efficacy of antitumor immunotherapeutics (7, 8). Similar strategies are required to enhance the efficacy of poorly immunogenic prophylactic infectious disease vaccines and for therapeutic vaccination in chronic infections.

New approaches to tumor immunotherapy and vaccination have focused on enhancement of effector T cell responses by targeting innate immune cells and the receptors that mediate their activation. Pathogen recognition receptors, in particular TLRs, have been the primary targets for activation of innate immune cells. Indeed, it has already been demonstrated that TLR agonists are potent adjuvants for infectious disease Ags and have also been used as immunotherapeutic agents in cancer patients (7, 10). TLR agonists activate maturation and promote inflammatory cytokines, including IL-12, from dendritic cells (DC) and thereby promote IFN-γ-secreting Th1 cells, NK cells, and CD8$^+$ CTL. With the exception of ligands for TLR2, which drive the induction of Th2 cells (11), most TLR agonists have been shown to promote Th1 cells (12–14). We and others have also demonstrated that certain pathogen-derived molecules, that enhance IL-10 and inhibit IL-12 production by DC, promote the induction of IL-10-secreting Treg cells (15–19).

In this study, we demonstrate that TLR agonists simultaneously induce IL-10 and IL-12 production by DC and promote the generation of both Th1 and IL-10-secreting Treg cells. We found that IL-10, but not IL-12, production by DC was mediated by TLR

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signaling through the MAPK p38. Selective inhibitors of p38 suppressed IL-10 and enhanced IL-12 production from DC and consequently enhanced Th1 responses and suppressed their ability to direct the induction of Treg cells. When applied to disease models, we found that p38 inhibition improved the therapeutic efficacy of TLR ligand-activated DC immunotherapy against tumors and protective immunity induced with a vaccine against *Bordetella pertussis*.

Materials and Methods

Animals and immunizations

C57BL/6 and BALB/c mice were obtained from Harlan U.K. IL-10$^{-/-}$ and DO11.10 OVA TCR-transgenic (Tg) mice were obtained from The Jackson Laboratory and bred in-house. Animal experiments and maintenance were approved and regulated by the university ethics committee and the Irish Department of Health. Mice were immunized s.c. with keyhole limpet hemocyanin (KLH; 5 μg), KLH and CpG (CpG-oligodeoxynucleotide (ODN) 1668; 5′-tccatgacgttccgatgct-3′; Sigma-Genosys; 25 μg), a control ODN with the reverse GpC sequence (20 μg), LPS (20 μg), poly(I:C) (25 μg), or cholera toxin (CT; 10 ng). Alternatively, mice were immunized i.p. with an acellular pertussis vaccine (Pa) and alum or 0.01–0.2 of human doses of Pa alone or with 5 or 50 μg of CpG or with a 0.2-human dose of a whole-cell pertussis vaccine (Pw).

Tumor challenge model

The B16F10 tumor cell line was maintained in DMEM supplemented with 10% heat-inactivated FCS and forms solid tumors in C57BL/6 mice when challenged s.c. Mice were injected s.c. with 2 × 10^5 tumor cells. Mice were routinely monitored for tumor growth. Tumor size was measured in two dimensions by calipers and determined by the following formula: (width^2) × length) × π/6, where width is the lesser value. Mice were killed when tumor length measured >15 mm.

Bordetella pertussis challenge model

Mice were challenged by aerosol exposure to live *B. pertussis* 14 days after two immunizations and *B. pertussis* CFU in lungs determined at intervals after infection as previously described (20).

Ag-specific cytokine production

Lymph node (LN) cells (2 × 10^6/ml) removed 7 days after immunization were cultured with KLH (50 μg/ml), sonicated *B. pertussis* (5 μg/ml), or medium only. Supernatants were removed after 72 h and IL-4, IL-10, and IFN-γ concentrations were determined by ELISA.

Ag-specific T cell lines

KLH-specific CD4$^+$ T cell lines were established from mice immunized with KLH and CpG by stimulating spleen or LN cells with Ag (10 μg/ml KLH) as previously described (15). This protocol generated conventional Th1 cells (IFN-γ^+IL-10$^-$IL-4$^-$CD4$^+$), but also a population of IFN-γ^+IL-10$^+$IL-4$^-$CD4$^+$ T cells (designated Th1Tr cells). To generate Tr1-type T cells (IFN-γ^-IL-10$^+$IL-4$^-$CD4$^+$), spleen or LN cells were initially stimulated with Ag (KLH) in the presence of anti-IFN-γ-neutralizing Ab, followed by the addition of IL-2 on day 5. On days 10–12, cells were washed and restimulated with Ag and APC (2 × 10^6/ml irradiated spleen cells) in the absence of added Ab. T cell lines, established after two to three rounds of Ag restimulation, were cultured with KLH (10 μg/ml) and APC and, after 3 days, the concentrations of IFN-γ, IL-4, and IL-10 in supernatants were determined by ELISA.

Suppressor assay

KLH-specific Th1 (IFN-γ^+IL-10$^-$IL-4$^-$CD4$^+$), Tr1 (IFN-γ^-IL-10$^+$IL-4$^-$CD4$^+$), and Th1Tr (IFN-γ^+IL-10$^+$IL-4$^-$CD4$^+$ cell lines were established from mice immunized with KLH and CpG as described above. The Th1 cell line (1 × 10^5/ml) was cultured with APC (2 × 10^6/ml) and KLH alone or in the presence of Tr1 or Th1Tr cells at a ratio of 1:3, 1:1, or 3:1. Supernatants were removed after 3 days and the concentration of IFN-γ was tested by ELISA.

Detection of intracellular cytokines

LN cells were cultured with Ag (KLH) and, after 6 days, cells were restimulated for 6 h with PMA (10 ng/ml) and ionomycin (1 μg/ml); brefeldin A (10 μg/ml) was added for the final 4 h. Alternatively, single-cell

suspensions were prepared from LNs or tumors. Tumors were digested in HBSS with 0.1% collagenase D (Sigma-Aldrich). Cells were stimulated with PMA and ionomycin for 1 h, then brefeldin A was added for 4 h at 37°C. Cells were resuspended with Abs specific for either CD4 (Caltag Laboratories) or CD8 (BD pharMingen). Cells were then fixed, permeabilized, and incubated with anti-IFN-γ or anti-IL-10 Abs (BD Pharmingen) according to the manufacturer's instructions (Fix & Perm Cell Permeabilization Kit; Caltag Laboratories). Intracellular Foxp3 staining was performed according to the manufacturer's instructions (eBioscience). Briefly, cells were stained for Abs to CD4 and CD25 at 4°C for 30 min. Cells were then fixed, blocked, and permeabilized with anti-mouse/rat Foxp3 (FJK-16s) at 4°C for 30 min and washed. Immunofluorescence was analyzed using CellQuest software on a FACSCalibur (BD Biosciences).

DC activation

Mouse bone-marrow derived immature DC were generated as previously described (20). DC were incubated with 1 ng/ml to 10 μg/ml of the TLR agonists LPS (*Escherichia coli* R515 Re; Alexis), palmitoyl-3-cysteine-serine-lysine-4 (Pam$_3$CSK$_4$), zymosan, flagellin, poly(I:C) (all from Invitrogen Life Technologies), CpG-ODN 1668, or medium only, with or without the p38 inhibitor SB203580 (0.1–10 μM) added 1 h earlier. After 24 h, supernatants were removed and IL-10, IL-12p40, and IL-12p70 concentrations were determined by ELISA. Human DC were expanded from human monocytes, purified from PBMC using magnetic bead separation (Miltenyi Biotec), by culture for 7 days in the presence of 50 ng/ml GM-CSF and 70 ng/ml IL-4 (both from R&D Systems). Human DC were cultured with 1 and 10 ng/ml LPS (*E. coli* R515 Re; Alexis), 10 and 50 μg/ml poly(I:C) (Sigma-Aldrich), 100 and 500 ng/ml Pam$_3$CSK$_4$ (Invitrogen Life Technologies), or 25 and 50 μg/ml CpG-B (ODN 2006, also known as K-type ODN; 5′-tcgtcgttttgtcgttttgtcgtt-3′; Sigma Genosys). A p38 inhibitor (1 μM SB203580) was added 2 h before the TLR agonists. After 24 h, supernatants were removed and analyzed by ELISA for the concentration of IL-12p40, IL-12p70, and IL-10 (all from BD Pharmingen).

