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Andrew L. Mellor

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IDO Activates Regulatory T Cells and Blocks Their Conversion into Th17-Like T Cells

Babak Baban,*‡ Phillip R. Chandler,*§ Madhav D. Sharma,*¶ Jeanene Pihkala,† Pandelakis A. Koni,*§ David H. Munn,*¶ and Andrew L. Mellor2*§

TLR ligands are effective vaccine adjuvants because they stimulate robust proinflammatory and immune effector responses and they abrogate suppression mediated by regulatory T cells (Tregs). Paradoxically, systemic administration of high doses of CpGs that bind to TLR9 ligands stimulated Tregs in mouse spleen to acquire potent suppressor activity dependent on interactions between programmed death-1 and its ligands. This response to CpG treatment manifested 8–12 h and was mediated by a rare population of plasmacytoid dendritic cells (CD19+ pDC) induced to express the immunosuppressive enzyme IDO after TLR9 ligation. When IDO was blocked, CpG treatment did not activate Tregs, but instead stimulated pDCs to uniformly express the proinflammatory cytokine IL-6, which in turn reprogrammed Foxp3-lineage Tregs to express IL-17. Thus, CpG-induced IDO activity in pDCs acted as a pivotal molecular switch that induced Tregs to acquire a stable suppressor phenotype, while simultaneously blocking CpG-induced IL-6 expression required to reprogram Tregs to become Th17-like effector T cells. These findings support the hypothesis that IDO dominantly controls the functional status of Tregs in response to inflammatory stimuli in physiological settings. The Journal of Immunology, 2009, 183: 2475–2483.

The TLRs are critical in host defense to infectious microorganisms (1, 2). TLR ligands displayed by pathogens stimulate rapid proinflammatory responses by host cells of the innate and adaptive immune systems. For this reason, pathogen-derived TLR ligands are critical components of vaccines and vaccine adjuvants, and synthetic TLR ligands are under investigation as potential vaccine adjuvants to stimulate more effective immunity in the treatment of cancer, infections, and allergies (3). In mice, LPS and oligonucleotides containing unmethylated CpG motifs that bind to TLR4 and TLR9, respectively, trigger plasmacytoid dendritic cells (pDC) and other cell types to mature, acquire potent T cell-stimulatory functions, and produce proinflammatory cytokines such as type I IFNs (IFN-α) and IL-6. TLR ligands also promote proinflammatory outcomes by attenuating suppression mediated by regulatory T cells (Treg). Thus, TLR ligands block de novo Treg differentiation, promoting differentiation of effector/helper T cells instead, and abrogate suppressor activity of Tregs (4–8). Although some TLR ligands may affect Tregs directly, TLR9 ligands block Treg development and function indirectly by inducing other immune cells to produce proinflammatory cytokines such as IL-6 (4). IL-6, in concert with other cytokines such as TGFβ and IL-23, is essential to induce naive CD4+ T cells to differentiate into effector Th17 T cells expressing the cytokine IL-17 (9–11). Even mature, lineage-committed Foxp3+ Tregs are more plastic than previously thought because exposure to IL-6 and other proinflammatory cytokines reprogrammed mature Tregs to acquire a phenotype resembling proinflammatory Th17 T cells (12). Thus, by binding to TLR9, CpGs possess potent proinflammatory properties widely assumed to overcome counterregulatory mechanisms that may impede beneficial therapeutic outcomes in clinical settings such as cancer and chronic infections where hypoimmunity contributes to disease progression.

Paradoxically, TLR ligands displayed by some pathogens can also stimulate immune counterregulatory mechanisms that suppress T cell responses, which may stabilize chronic infections in immunocompetent hosts (13). However, the mechanisms by which TLR ligands promote counterregulatory over proinflammatory outcomes in physiological settings are poorly defined. One potential mechanism involves induced expression of the intracellular enzyme IDO, which catalyzes tryptophan and triggers cellular stress responses that may modify cellular responses to proinflammatory stimuli (14). IDO activity correlates with immune counterregulation in a range of clinically significant syndromes such as chronic infections, autoimmunity, cancer, and allograft resistance to destructive host immunity (14). Phenotypically distinctive sub-sets of pDCs in mice and humans are competent to express IDO in response to certain inflammatory cues. IDO-expressing pDCs possess potent T cell-regulatory properties that block T cell responses to antigenic stimulation, and induce naive T cells to differentiate into Tregs ex vivo. Constitutive IDO expression by pDCs blocks T cell responses to nominal tumor Ags in draining lymph nodes (dLN) associated with sites of melanoma growth (15). Moreover, exposing mouse skin to the proinflammatory phorbol ester PMA, which also possesses tumor-promoting properties, caused pDCs in inflamed skin dLNs to express IDO and to acquire potent T cell.
regulatory properties as a consequence (16). Thus, induced IDO activity at sites of inflammation may attenuate the proinflammatory effects of TLR ligands.

