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Human Plasmacytoid Dendritic Cells Activated by CpG Oligodeoxynucleotides Induce the Generation of CD4⁺CD25⁺ Regulatory T Cells¹

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Plasmacytoid dendritic cells (PDCs) are key effectors in host innate immunity and orchestrate adaptive immune responses. CpG oligodeoxynucleotides (ODN) have potent immunostimulatory effects on PDCs through TLR9 recognition and signaling. Little is known about the effects of CpG ODN on human PDC-mediated T cell priming. Here we show that type B CpG ODN effectively promotes PDCs to prime allogeneic naive CD4⁺CD25⁻ T cells to differentiate into CD4⁺CD25⁺ regulatory T (Treg) cells. The CD4⁺CD25⁺ T cells induced by CpG ODN-activated PDCs express forkhead transcription factor 3 and produce IL-10, TGF- β , IFN- γ , and IL-6, but low IL-2 and IL-4. These CD4⁺CD25⁺ T cells are hyporesponsive to secondary alloantigen stimulation and strongly inhibit proliferation of autologous or allogeneic naive CD4⁺ T cells in an Ag-nonspecific manner. CpG ODN-activated PDCs require direct contact with T cells to induce CD4⁺CD25⁺ Treg cells. Interestingly, IL-10 and TGF- β were undetectable in the supernatants of CpG ODN-stimulated PDC cultures. Both CpG-A and CpG-C ODN-activated PDCs similarly induced the generation of CD4⁺CD25⁺ Treg cells with strong immune suppressive function. This study demonstrates that TLR9 stimulation can promote PDC-mediated generation of CD4⁺CD25⁺ Treg cells and suggests PDCs may play an important role in the maintenance of immunological tolerance. *The Journal of Immunology*, 2004, 173: 4433–4442.

Dendritic cells (DCs)³ are specialized APCs that play a unique role in orchestrating both innate and adaptive immunity. Multiple DC subsets have been identified (1). These subsets may have unique roles in regulating immunity and tolerance (2–4). One of these DC subsets is the lineage⁻ (lin⁻) HLA-DR⁺CD11c⁻CD123⁺BDCA2⁺BDCA4⁺ plasmacytoid DCs (PDCs), also known as IFN- $\alpha\beta$ -producing cells (5–7). Upon viral or bacterial stimulation, PDCs in human blood and peripheral lymphoid tissues rapidly produce large amounts of IFN- $\alpha\beta$, differentiate into mature CD11c⁻ DCs and stimulate T cell-mediated adaptive immune responses. PDCs are known to play an important role in immunological tolerance. Blood PDCs matured with CD40 ligand (CD154) induce the generation of Th2 cells producing IL-4, IL-5, and IL-10 (8, 9). Naive CD8⁺ T cells primed with CD154-matured PDCs differentiated into IL-10-producing CD8⁺ regulatory T (Treg) cells that strongly inhibit allospecific proliferation of naive CD8⁺ T cells in a primary MLR (10). Studies in mice suggest that PDCs may have a role in the generation of CD4⁺ Treg

cells (11–13). PDCs have also been reported to stimulate T cells and drive Th1 polarization (14). These findings suggest that the distinct capacity of PDCs to induce a Th1, Th2, or Treg response may largely depend upon the signals that induce their activation and maturation (2).

Recent studies of bacterial DNA containing unmethylated CpG motifs as immunostimulatory agents demonstrated that certain CpG DNA sequences can rapidly activate human PDCs isolated from blood, promoting their maturation and survival (15–17). Although the exact mechanism of CpG motif recognition and downstream signaling is not known, the receptor for CpG DNA has been identified as TLR9, a member of the Toll receptor family that comprises an elegant pathogen recognition system for host defense in innate immunity (18). Human TLR9 is found on PDCs but not on myeloid DCs (19, 20), thus, human PDCs but not myeloid DCs are directly responsive to CpG DNA stimulation (17, 19). To mimic the stimulatory capacity of bacterial CpG DNA, synthetic oligodeoxynucleotides (ODN) containing the signature CpG dinucleotides (CpG ODN) and various flanking sequences and backbones have been used in both human and mouse studies (21). At least three distinct classes of CpG ODNs with structural and functional differences have been identified (22–24). The CpG-A (also known as D type) ODN (2216) consists of a chimeric phosphorothioate and phosphodiester backbone, one or more central CpG dinucleotides arranged in a palindrome, and poly-G motifs (“G-tetrads”) at either or both the 5′ and 3′ ends (22, 25, 26). The CpG-B (also known as K type) ODN (2006) consists of one or more CpG motifs on a phosphorothioate backbone (27, 28). CpG-C ODN (2395) consists of a 5′ stimulatory hexameric CpG motif linked by a T spacer to 3′ palindromic sequences that are preferably GC-rich (23, 24). All three classes of CpG ODN are known to potentially activate human PDCs. The major differences between these classes of CpG ODN include that CpG-A and CpG-C ODN, but not CpG-B ODN, can effectively induce high

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³ Abbreviations used in this paper: DC, dendritic cell; PDC, plasmacytoid DC; Treg, T regulatory cell; GVHD, graft-versus-host disease; ODN, oligodeoxynucleotide; BDCA, blood DC Ag; HCT, hemopoietic cell transplant.

levels of IFN- α production from PDCs (22) and that CpG-B ODN and CpG-C ODN, but not CpG-A ODN, are strong stimulators of human B cells (23). However, little is known about the effects of these CpG ODN on human PDC-mediated T cell priming.