RT-PCR

RNA was extracted from DC using TriReagent (Sigma-Aldrich) and reverse transcribed using Superscript II RT (Invitrogen Life Technologies) and oligo(dT)$_{12–18}$ primers (Invitrogen Life Technologies). Primers specific for murine cyclooxygenase 2 (COX-2) (sense, GTATCAGAACC GCATTGCCTCTGA; antisense, CGGCTTCCAGTATTGAGGAGAA CAGAT) and β-actin (sense, GGACTCCTATGTGGGTGACGAGG; antisense, TGCCAATAGTGATGACTTGGC) were used with 2 μg of sample cDNA and amplified with *Taq* polymerase (Promega) using a Peltier Thermal Cycler.

Western blot analysis

DC were cultured at 1 × 10^6/ml with TLR ligands for 15 min to 9 h. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with Abs specific for phospho-p38 (Cell Signal Technology) and a HRP-linked secondary Ab. The nitrocellulose was stripped and probed with Abs specific for total p38.

DC transfer experiments

Bone marrow-derived DC from C57BL/6 mice were incubated with CpG (5 μg) and KLH (5 μg) and pretreated with the p38 inhibitor SB203580 (1 μM). Treated cells (2.5 × 10^5) were injected into each footpad. Popliteal LNs were removed after 5 days and single-cell suspensions were restimulated with KLH (2, 10, and 50 μg/ml). Supernatants were removed after 3 days and IFN-γ and IL-10 concentrations were quantified by ELISA. For tumor experiments, C57BL/6 or IL-10$^{-/-}$ mice were challenged with 2 × 10^5 B16 tumor cells s.c. and treated with three s.c. injections of treated DC (1–5 × 10^5) 1 wk apart, starting on day 3 into the tumor site. DC were loaded with heat-shocked and irradiated B16 tumor cells with CpG (5 μg/ml) with and without the p38 inhibitor SB203580 (1 μM) or COX-2 inhibitor NS-398 (1 μM) for 24 h. Heat-shocked and irradiated tumor cells were prepared by incubation of B16 tumor cells at 43°C for 1 h, irradiation at 200 Gy, and then incubation at 37°C for 4 h. Cells were added to DC at a ratio of 1:1.

DC activation of T cell responses in vitro

Bone marrow-derived DC were cultured for 24 h with OVA peptide (50 μg/ml) and CpG (1 μg/ml) in the presence or absence of the p38 inhibitor SB203580 (1 μM). Cells were washed and cultured at 1 × 10^5/ml with CD4$^+$ T cells purified from DO11.10 TCR Tg mice (1 × 10^6/ml). After 4 days, fresh medium and IL-2 were added to the cultures and fresh DC, preincubated for 24 h with OVA peptide and modulators as for the primary
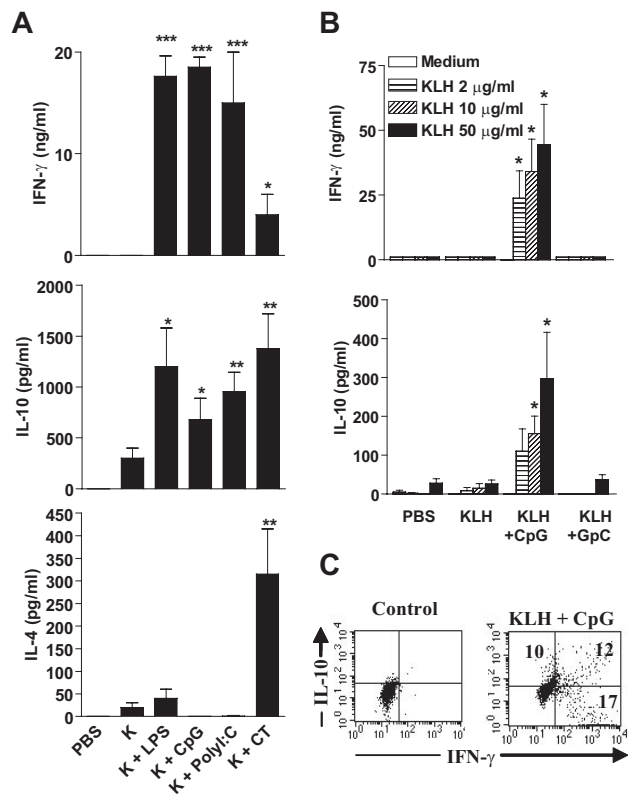
FIGURE 1. TLR ligands promote the induction of Ag-specific T cells that secrete IFN-γ, IL-10, or both cytokines. *A,* Mice were immunized s.c. with PBS only, 5 μg of KLH (K) or 25 μg of KLH and CpG, 1 μg of LPS, 25 μg of poly(I:C), or 10 ng of CT. After 7 days, draining LN cells were stimulated with KLH (50 μg/ml) and IFN-γ, IL-4, and IL-10 concentrations were determined by ELISA after 3 days. *B,* Mice were immunized s.c. with PBS only, KLH (5 μg), KLH and CpG (25 μg), or KLH and a control GpC ODN (25 μg). After 7 days, draining LN cells were stimulated with KLH (2, 10, and 50 μg/ml) and IFN-γ and IL-10 concentrations were determined by ELISA after 3 days. *C,* LN cells from mice immunized with PBS (control) or KLH and CpG were stimulated with KLH (50 μg/ml) and, after 6 days, cells were restimulated for 6 h with PMA and ionomycin. Brefeldin A was added for the final 4 h. Immunofluorescence analysis was performed for intracellular IL-10 and IFN-γ after gating on CD4+ cells. Representative of three experiments. $*, p < 0.05$; $**, p < 0.01$; and $***, p < 0.001$ vs KLH alone, ANOVA.

FIGURE 2. Ag-specific IL-10-secreting CD4+ T cells induced by immunization with Ag in the presence of a TLR ligand suppresses IFN-γ production by Th1 cells. *A,* CD4+ T cell lines established from individual mice immunized with KLH and CpG were stimulated with KLH (10 μg/ml) and APC (irradiated spleen cells 2×10^6/ml). Lines 6 and 7 were initially stimulated with Ag in the presence of anti-IFN-γ (added at the initiation of the culture and removed by washing at several reculture steps) to prevent outgrowth of IFN-γ-secreting T cells. T cell lines were tested for IFN-γ, IL-4, and IL-10 production by stimulation with Ag and APC. *B,* A KLH-specific Th1 cell line (IFN-γ+IL-10−IL-4−CD4+ T cells) was established from mice immunized with KLH and CpG by culturing T cells in the presence of IL-12 and anti-IL-10. A KLH-specific Tr1 cell line (IFN-γ−IL-10+IL-4−CD4+ T cells) was established by initial culture in the presence of anti-IFN-γ (line 7 in *A*). A IFN-γ+IL-10+IL-4−CD4+ T cell line (designated Th1Tr) was established from mice immunized with KLH and CpG by culturing T cells in the presence of Ag only (line 4 in *A*). The Th1 cell line was cultured with APC and Ag alone or in the presence of IFN-γ−IL-10+IL-4−CD4+ T cells (Tr1 cells) or IFN-γ+IL-10+IL-4−CD4+ T cells (Th1Tr cells) at ratio of 1:3, 1:1, or 3:1. Supernatants were removed after 3 days and the concentration of IFN-γ was tested by ELISA. Results are expressed as percentage of the IFN-γ response of the Th1 cell line alone. $*, p < 0.05$; $**, p < 0.01$; and $***, p < 0.001$ vs Th1 alone, ANOVA. Means of triplicate assays.

stimulation, were added after a further 8 days. Surviving T cells were cultured at 1×10^5/ml with OVA peptide (5 μg/ml) and splenic APC (2×10^6/ml). Supernatants were removed after Ag stimulation and concentrations of IL-10 and IFN-γ were determined by ELISA.