Previously, we showed that administering high doses of CpG oligonucleotides to mice induced rare CD19<sup>−</sup>pDCs in spleen to express IDO and to acquire potent T cell suppressor activity ex vivo (17). This response to TLR9 ligation in vivo manifested rapidly, within 24 h of administering CpGs, and was dependent on IDO activity in CD19<sup>−</sup>pDCs. However, it is unclear how a small population of IDO-expressing pDCs, and the inherently local effects of IDO, could create the dominant suppression that blocked all T cell-stimulatory activity in mouse spleen after high dose CpG treatment. We hypothesized that CpG-induced IDO acts on Tregs to activate and stabilize a suppressor phenotype, which is resistant to TLR9-mediated deactivation of Tregs and mediates dominant counterregulation in spleen. We report that IDO acts as a molecular switch driving mature, quiescent Tregs into diametrically opposite functional states during CpG-induced innate immune responses, eliciting dominant Treg-mediated suppression when IDO is active, or allowing CpG-induced inflammatory stimuli to drive IL-6-dependent Treg conversion into Th17-like T cells when IDO is blocked.

**Materials and Methods**

**Mice**

All mice were bred in a specific pathogen-free facility. A1 TCR-transgenic, BM3 TCR-transgenic, OT-1 TCR-transgenic, CBK-transgenic, IDO-deficient (IDO1-KO), general control nonderepressible-2 (GCN2)-deficient (GCN2-KO), C/EBP-homologous protein-deficient (CHOP-KO), transgenic mice expressing membrane-bound chicken ovalbumin Act-mOVA, Foxp3<sup>GFP</sup>, IFN type I receptor (IFNAR)-KO, and IFNγ-yr-KO mice were described previously (17–21). IL-6-KO mice were purchased from The Jackson Laboratory. All gene-deficient mice were fully backcrossed (**10*/H11022*).

**CpG**

CpG-B (TCTAGACGTTCCTGAGCGT) and sequence-matched non-CpG B (TCTAGAGCTTCCTGACGTG), CpG-C (TGCCTGCTTTTGGCGGCNGCCG) and sequence-matched non-CpG C (TGCCTGCTTGTGCTTTTGCGCTT) with fully phosphorothioate backbones were a gift from Coley Pharmaceuticals. Mice were injected with comparatively high doses of oligonucleotides (ODN, 50 or 100 μg/mouse i.v.) as described (17). The class of CpG used was dependent on the background strain of mice.

**1-Methyl-D-tryptophan (D-1MT)**

D-1MT (Sigma-Aldrich and clinical grade Newlink Genetics) was prepared as a 20 mM stock solution in 0.1 M NaOH, adjusted to pH 7.4, and stored at −20°C protected from light. For in vitro use, D-1MT was added at a final concentration of 100 μM. For in vivo treatment, slow-release polymer pellets (5 mg/day) containing D-1MT or vehicle alone (1/50) anti-IL-6 (ABCM) or anti-IL-17 (eBioscience) Ab overnight at 4°C in the dark. After one washing in PBS, cells were incubated with (1/50) anti-IL-6 (ABCM) or anti-IL-17 (eBioscience) Ab overnight at 4°C in the dark. After another wash in PBS, cells were analyzed as above.

**Immunohistochemistry**

Sections were stained for IDO with a rabbit polyclonal Ab generated using a synthetic IDO peptide according to previously published methods (24). For IL-6, sections were first exposed to 0.5% Triton X for 15 min at room temperature and washed with PBS before addition of the primary anti-IL-6 Ab (1/100; ABCAM) and processed as for IDO.

**Treg suppression assays**

Treg suppression (readout) assays were performed in 96-well V-bottom plates by adding graded numbers (maximum concentration, 10 × 10⁴) of Mo-Flo sorted or MACS-enriched CD4<sup>+</sup>CD25<sup>+</sup> cells to T cell proliferation (72-h [³H]thymidine incorporation) assays containing 1 × 10⁸ or 5 × 10⁴ responders, either 1) H-2K<sup>b</sup>-specific T cells (nylon-wool enriched) from BM3 TCR-transgenic mice and 2 × 10⁶ CD11c<sup>+</sup> APCs (Mo-Flo sorted or MACS enriched) from CBK (H-2K<sup>b</sup>-transgenic CBA) mice prepared as described (17) or 2) H-Y-specific T cells (CD4<sup>+</sup> MACS enriched) responders from A1 TCR-transgenic mice, CD11c<sup>+</sup> APCs (MACS enriched) from female CBA spleen and H-Y<sup>+</sup> cognate peptide as described (19). A mixture of programmed death-1 (PD-1), programmed death-1 ligand (PD-L1), and PD-L2 Abs was used as described previously (19).