Although studies suggest that PDCs play an important role in immunological tolerance induction (29), it is unknown whether human PDCs may play a role in the generation and function of CD4⁺CD25⁺ Treg cells. Recent studies have shown that CD4⁺CD25⁺ T cells can be induced to differentiate into CD4⁺CD25⁺ T cells with regulatory cell function by cytokines (e.g., IL-10, TGF- β) (30–32), stimulation with immature myeloid DCs (33–35), or immunomodulatory drugs such as vitamin D3 (36). In this study, we assessed the effects of CpG ODN on purified human PDC-mediated priming of allogeneic naive CD4⁺ T cells. We demonstrate that CpG ODNs promote human PDC-mediated priming of naive CD4⁺CD25⁺ T cells to differentiate into CD4⁺CD25⁺ Treg cells that potently suppress autologous and allogeneic T cell proliferation.

Materials and Methods

PBMC preparation, PDC, and T cell purifications

Human PBMC were isolated from apheresis products of healthy blood donors (Memorial Blood Centers of Minnesota, Minneapolis, MN) by Ficoll-Paque density gradient centrifugation. Blood PDCs were enriched from PBMC using blood DC Ag (BDCA)-4 cell isolation kits and the MACS system. The BDCA-4⁺ cell-enriched preparation was then stained with a mixture of FITC-conjugated mouse anti-human Abs directed against lineage (Lin) markers (CD3, CD14, CD16, CD19, CD20, and CD56), allophycocyanin-conjugated anti-CD11c, and PE-conjugated anti-CD123 (IL-3R α) Abs. The labeled cells were sorted on a FACS Vantage SE (BD Biosciences, San Jose, CA) to collect the Lin⁻CD11c⁻CD123⁺ PDCs. The purity of sorted PDCs was consistently higher than 98%. CD4⁺CD45RA⁺ naive T cells were isolated from PBMC by using CD4 T cell isolation kits followed by positive selection with CD45RA microbeads. The purity of naive CD4⁺ T cells was higher than 95% for CD4⁺CD45RA⁺ expression and <0.5% for CD25⁺ expression. Blood CD4⁺CD25⁺ natural-arising Treg cells were purified from CD4⁺ selected T cells by labeling cells with CD25 microbeads and positively selecting CD4⁺CD25⁺ T cells. B cells were purified from PBMC by labeling cells with CD19 microbeads and positively selecting CD19⁺ B cells. All cell isolation kits and microbeads were from Miltenyi Biotec (Bergisch Gladbach, Germany).

Oligodeoxynucleotides

Phosphorothioate-modified CpG ODNs were obtained from Coley Pharmaceutical Group (Wellesley, MA). CpG-A ODN 2216: ggGGGAC GATCGTcgggggG; CpG-B ODN 2006: **tcgtcgtttgctgctttgctg**T; CpG-C ODN 2395: tcgtcgttttcggcgcgcgcg (sequences are shown 5' to 3'; lower case letters, phosphorothioate linkage; capital letters, phosphodiester linkage 3' of the base; bold, CpG dinucleotides). No endotoxin could be detected in CpG ODN preparations (<0.03 EU/ml, *Limulus* ameobocyte lysate assay; BioWhittaker, Walkersville, MD). All three classes of CpG ODN were resuspended in TE buffer, diluted in PBS, and used at a final concentration of 3 μ g/ml.

In vitro priming of naive CD4⁺ T cells

Purified CD4⁺CD45RA⁺ T cells were incubated with allogeneic PDCs, CD11c⁺ DCs, or irradiated B cells (30 Gy) at a 10:1 ratio in 24-well plates (e.g., 2 \times 10⁶ CD4⁺CD45RA⁺ T cells plus 2 \times 10⁵ PDCs per well) with or without the designated CpG ODN in complete medium (RPMI 1640 supplemented with 10% human AB serum, 2 mM glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 μ M 2-ME). As controls, naive CD4⁺ T cells were cultured in complete medium with or without CpG ODN. In Transwell experiments, naive CD4⁺ T cells were incubated with Transwells containing sorted allogeneic PDCs at a 10:1 ratio in 24-well plates (2 \times 10⁶ naive CD4⁺ T cells plus a Transwell containing 2 \times 10⁵ PDCs) in complete medium with or without CpG ODN. After 6–7 days, primed T cells were harvested, analyzed for their cell surface phenotype and cytokine production profile, and assessed for their function in MLR assays.