Statistical analysis

Statistical analysis was performed using GraphPad PRISM. Student's *t* test was used to compare the mean values between two groups. Statistical differences in mean values between more than two experimental groups were determined by ANOVA. Values of $p < 0.05$ were considered significant. Kaplan-Meier curves were analyzed using a log rank test.

Results

TLR ligands induce Treg as well as Th1 cells

We examined the role of TLR ligands in directing T cell responses to a model bystander Ag in vivo. Immunization of mice with KLH alone generated T cells that secreted IL-10 and low concentrations of IL-4, but no IFN-γ. Coadministration with the TLR ligands CpG, LPS, or poly(I:C) generated T cells that secreted high concentrations of IFN-γ, but low or undetectable IL-4, a cytokine profile consistent with the induction of Th1 cells (Fig. 1*A*). How-
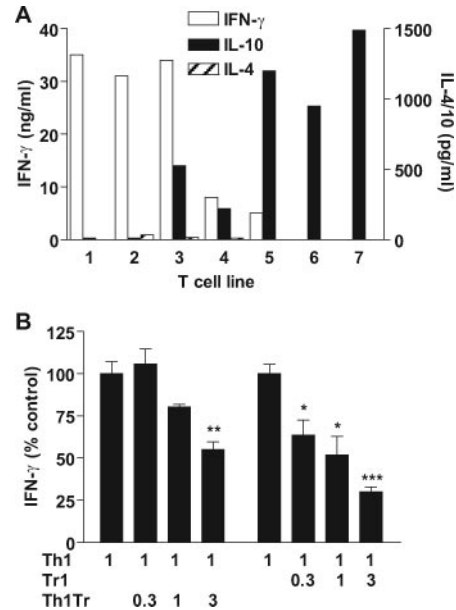
ever, in addition to IFN-γ, significant IL-10, but not IL-4, was also detected in supernatants from Ag-stimulated LN cells from mice immunized with KLH in the presence of TLR ligands (Fig. 1*A*). In comparison, LN cells from mice immunized with KLH in the presence of CT secreted IL-4 and IL-10, but low concentrations of IFN-γ. Ag-specific IL-10 and IFN-γ was detected in LN cells from mice immunized with KLG and CpG following in vitro restimulation with a range of doses of Ag, but not in mice immunized with PBS, KLH alone, or KLH and a control GpC-containing ODN (Fig. 1*B*). Intracellular cytokine staining on CD4+ T cells from mice immunized with KLH and CpG also revealed distinct populations of cells that secreted IFN-γ or IL-10 alone or both IFN-γ and IL-10 (Fig. 1*C*). These T cells did not express Foxp3 (CD4+Foxp3+, 2.6–3.3%; CD4+Foxp3+IFN-γ+, 0.1–0.2%; CD4+Foxp3+IL-10+, 0.1–0.3%).

The generation of KLH-specific CD4+ T cell lines from immunized mice showed that immunization with KLH and CpG induced conventional Th1 cells (IFN-γ+IL-10−IL-4−CD4+), but also a population of T cell that secreted both IFN-γ and IL-10 (Fig. 2*A*). Tr1 cells are normally difficult to culture in vitro and are easily outgrown

by Th1 cells; therefore, to confirm the induction of Tr1-type T cells (IFN-γ^-IL-10$^+$IL-4$^-$CD4$^+$), T cell lines were generated from mice immunized with KLH and CpG by initial culture in the presence of anti-IFN-γ to block expansion of Th1 cells (and removed through several wash and reculture cycles). Two KLH-specific T cell lines generated in this way secreted IL-10 but not IFN-γ or IL-4 (Fig. 2A).

We next examined the suppressor function of the TLR agonist-induced T cells. A conventional KLH-specific CD4$^+$ Th1 cell line established from a mouse immunized with KLH and CpG that secreted high concentrations of IFN-γ in response to stimulation with Ag and APC was used as the effector T cell in this assay. IFN-γ^-IL-10$^+$IL-4$^-$CD4$^+$ (Tr1) and IFN-γ^+IL-10$^+$IL-4$^-$CD4$^+$ (Th1Tr) cell lines (described in Fig. 2A) from a mouse immunized with KLH and CpG (the former initially cultured in the presence of anti-IFN-γ as described above) were used as the suppressor cells. The Tr1-type cells significantly suppressed IFN-γ production by Th1 cells at a ratio of 3:1, 1:1, and 1:3, with the greatest expression observed with the highest number of Tr1 cells (Fig. 2B). The IFN-γ^+IL-10$^+$IL-4$^-$CD4$^+$ cells also suppressed IFN-γ production by Th1 cells but only at ratios of 1:1 and 3:1 and the suppression was not as great as that observed with the Tr1-type cells. These findings demonstrate that in addition to promoting the induction of conventional IFN-γ-secreting Th1 cells, TLR ligands simultaneously generate distinct populations of IFN-γ^+IL-10$^+$IL-4$^-$CD4$^+$ T cells (21, 22) and IFN-γ^-IL-10$^+$IL-4$^-$CD4$^+$ T cells (Tr1-type cells) (15, 23) and that the latter T cell populations, which do not express Foxp3, have suppressor activity against conventional Th1 cells.

Inhibition of p38 suppresses TLR ligand-induced IL-10 and PGE$_2$, enhances IL-12 production by DC, and enhances their ability to induce Th1 over Tr1 cells

Because IL-10 and IL-12 production by innate cells promotes the induction of Tr1 and Th1 cells, respectively (12–15, 23), we examined the ability of TLR agonists to stimulate production of these regulatory cytokines from DC. Each of the TLR ligands examined, Pam$_3$CSK$_4$ (TLR2), zymosan (TLR2), poly(I:C) (TLR3), LPS (TLR4), flagellin (TLR5), and CpG (TLR9) induced IL-10, IL-12p40, and IL-12p70 production from immature bone marrow-derived mouse DC (Fig. 3A). A control GpC-ODN did not stimulate cytokine production by murine DC (data not shown). The TLR agonists Pam$_3$CSK$_4$, LPS, poly(I:C), and CpG also promoted IL-10 and IL-12 production by monocyte-derived human DC (Fig. 3B).

The induction of IL-10 and IL-12 has been linked to ERK (11) and p38 (24) signaling, respectively. We found that each of the TLR ligands examined induced phosphorylation of p38 (Fig. 4A) and ERK (data not shown). TLR agonist-induced p38 phosphorylation was detectable after 20–30 min, was maximal at 60 min, but was still evident for up to 6–9 h. Preincubation of DC with the p38 inhibitor SB203580 suppressed IL-10 and enhanced IL-12 production in response to CpG or LPS (Fig. 4B). We confirmed these findings with three distinct inhibitors of p38 over a range of concentrations (data not shown). Suppression of p38 also attenuated CpG-induced PGE$_2$ production (Fig. 4C). Furthermore, p38 inhibition suppressed LPS or poly(I:C)-induced IL-10 production by human DC (Fig. 4D).