**Co-adaptive transfers**

Mo-Flo-sorted or MACS-enriched Tregs (from 0.4 to 1.25 × 10⁶/reipient) were isolated from spleens of donor B6 (or CBA) mice treated for 24 h with CpGs or control ODNs and mixed with MACS-enriched (or nylon wool-purified) OT-1 Thyl.1 (or BM3) T cells (from 1.25 to 10 × 10⁶/reipient) and coinfected into Act-mOVA-transgenic (or CBK) recipients (three mice per group). OT-1 Thyl.1 T cells were prelabeled with tracking dye (CFSE) before transfer. Positive control mice received OT-1 Thyl.1 (or BM3) T cells without Tregs; negative control mice were B6 (or CBA) mice that did not express the target Ag for OT-1 Thyl.1 (or BM3) T cells. After 96 h, mice were sacrificed, and spleen cells were stained with Abs to detect donor T cells; anti-CD8α and anti-Thyl.1 or anti-clonotypic Ab Ti98 (for BM3 visualized with streptavidin allophycocyanin; Ref. 25).

**Statistical analysis**

Analyses were performed using Student’s t test to compare data from triplicate wells within a group.

**Results**

**High-dose CpGs rapidly activate resting splenic Tregs**

Previously, we showed that systemic treatment with high doses (>50 μg i.v.) of CpGs induced splenic CD19<sup>+</sup>pDCs to express IDO (17, 26). To test the hypothesis that CpG-induced IDO activates splenic Tregs, we treated CBA mice with CpGs or sequence-matched control ODNs, and 24 h later sorted splenic CD4<sup>+</sup>CD25<sup>+</sup>T cells (Tregs) from treated mice. Graded numbers of sorted Tregs were added to readout assays containing responder H-2K<sup>b</sup>-specific (CD8<sup>+</sup>) T cells from B6M3 TCR-transgenic mice, and stimulatory APCs from H-2K<sup>b</sup>-transgenic (CBK) mice (17). As shown in Fig. 1A, adding only 5000 sorted Tregs from CpG-treated CBA mice abrogated BM3 T cell responses completely (Treg to T cell ratio, 1:20). In contrast, adding up to 10,000 sorted Tregs from control ODN-treated mice had no significant effect on BM3 T cell proliferation, indicating that TLR9 ligation was essential to stimulate resting Tregs to acquire suppressor activity.

Next, we confirmed CpG-induced Treg activation using a second readout assay, in which sorted Tregs from B6 mice were...
MHC-mismatched with respect to stimulatory APCs from CBA mice used to stimulate H-Y-specific responder T cells from A1 TCR-transgenic mice (19). This approach ensures that Treg suppressor activity is not activated during the readout assay itself. When sorted Tregs from B6 mice treated with high-dose CpG for 18–48 h were added to readout assays in the presence of H-Y peptide (Fig. 1B), as few as 2000 Tregs completely suppressed proliferation of A1 readout cells (Treg to T cell ratio, 1:50), but suppression was not detected 12 h after CpG treatment (Fig. 1B). Treg suppressor activity in readout assays was abrogated completely by adding a mixture of mAbs that block the PD-1 pathway (19). In this regard, suppression mediated by CpG-activated Tregs resembled IDO-activated Tregs from tumor dLNs, which also showed dependence on the PD-1 pathway (19).

