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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

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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-12-secretory regulatory T (Treg) cells through p38 MAPK-induced IL-10 production by dendritic cells (DC). Inhibition of p38 suppressed TLR-induced IL-10 and PGE2 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.

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5 Abbreviations used in this paper: Treg, regulatory T; DC, dendritic cell; Tg, transgenic; CT, cholera toxin; Pa, acellular pertussis vaccine; Pw, whole-cell pertussis vaccine; KLH, keyhole limpet hemocyanin; ODN, oligodeoxynucleotide; LN, lymph node; Pam3CSK4, palmitoyl-3-cysteine-serine-lysine-4; COX-2, cyclooxygenase 2.

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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 Laboratories 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′-tcattgcgtcctgacct-3′; Sigma-Genosys; 25 μg), a control ODN with the reverse CpG 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 × 106 tumor cells. Mice were routinely monitored for tumor growth. Tumor size was measured in two dimensions by calipers and determined by the following formula: \(\text{area} = \pi \times \frac{\text{width}^2}{4}\). Width (x) × length (y) × w/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 × 106/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+ T cells) (designated Th1Tr cells). To generate Th1-type T cells (IFN-γ IL-10 IL-4 CD4+ T cells), 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 × 106/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+), Th1 (IFN-γ IL-10 IL-4 CD4+), and Th1Tr (IFN-γ IL-10 IL-4 CD4+ T cells) cell lines were established from mice immunized with KLH and CpG as described above. The Th1 cell line (1 × 106/ml) was cultured with APC (2 × 106/ml) and KLH alone or in the presence of Th1 or Th1Tr cells at a ratio of 1:1, 1:2, or 1:4. 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 with 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 LN 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 CelleQuest software on a FACScanCalibur (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-CSK), 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), and 10 and 50 μg/ml poly(I:C) (Sigma-Aldrich, 100 and 500 ng/ml Pam-CSK, Invitrogen Life Technologies), or 25 and 50 μg/ml CpG-B (ODN 2006, also known as K-type ODN; 5′-tcattgcgtcctgacct-3′; Sigma Genosys). A p38 inhibitor (1 μM SB203580) was added 2 h before the TB challenge. 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 cytokines IL-2 (200 ng/ml) from ATCC, human IL-10 (10 ng/ml) from R&D Systems, and a competition primer (K-type ODN; 5′-tcattgcgtcctgacct-3′; Sigma Genosys). A p38 inhibitor (1 μM SB203580) was added 2 h before the TB challenge. 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).

Western blot analysis

DC were cultured at 1 × 106/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 according to the manufacturer's instructions (eBioscience). Briefly, 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 × 105) 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, 7, and 10 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 × 105 B16 tumor cells i.c. and treated with three s.c. injections of treated DC (1–5 × 105) 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 × 105/ml with CD4+ T cells purified from DO11.10 TCR Tg mice (1 × 105/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
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. 1A). However, 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. 1A). In contrast, 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 the presence of IFN-γ IL-10 IL-4 CD4+ T cells (Th1 cells) or IFN-γ IL-10 IL-4 CD4+ T cells (ThTr cells) at ratio of 1:3, 1:1, or 3:1. Supernatants were removed after 3 days and the concentration of IFN-γ was determined 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.

**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 IL-10, 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 x 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 Ag and Ag alone or in the presence of IFN-γ IL-10 IL-4 CD4+ T cells (Th1Tr cells) or IFN-γ IL-10 IL-4 CD4+ T cells (ThTr cells) at ratio of 1:3, 1:1, or 3:1. Supernatants were removed after 3 days and the concentration of IFN-γ was determined 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.
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 PGE2, enhances IL-12 production by DC, and enhances their ability to induce Th1 over Th1 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, Pam3CSK4 (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 Pam3CSK4, 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 PGE2 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. A control GpC-ODN did not stimulates cytokine production by murine DC (data not shown). The TLR agonists Pam3CSK4, LPS, poly(I: C), and CpG also promoted IL-10 and IL-12 production by monocyte-derived human DC (Fig. 3B).

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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 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...
The frequency of CD4+ T cells secreting IFN-γ was determined by immunofluorescence analysis with anti-CD4, CD25, and Foxp3 antibodies. The frequency of IFN-γ-secreting CD4+ 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 6. A p38 inhibitor enhances the antitumor therapeutic efficacy of DC pulsed with heat-shocked tumor cells and CpG. Mice were challenged with 2 × 10⁵ B16 tumor cells and injected s.c. in the region of the tumor 3, 10, and 17 days later with 1–5 × 10⁵ 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) 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. A, Mice were challenged with 2 × 10⁵ B16 tumor cells and injected s.c. in the region of the tumor 3, 10, and 17 days later with 1–5 × 10⁵ 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. B, Untreated, wild-type DC + B16Ag + CpG, IL-10-/- DC + B16Ag + CpG, and IL-10-/- DC + B16Ag + CpG + p38i. Results are representative of two experiments, with six mice per experimental group.

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. 6A) and the most survivors (Fig. 6B), 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. 6C). 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. 6D). 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. 6D).
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. 7A). Furthermore, active immunotherapy with DC pulsed with the B16 vaccine with CpG was enhanced using DC from IL-10\(^{−/−}\) mice (Fig. 7B), 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. 8A) and PGE\(_2\) (Fig. 8B) in DC and addition of a COX-2 inhibitor suppressed CpG-induced PGE\(_2\) (Fig. 8B). IL-10, and TGF-\(\beta\)}
Reduced with a TLR agonist. Our findings demonstrate that the combination 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 Th 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^+^ Th1 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 Th1-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 Th1 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 Th2 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 Th1 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
Kingston Mills is a cofounder and shareholder in Opsona Therapeutics Ltd., a university start-up company that focuses on regulation of the human immune system.

References