We next examined the influence of the p38 inhibitors on the ability of CpG-activated DC to induce T cell responses in vitro and in vivo. Coincubation of DC with CpG and a p38 inhibitor suppressed their ability to promote Ag-specific IL-10 production by CD4$^+$ T cells from OVA TCR Tg mice, with reciprocal enhancement of T cell IFN-γ production (Fig. 5A). Adoptive transfer of DC pulsed with KLH in the presence of CpG induced IL-10 and IFN-γ-secreting T cells in vivo. Addition of a p38 inhibitor to the DC during the in vitro stimulation with CpG and Ag significantly suppressed KLH-specific IL-10 and resulted in a nonsignificant increase in IFN-γ production
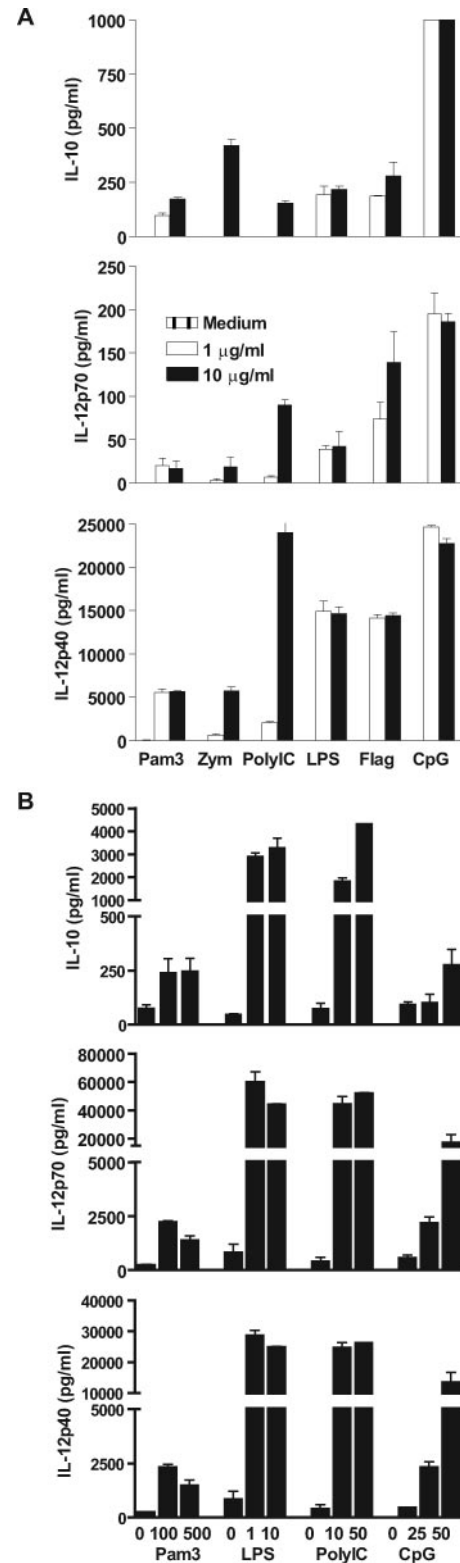

FIGURE 3. TLR ligands induce IL-10 and IL-12 production by murine and human DC. *A*, Mouse bone marrow-derived DC were incubated with 1 or 10 μg/ml Pam$_3$CSK$_4$ (Pam3), zymosan (Zym), poly(I:C), LPS, flagellin (Flag), CpG, or medium only. *B*, Human monocyte-derived DC were incubated with 100 and 500 ng/ml Pam$_3$CSK$_4$, 1 and 10 ng/ml LPS, 10 and 50 μg/ml poly(I:C), 25 and 50 μg/ml CpG-B. or medium only. After 24 h, supernatants were removed and IL-10, IL-12p40, and IL-12p70 concentrations were determined by ELISA.
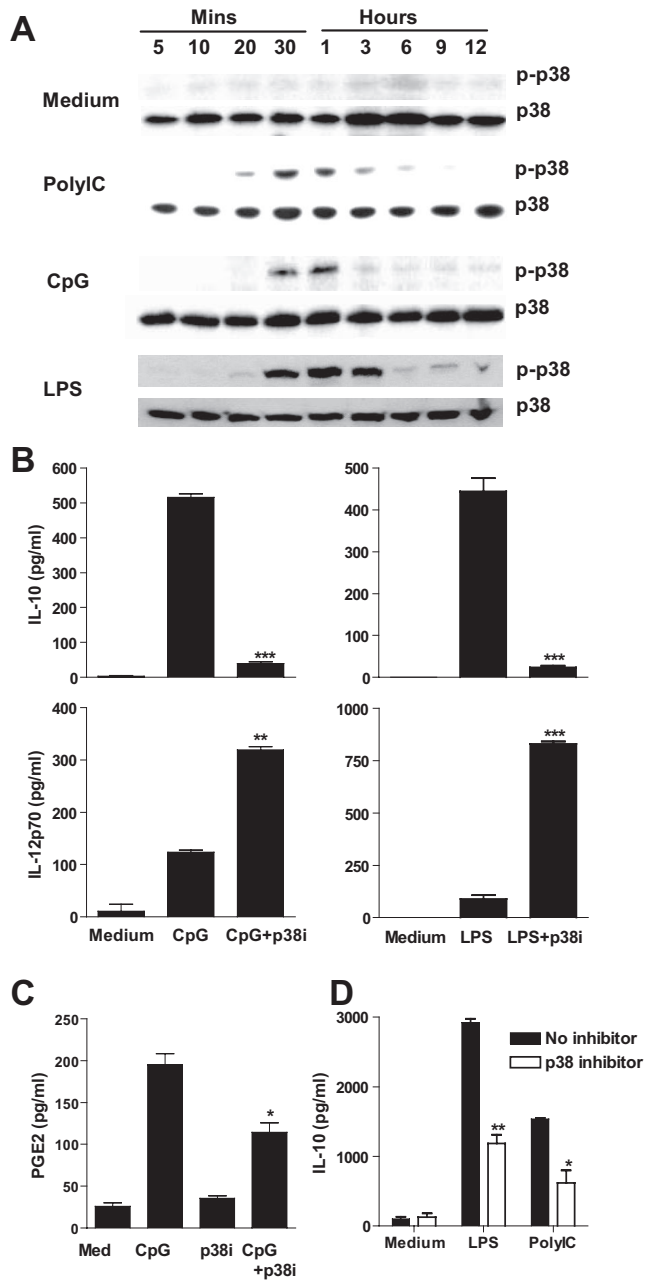
FIGURE 4. TLR ligands induce p38 phosphorylation in DC and inhibition of p38 suppresses IL-10 and PGE$_2$ production by DC. *A,* Bone marrow-derived murine DC were incubated for 5, 10, 20, or 30 min or 1, 3, 6, 9, or 12 h with medium only, poly(I:C) (20 μg/ml), CpG (20 μg/ml), or LPS (10 ng/ml). Cells were lysed and Western blots performed using Abs specific for phospho-p38 or total p38. *B,* Murine DC were preincubated with medium only (control) or the p38 inhibitor (p38i) SB203580 (1 μM) for 1 h before the addition of CpG (5 μg/ml), LPS (100 ng/ml), or medium only and, 24 h later, IL-10 and IL-12p70 and IL-12p40 concentrations were determined by ELISA. *C,* Murine DC were preincubated with medium only or the p38 inhibitor SB203580 (1 μM) for 1 h before the addition of CpG (5 μg/ml). After 24 h, PGE$_2$ was determined by ELISA. *D,* Human monocyte-derived DC were preincubated with medium only or the p38 inhibitor SB203580 (1 μM) for 1 h before the addition of LPS (100 ng/ml), poly(I:C) (1 μg/ml), or medium only and, 24 h later, IL-10 production was determined by ELISA. $*$, $p < 0.05$; $**$, $p < 0.01$; and $***$, $p < 0.001$, TLR agonist + p38 inhibitor vs TLR agonist alone, Student's *t* test.