Next, we tested if IDO was required for CpG-induced Treg activation. IDO-sufficient CBA and background-matched IDO-deficient (IDO1-KO) mice were treated with high-dose CpGs or control ODNs. After 24 h, splenic CD4+CD25+ Tregs were sorted and added (5000/well) to readout assays containing BM3 responder T cells and stimulatory APCs from CBK mice. As before, Tregs from CpG-treated wild-type (IDO-sufficient) CBA mice were potently suppressive, but Tregs from CpG-treated IDO1-KO mice exhibited no significant suppressor activity relative to mice treated with control ODNs (Fig. 2A). Similarly, Tregs exhibited no CpG-induced suppressor activity when obtained from wild-type mice pretreated to inhibit IDO pharmacologically using slow-release pellets impregnated with the IDO inhibitor drug D-1MT (Fig. 2B). Control mice pretreated with pellets containing vehicle alone showed potent CpG-induced Treg suppressor activity. Naive splenic CD4+CD25+ T cells from CpG-treated mice had no effect on BM3 T cell proliferation in readout assays (data not shown), indicating that CpG-induced suppressor activity was induced selectively in the CD4+CD25+ Treg subset. Similar outcomes were obtained in readout assays containing A1 responder T cells; Tregs from CpG-treated IDO1-KO mice (with B6 backgrounds) did not mediate suppression, whereas Tregs from B6 mice mediated potent PD-1-dependent suppression in this readout assay (data not shown). These findings revealed that functional IDO1 expression was essential to
activate the Treg suppressor phenotype following CpG administration in vivo.

IFN type I and GCN2 signaling is essential for CpG-mediated Treg activation

We have previously shown that signaling via IFNAR was essential to stimulate splenic CD19<sup>+</sup> pDCs to express IDO, whereas signaling via IFN type II receptors (IFN-γR) was not required (17, 27). To elucidate requirements to activate Tregs after TLR ligation, we treated IFNAR-deficient (IFNAR-KO) and IFN-γR-deficient (IFN-γR-KO) mice and measured Treg suppressor activity. As expected, control Tregs (1200/well) from CpG-treated wild-type (BALB/c) mice mediated potent suppression dependent on PD-1 interactions (Fig. 3A). In contrast, Tregs from CpG-treated IFNAR-KO exhibited no suppressor activity (Fig. 3B). However, Tregs from CpG-treated IFN-γR-KO mice mediated potent PD-1 dependent suppression comparable with wild-type (129) controls (Fig. 3C). These outcomes were thus consistent with our previous reports that IDO induction in CD19<sup>+</sup> pDCs was dependent on signaling via IFNAR, but not IFN-γR signaling (17).

We have also shown that susceptibility of T cells and Tregs to the biochemical effects of tryptophan catabolism is critically dependent on intact GCN2 kinase stress response pathways (18, 19). To elucidate whether intact GCN2 was required for CpG-induced splenic Treg activation, we assessed suppressor activity of splenic Tregs from CpG-treated GCN2-deficient (GCN2-KO) mice. Tregs from CpG-treated IFN-γR-KO mice possessed no significant suppressor activity (Fig. 3D). Similarly, Tregs from mice with a defective gene encoding CEBP-homologous protein (CHOP-KO mice), a downstream target of activated GCN2 kinase, did not acquire suppressor activity in response to high dose CpGs (Fig. 3E).

IDO-activated Tregs suppress alloreactive T cell responses elicited in vivo

To evaluate whether IDO-activated Tregs inhibited effector T cell responses elicited in vivo, we sorted Tregs from donors (Thy1.2), mixed them with CD8<sup>+</sup> T cells from OT-1 TCR transgenic (Thy1.1) mice prelabeled with tracking dye (CFSE), and coinfected Treg-T cell mixtures into Act-mOVA (Thy1.2)-transgenic mice (21), which express OVA constitutively as a nominal tissue alloantigen (Fig. 4A). OT-1 T cell responses were assessed 96 h after Treg/T cell coadoptive transfers. In this in vivo system, OT-1 T cells injected alone into Act-mOVA recipients underwent multiple rounds of cell division, as evidenced by extensive dilution of CFSE levels (Fig. 4C). OT-1 T cells also underwent extensive division when cotransferred into Act-mOVA mice with resting Tregs from untreated B6 mice. In contrast, OT-1 T cells injected into Act-mOVA recipients cotransferred with IDO-activated Tregs underwent minimal division, indicating these Tregs efficiently suppressed OT-1 T cell responses (Fig. 4D). These outcomes were thus consistent with our previous reports that IDO induction in CD19<sup>+</sup> pDCs was dependent on signaling via IFNAR (17).
mice (Fig. 4D). In striking contrast, OT-1 T cells did not undergo division and retained uniformly high levels of CFSE when cotransferred with activated Tregs from CpG-treated B6 (wild-type) mice (Fig. 4E). However, when Tregs originated from CpG-treated IDO1-KO donors, CFSE levels on donor OT-1 T cells were extensively diluted (Fig. 4F), indicating that Tregs did not possess a suppressor phenotype. Analyses of the total numbers of OT-1 T cells in recipients revealed that OT-1 T cells underwent clonal expansion (8- to 10-fold relative to control B6 recipients without target Ag) when no Tregs were cotransferred (data not shown) and when Tregs originated from untreated B6 mice (Fig. 4G) or CpG-treated IDO1-KO mice (Fig. 4H). In contrast, total numbers of OT-1 T cells in spleens of mice that received Tregs from CpG-treated B6 mice were significantly lower, comparable with the numbers of OT-1 T cells in spleens of B6 mice that received OT-1 T cells only (Fig. 4, G and H). Thus, the ability of splenic Tregs to block in vivo clonal expansion of alloreactive OT-1 T cells depended on CpG treatment and on induced IDO activity in Treg donor mice.

Suppression of in vivo T cell clonal expansion was also observed when CpG-activated Tregs from wild-type (CBA) mice were cotransferred with H-2Kb-specific BM3 T cells into H-2Kb-transgenic (CBK) mice, whereas spleens from CBK mice that received BM3 T cells and Tregs from control ODN-treated mice were significantly smaller, contained large cohorts of BM3 T cells, and exhibited extensive pathology. Thus, IDO-activated Tregs suppressed clonal expansion and differentiation of alloreactive effector T cells capable of causing tissue pathology.

**Induced IDO activity blocks IL-6 production after high-dose CpG treatment**

The preceding studies suggested that IDO induced by high CpG doses antagonized proinflammatory responses to CpGs. IL-6 is a classic proinflammatory cytokine produced by several cell types following TLR9 ligation (4). We therefore asked whether IDO attenuated IL-6 expression in mice treated with high CpG doses. Consistent with this hypothesis, no cells expressing IL-6 were detected in spleen of wild-type (IDO-sufficient) mice following high-dose CpG treatment (Fig. 5A, lower left). In striking contrast,

4 Consistent with these outcomes, spleen histology was normal in CBK mice that received BM3 T cells and IDO-activated Tregs from CpG-treated mice, whereas spleens from CBK mice that received BM3 T cells and Tregs from control ODN-treated mice were significantly smaller, contained large cohorts of BM3 T cells, and exhibited extensive pathology. Thus, IDO-activated Tregs suppressed clonal expansion and differentiation of alloreactive effector T cells capable of causing tissue pathology.
spleens from CpG-treated IDO1-KO mice showed many IL-6-expressing cells distributed widely in lymphoid follicles and in non-lymphoid regions of spleen (Fig. 5A, lower right). IDO expression in response to CpG treatment was confirmed by immunohistochemistry (Fig. 5A, top).

Flow cytometric analyses of gated splenic pDCs (CD11c^-B220^-) to detect intracellular IL-6 showed that pDCs from CpG-treated IDO-sufficient mice were not induced to express IL-6, whereas pDCs from IDO1-KO mice uniformly expressed IL-6 (Fig. 5B). However, splenic myeloid DCs (CD11c^-B220^-) from CpG-treated mice showed no IL-6 expression, irrespective of whether they originated from IDO-sufficient or IDO1-KO mice (data not shown), indicating that IL-6 production was a selective response to TLR9 ligation by pDCs among DCs. Levels of CD80 and PD-L2 surface expression by splenic DCs were unaffected by CpG treatment, whereas levels of CD86 and PD-L1 were elevated following CpG treatment (supplemental Fig. 2), indicating that CpG treatment had some effect on expression of positive and negative costimulatory markers by DCs. Other splenocyte populations such as subsets of B cells also expressed IL-6 in response to CpG treatment, but only when IDO was not active (data not shown). These data suggested that multiple cell types, including the majority of pDCs, were competent to express IL-6 in response to TLR9 ligation, but all these cells were prevented from expressing IL-6 when CpGs also coinduced CD19^- pDCs to express IDO.