(Fig. 5*B*). These findings demonstrate that inhibition of p38 activation suppresses the ability of TLR agonist-activated DC to drive Ag-specific Tr1 cells, while enhancing Th1 responses.
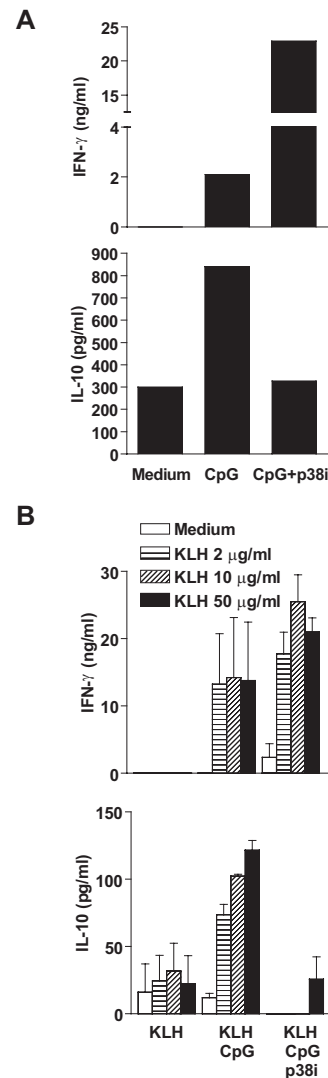

FIGURE 5. Inhibition of p38 suppresses the ability of TLR ligand-activated DC to induce IL-10-secreting T cells. *A,* Murine bone-marrow derived DC cultured for 24 h with OVA peptide (50 μg/ml) and CpG (1 μg/ml) in the presence or absence of the p38 inhibitor SB203580 (1 μM) were cultured with CD4$^+$ T cells from DO11.10 TCR Tg mice. After restimulation with Ag, supernatants were removed and IL-10 and IFN-γ concentrations were determined by ELISA. *B,* DC were pulsed for 18 h with KLH (5 μg/ml) in the presence or absence of CpG (5 μg/ml) with or without the p38 inhibitor SB203580 (1 μM). Cells were washed and injected into the footpad of recipient mice. After 7 days, the draining LNs were removed and LN cells were restimulated with KLH (2–50 μg/ml), and IFN-γ and IL-10 concentrations in the supernatants were determined by ELISA 3 days later. $***$, $p < 0.001$, KLH + CpG vs KLH + CpG + p38i, ANOVA.

CpG with a p38 inhibitor enhanced the protective efficacy of a DC tumor vaccine

Having demonstrated that inhibition of p38 signaling in DC can increase the ratio of effector to regulatory T cells induced with a TLR agonist, we tested the hypothesis that this approach could have therapeutic potential against tumors. We used the poorly immunogenic B16 tumor model and a DC therapy approach. Following s.c. tumor challenge, mice were treated with DC pulsed with irradiated heat-shocked whole tumor cells and CpG, with and without the p38 inhibitor. The results demonstrate that immunotherapy with DC pulsed with the whole-cell tumor vaccine has only a modest antitumor effect which is not enhanced by costimulation with
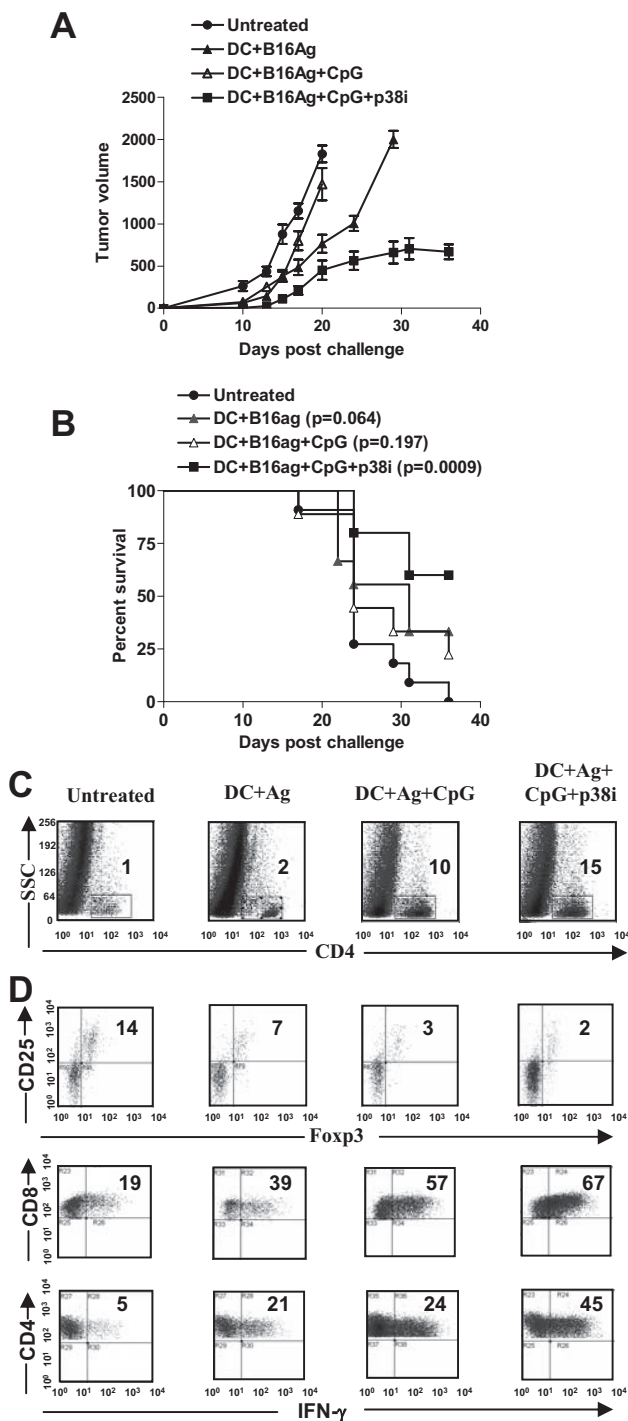
FIGURE 6. A p38 inhibitor enhances the antitumor therapeutic efficacy of DC pulsed with heat-shocked tumor cells and CpG. Mice were challenged with 2×10^5 B16 tumor cells and injected s.c. in the region of the tumor 3, 10, and 17 days later with $1–5 \times 10^5$ DC pulsed for 24 h with heat-shocked and irradiated tumor cells (ratio 1:1) with or without CpG (5 μg/ml) in the presence or absence of the p38 inhibitor SB203580 (1 μM). Tumor volume (*A*) and survival (*B*) were monitored. Statistical significance (in figure) by log rank test. Tumors were removed from treated and untreated mice 22 days after challenge. *C,* Infiltrating CD4^{+} T cells were determined by immunofluorescence analysis with anti-CD4. *D,* The frequency of CD4^{+}CD25^{+}Foxp3^{+} cells was determined by surface labeling with anti-CD4 and anti-CD25 and intracellular labeling with anti-Foxp3. The frequency of IFN-γ secreting CD4^{+} and CD8^{+} T cells was determined by stimulating T cells purified from the tumor with PMA and ionomycin for 1 h, then brefeldin A for 4 h, followed by staining for surface CD4 and CD8 and intracellular IFN-γ. Results are representative of three experiments, with six mice per experimental group.
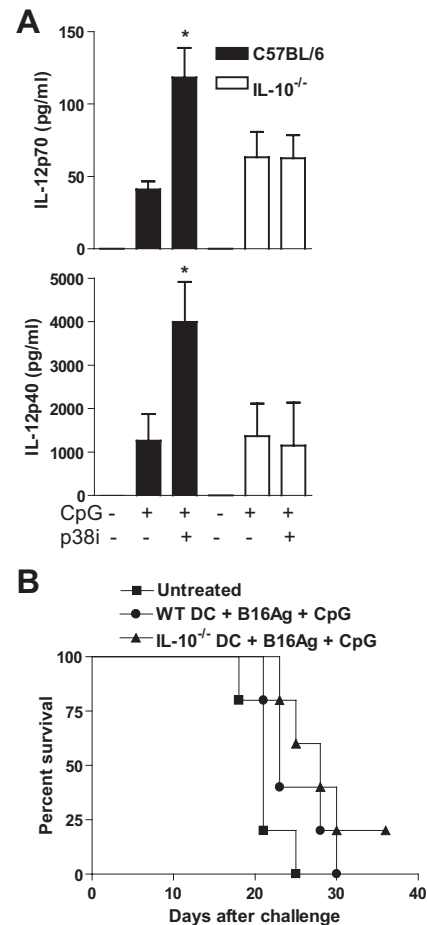