CpG treatment reprograms splenic Tregs to express IL-17 in the absence of IDO

In concert with a mixture of other cytokines (such as IL-1 and IL-23), IL-6 has been reported to reprogram Foxp3 lineage-committed Tregs to express the inflammatory cytokine IL-17 in vitro (11, 12, 28). We asked whether CpG-induced IL-6 expression in IDO-deficient mice reprogrammed Tregs to express IL-17. Consistent with this hypothesis, a subset (6–7%) of splenic CD4^+ cells expressed intracellular IL-17 in IDO1-KO mice treated with high-dose CpG; in contrast, no IL-17-expressing cells were detected in spleens of IDO-sufficient (B6) mice after high-dose CpG treatment (Fig. 6A). IL-17 expression in this subset of CD4^+ cells was induced rapidly, with maximal IL-17 expression occurring 6–9 h after CpG administration (Fig. 6B). Further analysis revealed that the CD4^+CD25^- subset expressed IL-17 uniformly, whereas the CD4^-CD25^- subset did not express IL-17 (Fig. 6C), suggesting that CpG treatment in the absence of IDO stimulated selective IL-17 expression in Tregs. To test this hypothesis, we used Foxp3^-GFP^ knock-in mice that express a Foxp3-GFP fusion protein (20). Foxp3^-GFP^ mice were treated with oral IDO inhibitor (D-IMT in drinking water) beginning 2 days before CpG treatment. At baseline (<6 h after CpG treatment) no GFP^+ Tregs expressed IL-17, but 24 h after CpG treatment the majority of GFP^+ Tregs (>80%) coexpressed IL-17 (Fig. 6D). Moreover, the population of IL-17^-cells corresponded with the GFP^+ Treg population. Consistent with this finding, CpG treatment induced CD4^+CD25^- cells from IDO1-KO mice to express IL-17 uniformly, whereas CD4^-CD25^- cells from CpG-treated B6 mice did not express IL-17 (supplemental Fig. 3); however, induced IL-17 expression correlated with loss of intracellular Foxp3 staining by CD4^-CD25^- cells, suggesting that Foxp3 protein may be sequestered or may undergo conformational changes leading to loss of Ab-binding sites, despite apparent retention of the Foxp3-GFP fusion protein in Foxp3^-GFP^ knock-in mice. Because IL-17 expression by Tregs occurred rapidly after CpG treatment and showed no change in the total number of Tregs (Fig. 6 and supplemental Fig. 3), we hypothesized that CpG treatment in the absence of IDO directly reprogrammed existing, preformed Foxp3^- Tregs, and injected them into IDO-sufficient (B6) or IDO1-KO recipients. Two days after Treg transfer recipients were treated with high-dose CpG, and IL-17 expression was assessed in the transferred GFP^+ cells 24 h later. Analysis of gated GFP^+ cells revealed that donor Tregs uniformly up-regulated IL-17 in CpG-treated IDO1-KO recipients (Fig. 6E), whereas GFP^+ donor cells injected into CpG-treated IDO-sufficient recipients did not express IL-17. Collectively, these outcomes support the hypothesis that preformed, Foxp3-lineage-committed Tregs

![Figure 6](http://www.jimmunol.org/)
were directly converted into IL-17-expressing cells by CpG treatment in the absence of IDO, whereas reprogramming was blocked when IDO was active.

**Treg reprogramming to express IL-17 is dependent on CpG dose and IL-6**

Next, we evaluated requirements to reprogram splenic Tregs to express IL-17 following CpG treatment. Consistent with the outcomes in IDO1-KO mice, high-dose CpG treatment of IDO-sufficient B6 mice stimulated CD4⁺CD25⁺ splenocytes to express IL-17 when IDO was blocked by oral IDO inhibitor (D-1MT; Fig. 7B), but not when IDO was active (Fig. 7A). Administering lower doses (25 μg i.v.) of CpGs that fail to induce IDO (17) also stimulated IL-17 up-regulation by CD4⁺CD25⁺ cells (data not shown). Thus, selecting a lower dose of CpG to avoid inducing IDO stimulated purely proinflammatory responses (i.e., Treg conversion) without the need for D-1MT. Administering lower doses of CpGs that fail to induce IDO (17) also stimulated IL-17 up-regulation by CD4⁺CD25⁺ cells (data not shown). Thus, selecting a lower dose of CpG to avoid inducing IDO stimulated purely proinflammatory responses (i.e., Treg conversion) without the need for D-1MT. Administering lower doses of CpGs that fail to induce IDO (17) also stimulated IL-17 up-regulation by CD4⁺CD25⁺ cells (data not shown).

These data support the hypothesis that IDO actively suppresses IL-6-dependent reprogramming of Treg functionality, which would otherwise occur in response to TLR9 ligation.

**Discussion**

In this study, we show that IDO acts as a pivotal molecular switch controlling the functional status of Tregs following TLR9 ligation, leading to diametrically opposed counterregulatory or proinflammatory outcomes depending on whether IDO was active or inactive. Counterregulatory responses manifested only a few hours after CpG administration, and only at high CpG doses that induced pDCs to express IDO. Moreover, IDO-mediated counterregulation predominated over classic proinflammatory and T cell-stimulatory responses to TLR9 ligation induced concomitantly. CpG-induced IDO stimulated potent Treg bystander suppressor activity and simultaneously blocked IL-6 production required to convert Tregs into Th17-like T cells. Conversely, if IDO activity was blocked, CpG treatment elicited purely proinflammatory responses, inducing IL-6 expression that drove uniform conversion of mature Foxp3 lineage Tregs into a proinflammatory Th17-like phenotype. Thus, high doses of CpG are not intrinsically suppressive; rather, they trigger counterregulatory responses mediated by IDO that dominantly suppress or veto their underlying immunostimulatory and inflammatory effects. These findings suggest that under certain circumstances inducible or pre-existing IDO activity at local sites were directly converted into IL-17-expressing cells by CpG treatment in the absence of IDO, whereas reprogramming was blocked when IDO was active.
of inflammation may dominantly suppress proinflammatory processes and block effector/helper T cell responses to Ags encountered at such sites. Thus, sufficiently intense inflammation may elicit dominant counterregulation by IDO-activated Tregs to create local T cell suppression and immune privilege. Conversely, when IDO is absent, even strong proinflammatory stimuli do not elicit local Treg suppression, and Tregs are reprogrammed to acquire a proinflammatory Th17-like phenotype.

Splenic Tregs acquired potent suppressor activity rapidly (12–18 h) after TLR9 ligation. Activated Tregs blocked T cell proliferation ex vivo, and clonal expansion of allo-specific effector T cells in vivo. Rapid responses to CpG treatment were blocked completely by ablating the IDO1 gene and by pharmacological inhibition of IDO before CpG administration. Thus, intact IDO2 genes, which are closely related and linked to IDO1 genes (29, 30), did not compensate for loss of IDO1-regulatory functions. Ex vivo, the CpG-induced form of Treg suppressor activity was dependent on intact PD-1 signaling during suppressor assays. PD-1-dependent Treg suppression was also a distinctive feature of IDO-activated Tregs from inflamed lymph nodes draining sites of melanoma growth (19), and skin exposed to the proinflammatory tumor promoter phorbol ester (PMA; unpublished data). These models of counterregulation at sites of localized inflammation share with the CpG model the fact that Treg activation was dependent on IDO expression by pDCs in a physiological setting. The role of PD-1 in these in vivo systems remains to be elucidated, but the requirement for PD-1 to mediate suppression ex vivo is a characteristic feature of Tregs activated by IDO in all three models. A recent report identified requirements for PD-L1 to generate Tregs from naive T cells, implying that the PD-1 pathway is critical for Treg differentiation in vivo (31); however, this study did not address whether IDO was required to promote Treg differentiation and to stabilize the Treg suppressor phenotype.

CpG-induced Treg activation in the physiological setting of the spleen was also dependent on intact IFNAR signaling, but not IFN-γR signaling. These outcomes are consistent with our previous reports showing an obligatory requirement for IFNAR signaling to induce CD19<sup>+</sup> pDCs to express functional IDO following treatment with soluble CTLA4 (CTLA4-1g), and CpGs, which ligate B7 and TLR9, respectively (17, 27). We cannot exclude the possibility that IFNAR signaling may play a direct role in stimulating Treg suppressor activity, but the known role of IFNAR signaling upstream of IDO is sufficient to explain its importance in the high-dose CpG model. However, signaling via IFNAR was not required to reprogram Tregs to express IL-17 under IDO-deficient conditions.

IDO-mediated inhibition of IL-6 expression was a key novel finding in our study because this provides a plausible explanation for the ability of IDO to prevent Treg reprogramming into proinflammatory Th17-like cells, which is dependent on a mixture of cytokines, including IL-6 (8, 11, 12). Uniform expression of IL-6 and IL-17 by pDCs and Tregs, respectively, was induced rapidly (between 6 and 9 h) after TLR9 ligation in IDO-deficient mice. The mechanism of IDO-mediated blockade of IL-6 expression is not known but may involve autocrine and paracrine signaling mediated directly by IDO-expressing pDCs as the patterns of IDO and IL-6 expression induced by CpGs under IDO-sufficient and IDO-deficient conditions, respectively, were not identical. Metz (30) recently reported that induced IDO activity in transfected cell lines induced expression of liver-enriched inhibitory protein, an inhibitory isoform of the NF-IL-6 transcription factor required to promote IL-6 gene expression. Thus, molecular pathways exist by which IDO may directly suppress up-regulation of IL-6 gene expression. Whether by this direct mechanism or an alternative indirect route, our data unambiguously show that IDO blocked TLR9-induced IL-6 expression. CpG is widely used to induce IL-6 production by B cells and myeloid cells expressing TLR9, but our findings identify CpG dose as a critical factor, presumably because IDO expression by pDCs occurs only above a certain signaling threshold, which causes all splenic IDO-competent pDCs to up-regulate IDO simultaneously. Once this threshold was breached the counterregulatory effects of induced IDO were dominant, and CpG treatment failed to stimulate IL-6 production, unless IDO was absent.