FIGURE 7. The role of IL-10 in constraining the therapeutic effect of TLR-activated DC. *A,* DC from wild-type (WT) C57BL/6 or IL-10^{−/−} mice were preincubated with medium only or the p38 inhibitor (p38i), SB203580 (1 μM) for 1 h before the addition of CpG (5 μg/ml) and, 24 h later, IL-12p70 and IL-12p40 concentrations were determined by ELISA. *B,* Mice were challenged with 2×10^5 B16 tumor cells and injected s.c. in the region of the tumor 3, 10, and 17 days later with $1–5 \times 10^5$ DC from wild-type C57BL/6 or IL-10^{−/−} mice pulsed for 24 h with heat-shocked and irradiated tumor cells (ratio 1:1) and CpG (5 μg/ml). Mice were monitored for tumor growth and survival. *, $p < 0.05$, CpG vs CpG + p38i, Student's *t* test.

CpG, but is significantly augmented by coincubation with CpG and a p38 inhibitor. Mice treated with DC pulsed with B16 vaccine, CpG, and p38 inhibitor had the slowest tumor growth (Fig. 6*A*) and the most survivors (Fig. 6*B*), and these were significantly greater than the untreated mice. Therapeutic administration of DC pulsed with killed tumor cells, CpG, and p38 inhibitor enhanced the recruitment of CD4^{+} T cells into the growing tumor (Fig. 6*C*). In comparison to the untreated mice, treatment of mice with DC pulsed with Ag reduced the frequency of CD25^{+}Foxp3^{+} T cells infiltrating the tumor (Fig. 6*D*). Treatment with DC pulsed with Ag and CpG, with or without the p38 inhibitor, further reduced the frequency of Treg cells. However, it is difficult to quantify the full effect of the p38 inhibitor on T cell responses, since we were assessing T cells that infiltrate the tumor and the tumors resolved in many of the mice treated with DC pulsed with Ag, CpG, and p38 inhibitors. Therefore, the analysis was restricted to the mice that did develop tumors and therefore had responded most poorly within this group. In comparison to the untreated mice, treatment of mice with DC pulsed with Ag, TLR agonist, and p38 inhibitor enhanced the frequency of IFN-γ-producing CD4^{+} and CD8^{+} T cells in the tumor (Fig. 6*D*).
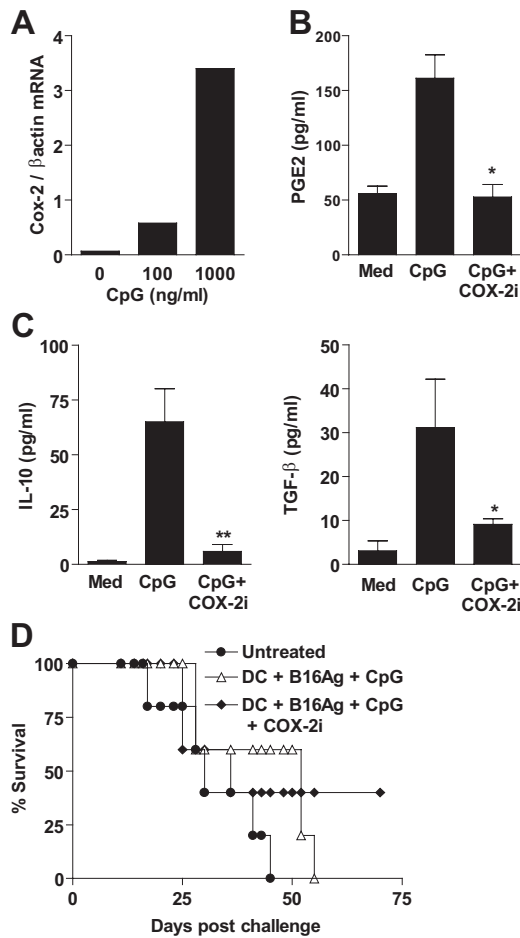
FIGURE 8. The role of COX-2 in constraining the therapeutic effect of TLR-activated DC. *A,* DC were stimulated with medium only or CpG (100 or 1000 ng/ml) and COX-2 mRNA expression was determined 6 h later by RT-PCR. Results are expressed relative to β-actin. *B* and *C,* DC were preincubated with medium only (Med) or the COX-2 inhibitor (COX-2i) NS-398 (1 μM) for 1 h before the addition of CpG (5 μg/ml) or medium only and, 24 h later, PGE$_2$ (*B*), IL-10, and TGF-β (*C*) concentrations were determined by ELISA. *D,* Mice were challenged with 2×10^5 B16 tumor cells and injected s.c. in the region of the tumor 3, 10, and 17 days later with 1–5×10^5 DC pulsed for 24 h with heat-shocked and irradiated tumor cells (ratio 1:1) and CpG (5 μg/ml) or the COX-2 inhibitor NS-398 (1 μM), or both. Mice were monitored for tumor growth and survival. *, < 0.05; **, $p < 0.01$, CpG vs CpG and COX-2 inhibitor, Student's *t* test.

Role of IL-10 and PGE$_2$ in compromising the efficacy of TLR-activated DC as cancer immunotherapeutics

Our finding demonstrates that inhibition of TLR agonist-induced p38 activation in DC enhances their efficacy as tumor immunotherapeutics. Since this appears to involve inhibition of IL-10 and PGE$_2$ and associated Treg cell expansion, with reciprocal enhancement of IL-12-mediated Th1 responses, we examined the possible role of IL-10 and PGE$_2$/COX-2. We first demonstrated that augmentation of CpG-induced IL-12 by the p38 inhibitor was abrogated in DC from IL-10$^{-/-}$ mice (Fig. 7*A*). Furthermore, active immunotherapy with DC pulsed with the B16 vaccine with CpG was enhanced using DC from IL-10$^{-/-}$ mice (Fig. 7*B*), but not to the same extent as that observed by p38 inhibition (Fig. 6).