Our finding that IDO predominates over the immunostimulatory and proinflammatory effects of CpG treatment has potentially important implications for understanding the role of IDO in clinically significant inflammatory disease processes and for treatment of such syndromes. We do not know what the human equivalent would be for the high-dose CpG used in our murine model. However, many natural infections and inflammatory conditions induce IDO in vivo (14), and CpGs are often administered locally as a vaccine adjuvant (3). If local or systemic levels were high enough to induce IDO, then paradoxical immunosuppression might ensue. The corollary of this, however, is that blocking IDO at the time of CpG treatment may allow IL-6 production, leading to local reprogramming of Tregs. A further consideration is that, unlike spleen where IDO has to be coinduced by CpG treatment, IDO is constitutively activated in some settings of chronic inflammation, including lymphoid tissues draining local microenvironments where tumors develop, and draining inflamed skin exposed to phorbol esters that promote tumor development (15, 16). In these settings, constitutive (preadjuvanted) IDO activity may preclude immunostimulatory and proinflammatory responses to a range of insults, including tumors, certain infectious pathogens that activate Tregs (32) and induce local IDO expression, and artificial immunostimulants such as vaccines and vaccine adjuvants (14). Moreover, the use of IDO inhibitors to enhance tumor vaccine efficacy in murine models of tumor growth correlated with loss of suppressor activity by Tregs, and concomitant IL-6-dependent conversion of Tregs into Th17-like T cells in tumor dLN (33), implying that the potent effects of IDO in spleens of CpG-treated mice are relevant to settings of chronic inflammation created by local tumor growth that induce constitutive IDO activity. IL-6 is known to synergize with other cytokines such as IL-1 and IL-23 to reprogram Foxp3 lineage Tregs to become Th17 T cells has been described and was observed in mice treated with a novel immune modulator (B7-DC XAb), and in some infectious disease settings (7, 8, 12). However, the physiological mechanisms that drive Treg to Th17 reprogramming remain obscure. The findings we report in this study identify IDO as a critical molecular switch that stimulates potent Treg suppressor functions and simultaneously blocks IL-6-mediated reprogramming of Tregs to generate proinflammatory effector T cells expressing IL-17, and other proinflammatory cytokines such as IFN-γ, TNF-α, and IL-2. Thus, our findings suggest that manipulating IDO may have profound effects on the balance of effector and counterregulatory suppressor functions during inflammation. This scenario is also consistent with a recent study showing that absence of functional IDO activity contributed to unregulated local proinflammatory responses to a pulmonary infection (34).

In summary, the striking dichotomy of physiological responses by splenic Tregs to high-dose CpG treatment under IDO-sufficient and IDO-deficient conditions suggests that IDO-competent CD19<sup>+</sup> pDCs are pivotal regulators of T cell responses at sites of inflammation. Thus, IDO emerges as a key molecular switch controlling the balance between suppressor and effector functions of T cells. Chronic activation of IDO has been reported in a diverse...
A range of clinically relevant syndromes, including persistent infections, and cancer (14). In these syndromes, chronic or excessive IDO activity may create paradoxical local immune suppression and privilege that promotes disease progression by blocking innate T cell immunity to pathogens, and tumor Ags. Conversely, a deficiency in IDO promotes excessive autoimmunity in mice prone to type I diabetes (35, 36), and allows exaggerated proinflammatory responses in infected mice genetically predisposed to chronic granulomatous disease (34). Thus, IDO appears to be a pivotal regulator of inflammation in certain settings: suppressing inflammation and maintaining the suppressive phenotype of Tregs when IDO is active; or allowing unchecked inflammation and reprogramming of Tregs when IDO is absent. This has significant implications for the pathogenesis of chronic inflammatory disorders and also has significant practical implications for the use of artificial ligands as vaccine adjuvants. Recently, IDO induced by treatment with the NKT (CD1d) ligand α-galactosylceramide was identified as the reason why α-galactosylceramide treatment did not stimulate a vaccine adjuvant effect in a murine model of influenza vaccination (37), suggesting that IDO-mediated attenuation of immune responses to vaccine adjuvants may not be restricted to TLR ligands. When it is desirable to achieve the maximal proinflammatory effect of immune agonists, it may be crucial to block the dominant counterregulatory effects of IDO. Alternatively, in settings where it is desirable to reduce excessive inflammation and T cell activation, enhancing IDO activity may be beneficial.

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