Treg cell induction by DC has also been linked with COX-2/PGE$_2$ production (25). We found that CpG induced COX-2 (Fig. 8*A*) and PGE$_2$ (Fig. 8*B*) in DC and addition of a COX-2 inhibitor suppressed CpG-induced PGE$_2$ (Fig. 8*B*), IL-10, and TGF-β
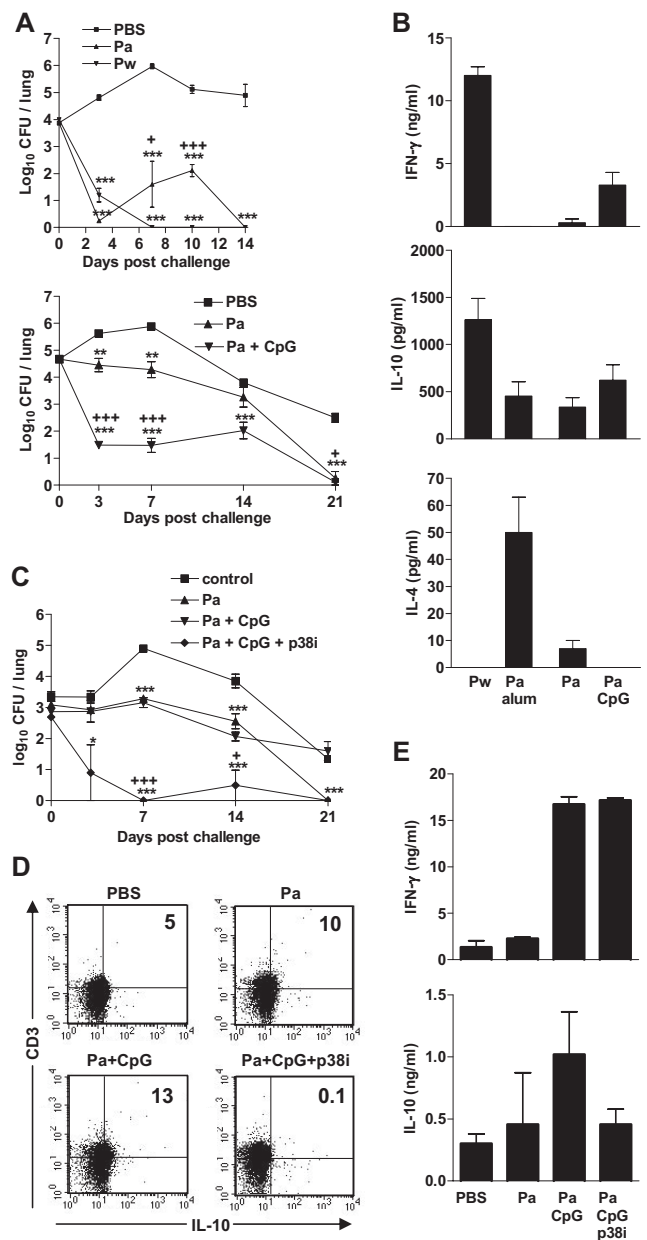

FIGURE 9. Formulation of a low-dose Pa with CpG and a p38 inhibitor enhances Th1 responses and protection against *B. pertussis. A,* Mice were immunized with 0.2-human dose Pa with alum or Pw (*upper graph*) or with 0.04-human dose Pa in PBS or Pa with 50 μg of CpG or PBS (*lower graph*). Mice were challenged by aerosol exposure to live *B. pertussis* 14 days after two immunizations and bacterial CFU were determined on lung homogenates. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs PBS; +, $p < 0.05$; +++, $p < 0.001$, Pa vs Pw or Pa + CpG, ANOVA. *B,* Spleen cells from immunized mice were stimulated with *B. pertussis* Ag and IFN-γ, IL-10, and IL-4 concretions in supernatants were quantified by ELISA 3 days later. *C–E,* Mice were immunized with 0.01-human dose Pa alone or with 5 μg of CpG with or without the p38 inhibitor SB203580 (50 μg). *C,* Mice were challenged by aerosol exposure to live *B. pertussis* 14 days after two immunizations and bacterial CFU were determined on lung homogenates. ***, $p < 0.001$ vs PBS; +, $p < 0.05$; and +++, $p < 0.001$, Pa + CpG vs Pa + CpG + P38i, ANOVA. *D,* LN cells from immunized mice were stimulated with *B. pertussis* Ag, followed 5 days later with PMA and ionomycin. Cells were labeled for surface CD3 and intracellular IL-10 and immunofluorescence analysis was performed. *E,* Spleen cells from immunized mice were stimulated with *B. pertussis* Ag and IFN-γ, IL-10, and IL-4 concretions in supernatants were quantified by ELISA 3 days later.

(Fig. 8*C*) production. Furthermore, a COX-2 inhibitor augmented the therapeutic efficacy of CpG-activated DC against B16 tumors (Fig. 8*D*). Our finding demonstrates that the antitumor therapeutic efficacy of CpG-activated Ag-pulsed DC can be significantly augmented by blocking the p38 signaling in DC and that this is mediated in part by suppression of IL-10 and PGE_2 induction.

CpG with a p38 inhibitor enhanced the protective efficacy of a pertussis vaccine

We next examined the hypothesis that the protective efficacy of an infectious disease vaccine might be enhanced using a p38 inhibitor in combination with a TLR agonist as adjuvant. Vaccines against whooping cough include the highly protective Pw and the less effective, but safer Pa administered with alum as adjuvant (26). We found that immunization of mice with Pw conferred a high level of protection with complete bacterial clearance by day 7 (Fig. 9*A*). In contrast, mice immunized with Pa in alum did not completely clear the bacteria until 14 days after challenge. Immunization with Pa in PBS (without an adjuvant) conferred limited protection against infection. However, addition of 50 μg of CpG significantly augmented its protective efficacy; the CFU counts after *B. pertussis* aerosol challenge were significantly lower in mice immunized with Pa and CpG than in mice immunized with Pa alone (Fig. 9*A*). Assessment of Ag-specific immune responses revealed that Pw induced Ag-specific IFN-γ and IL-10, but not IL-4, whereas Pa in alum induced a Th2-type response (Fig. 9*B*). Immunization with Pa without an adjuvant induced T cells that secreted IL-10 and low IL-4, but no IFN-γ. Addition of CpG augmented *B. pertussis*-specific IFN-γ, but also IL-10 production. (Fig. 9*B*), suggesting that this formulation promotes the induction of Tr1-type cells as well as Th1 cells. This was confirmed through the generation of a panel of T cell lines. *B. pertussis*-specific T cell lines from mice immunized with Pa and in the presence of CpG were either CD4$^+$IFN-γ^+IL-10$^-$IL-4$^-$ or CD4$^+$IFN-γ^+IL-10$^+$IL-4$^-$ and addition of neutralizing anti-IFN-γ Abs at the initiation of the culture allowed the expansion of Ag-specific T cells that secreted IL-10 only (data not shown). We next evaluated the effect of adding the p38 inhibitor using a suboptimal dose of Pa (0.01 human dose) and a suboptimal dose of CpG (5 μg). At these doses, the Pa alone or with CpG was poorly protective, but coadministration of a p38 inhibitor significantly enhanced protective efficacy; there were significantly lower *B. pertussis* CFU in the lungs in mice immunized with the vaccine in the presence of CpG and the p38 inhibitor (Fig. 9*C*). Addition of a p38 inhibitor to Pa with CpG reduced Ag-specific IL-10 production, detected by intracellular cytokine staining (Fig. 9*D*) or by cytokine concentration in supernatants (Fig. 9*E*). These data demonstrate that CpG enhances the protective efficacy of Pa by enhancing the induction of Ag-specific Th1 cells and that the dose of Ag can be spared by addition of a p38 inhibitor. Although we do not know what cell the p38 inhibitor is targeting in vivo, the overall effect was to reduce the induction of IL-10-secreting T cells.

Discussion

The findings of this study demonstrate that inhibition of p38 signaling in DC suppresses TLR agonist-induced IL-10 and PGE_2 production and their capacity to promote the induction of Treg cells. TLR ligands act as potent adjuvants, enhancing immune responses, especially Th1 responses to bystander Ags (12–14). In this study, we demonstrate that TLR ligands promote IL-10 and IL-12 production from DC and thereby induce Treg as well as effector Th1 cells. We found that IL-10 and PGE_2 production by DC was mediated by signaling through p38 and that inhibitors of p38 altered the ratio of IL-10 to IL-12 and Th1 to Treg cells induced with a TLR agonist. Our findings demonstrate that the com-

bination of a TLR agonist and a p38 inhibitor has considerable potential as an active immunotherapeutic approach against cancer and as an adjuvant for infectious diseases vaccines.

A number of previous reports have pointed to a role for TLR signaling in the induction or activation of Treg cells. We have demonstrated an association between defective induction of IL-10-secreting Treg cells and enhanced inflammatory pathology in the lungs of TLR4-deficient mice infected with *B. pertussis* (20). A TLR2 ligand, schistosomal lysophosphatidylserine, has been shown to promote the induction of IL-10-secreting Treg cells (19). It has also been reported that CpG can inhibit airway remodeling in a murine model of chronic allergen-induced asthma and this was associated with enhanced TGF-β production and possibly Tr cell induction (27). Furthermore, CD4$^+$CD25$^+$ Treg cells express TLR4, 5, 7, and 8 and exposure to LPS enhances their survival and proliferation. Finally, LPS can induce IL-10-secreting T cells that suppress CD8$^+$ T cell responses (28). Our data demonstrate that TLR ligands simultaneously induce regulatory and effector T cells through their interaction with cells of the innate immune system, in particular DC.

It has recently been reported that CD4$^+$ Th1 cells induced during parasite infections do not express Foxp3 but have suppressor activity (21, 22); intracellular cytokine staining on CD4$^+$ T cells from *Toxoplasma gondii*- or *Leishmania major*-infected mice revealed populations of T cells that secreted IFN-γ, IFN-γ and IL-10, or IL-10 only. Our data, based on intracellular cytokine staining analysis of Ag-specific T cells induced in the presence of a TLR agonist as adjuvant, are consistent with these findings. Furthermore, we demonstrated that Ag-specific T cell lines generated from mice immunized with Ag in the presence of TLR ligands secrete either IFN-γ only or IFN-γ with IL-10 and neutralization of IFN-γ at the initiation of culture allowed expansion of T cells that secreted IL-10 only. Assessment of the suppressor function revealed that a T cell line that secreted IFN-γ and IL-10 was capable of suppressing IFN-γ production by a conventional Th1 cell line (one that secreted IFN-γ only), but that the suppression was more significant with a Tr1-type T cell line that secreted IL-10 only. Thus, it appears that infection with certain pathogens or exposure to TLR agonists can induce regulatory as well as effector T cells, possibly as a means of controlling excessive inflammation, and that the ratio of IL-10 to IFN-γ may influence the outcome of the effector T cell response.

TLR agonists have major clinical applications as adjuvants for infectious disease vaccines and as immunotherapeutics for cancer (7, 10). Indeed the TLR7 ligand, imiquimod, is already in clinical use for basal cell carcinoma (29). Furthermore, TLR9 agonists have been evaluated in clinical trials as cancer immunotherapeutics and as vaccine adjuvants (10). However, a major obstacle to the development of successful immunotherapeutics against cancer and therapeutic vaccines is the immunosuppressive environment in patients with cancer or chronic infections. Tumors express a range of the immunosuppressive molecules, including COX-2, PGE_2, and TGF-β, which can activate or recruit Treg cells with suppressor activity (6, 30). Treg cells inhibit the function of protective T cells and this is believed to play a major part in the failure of many immunotherapies against cancer (7, 8). Depletion of Treg cells in mice enhances antitumor immunity and prolongs survival following tumor challenge (5, 6). Therefore, inhibition of Treg cells is an attractive approach to enhance the efficacy of cancer immunotherapeutics and tumor vaccines. We found that in addition to stimulating IL-12, which promotes Th1 and CTL responses, TLR agonists also induce IL-10 production. Pathogen-derived molecules that promote IL-10 and inhibit IL-12 selectively enhance Tr1 cells (15, 16). Our observation that all TLR ligands examined induced

both IL-10 and IL-12 production by DC is consistent with previous reports that LPS or CpG induced IL-10 production from macrophages (31, 32) and that the Th2-promoting TLR2 agonist Pam$_3$CSK$_4$ induces IL-10 production from DC (11) and may explain the simultaneous induction of Th1 and Tr1 cells.

It was previously suggested that the induction of IL-10 by LPS or CpG in macrophages was mediated by activation of ERK MAPK (31, 32). Induction of ERK has also been linked with Pam$_3$CSK$_4$ enhancement of IL-10 and inhibition of IL-12 production by DC and as a consequence induction of Th2 responses (11). Furthermore, it was proposed that ERK and p38 may differentially mediate IL-10 and IL-12 production (11, 31, 32). We found that all TLR ligands examined induced phosphorylation of p38 in DC. Inhibition of p38 suppressed IL-10 and PGE$_2$ production and enhanced IL-12 production. This is consistent with a report that the p38 inhibitor can reverse the suppressive effect of tumor-conditioned medium on IL-12 production by DC (30). In addition, recent studies have demonstrated that in addition to p38 (24), IFN regulatory factor 5 (33) and NF-κB (34) pathways are critical for IL-12p70 production. Taken together with the present study, these findings suggest that TLR ligands activate IL-10 through p38 phosphorylation and may simultaneously activate IL-12p70 production through alternative signaling pathways.

Our findings suggest that inhibition of p38 signaling in DC attenuates TLR agonist-induced IL-10 and PGE$_2$, but enhances IL-12 production, thus selectively suppressing the induction of Tr1 cells and allowing more polarized Th1 responses. IL-10, TGF-β, and PGE$_2$/COX-2 have been shown to promote differentiation or expansion of Treg cells in vivo (15, 25, 35). Consistent with this, we found that the use of DC from IL-10$^{-/-}$ mice enhanced the antitumor therapeutic efficacy of DC pulsed with heat-shocked tumor cells and CpG. The IL-10$^{-/-}$ DC vaccine was not as effective as DC treated with the p38 inhibitor. However TLR agonists also induced PGE$_2$ expression in DC and inhibiting p38, in addition to suppressing IL-10 production, also attenuated PGE$_2$ production. Furthermore, inhibition of COX-2 enhanced the efficacy of the DC vaccine, again not to the same extent as the p38 inhibitor. Therefore, it appears that TLR-induced signaling through p38 MAPK may lead to the production of IL-10, COX-2/PGE$_2$, and other unidentified immunosuppressive molecules, which may collectively dampen the inflammatory arm of the innate immune response. Thus, inhibition of TLR-activated p38 signaling may inhibit several mediators of Treg cell induction. The limited success of current vaccine and immunotherapeutics against cancer may be due to coinduction of Treg cells and may be compounded by the immunosuppressive environment created by the growing tumor. Our approach of specifically promoting effector T cells at the expense of Treg cells is a significant step forward, which we now plan to move into the clinic.

The demonstration that p38 MAPK inhibition may suppress the induction of IL-10-secreting T cells also has significant application for infectious disease vaccines. We provide proof-of-principle with an established mouse model of *B. pertussis* infection. IFN-γ is critical for protection against *B. pertussis,* but the current alum-adjuvanted Pa vaccine induces Th2 responses, and, although safer, is less effective than the Pw, which induces Th1 cells (36, 37). We found that CpG as the adjuvant significantly improved protection induced with Pa by promoting Th1 responses, and this effect is further enhanced at low vaccine and adjuvant doses by the addition of the p38 inhibitor. This has considerable application for Ag dose-sparing measures essential for vaccines, like pandemic influenza virus (38) and where there are a high number of nonresponders, as with the hepatitis B virus vaccine (39). However, perhaps the greatest application will be in therapeutic immunization during chronic infections, such as hepatitis C virus and HIV, where attenuation of Treg cells may facilitate the development of effector T cells required for effective pathogen elimination (40, 41).

Disclosures

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