Flow cytometry

FITC-, PE-, allophycocyanin-, or CyChrome-conjugated mouse anti-human Abs directed against CD3, CD4, CD11c, CD25, CD40, CD45,

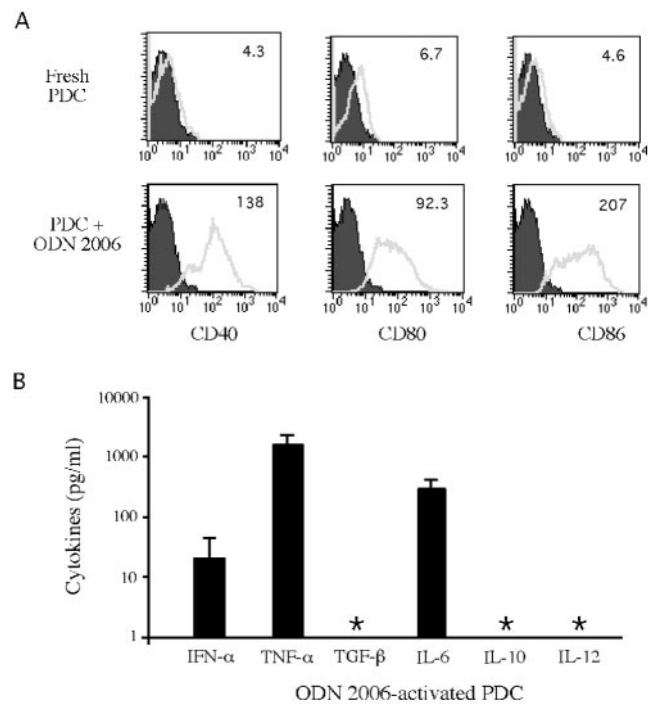


FIGURE 1. Cytokine profile of CpG ODN 2006-activated human PDCs. *A*, Sorted PDCs were cultured in serum-free medium (1.5×10^5 cells/600 μ l media/well) with ODN 2006 (3 μ g/ml) in 48-well plates for 48 h. Surface expression of CD40, CD80, and CD86 on PDCs pre- and post-CpG ODN stimulation was compared by staining with fluorescence-conjugated specific Abs (open histogram) or fluorescence-conjugated isotype-matched control Abs (filled histogram) and determined by flow cytometry. Mean fluorescent intensity is indicated. *B*, Culture supernatants collected from PDCs with or without ODN 2006 for 48 h were assayed for IFN- α , TNF- α , TGF- β , IL-6, IL-10, and IL-12p70 production by ELISA. The data shown are aggregated results from three independent experiments each from different donors and presented in the mean \pm SD. None of these cytokines were detected in the supernatants from PDCs cultured in medium alone (data not shown). *, Below the limits of detection.

CD45RA, CD45RO, CD80, CD86, CD123, HLA-DR, CTL-associated Ag-4 (CTLA-4: CD152), as well as isotype control Abs were from BD Pharmingen (San Diego, CA). FITC-conjugated mouse anti-human Abs directed against lineage (Lin) markers were from BD Immunocytometry Systems (San Jose, CA). Cells were stained with fluorescent Abs for 30 min on ice, washed twice with 0.2% FBS HBSS, and fixed with PBS containing 0.2% paraformaldehyde. Mean fluorescence intensity and positive cell percentages were determined by flow cytometry.

Cytokine production assays

Culture supernatants were collected from PDCs incubated in X-VIVO 15 serum-free media (BioWhittaker) with or without CpG ODN for 48 h. The levels of IFN- α , TNF- α , TGF- β , IL-6, IL-10, and IL-12p70 in the culture supernatants were determined by using ELISA kits according to the manufacturers' instructions. IFN- α ELISA kit was from BenderMed Systems (Vienna, Austria). TNF- α , TGF- β , IL-6, IL-10, and IL-12p70 ELISA kits were from R&D Systems (Minneapolis, MN). The lower limits of detection were as following: IFN- α , 4.8 pg/ml; TNF- α , 4.4 pg/ml; TGF- β , 7.0 pg/ml; IL-6, 0.7 pg/ml; IL-10, 3.9 pg/ml; and IL-12p70, 15 pg/ml.

The cytokine profile of PDC-primed CD4⁺ T cells were determined by incubating naive CD4⁺ T cells and allogeneic PDCs with or without CpG ODN. Naive CD4⁺ T cells cultured in complete medium alone were used as control. After 6–7 day, cells were harvested, washed, and restimulated at $1-2 \times 10^6$ cells/ml in serum-free medium in plates coated with immobilized anti-CD3 Ab (5 μ g/ml, UCHT1; BD Pharmingen) and soluble anti-CD28 Ab (1 μ g/ml, L293; BD Immunocytometry Systems) for 24 h. Culture supernatants were collected and cytokine profiles (IFN- γ , IL-2, IL-4, IL-6, and IL-10) of primed T cells were assayed with the Fluorokine MAP Immunoarray (R&D Systems) using the Luminex 100 analyzer. The levels

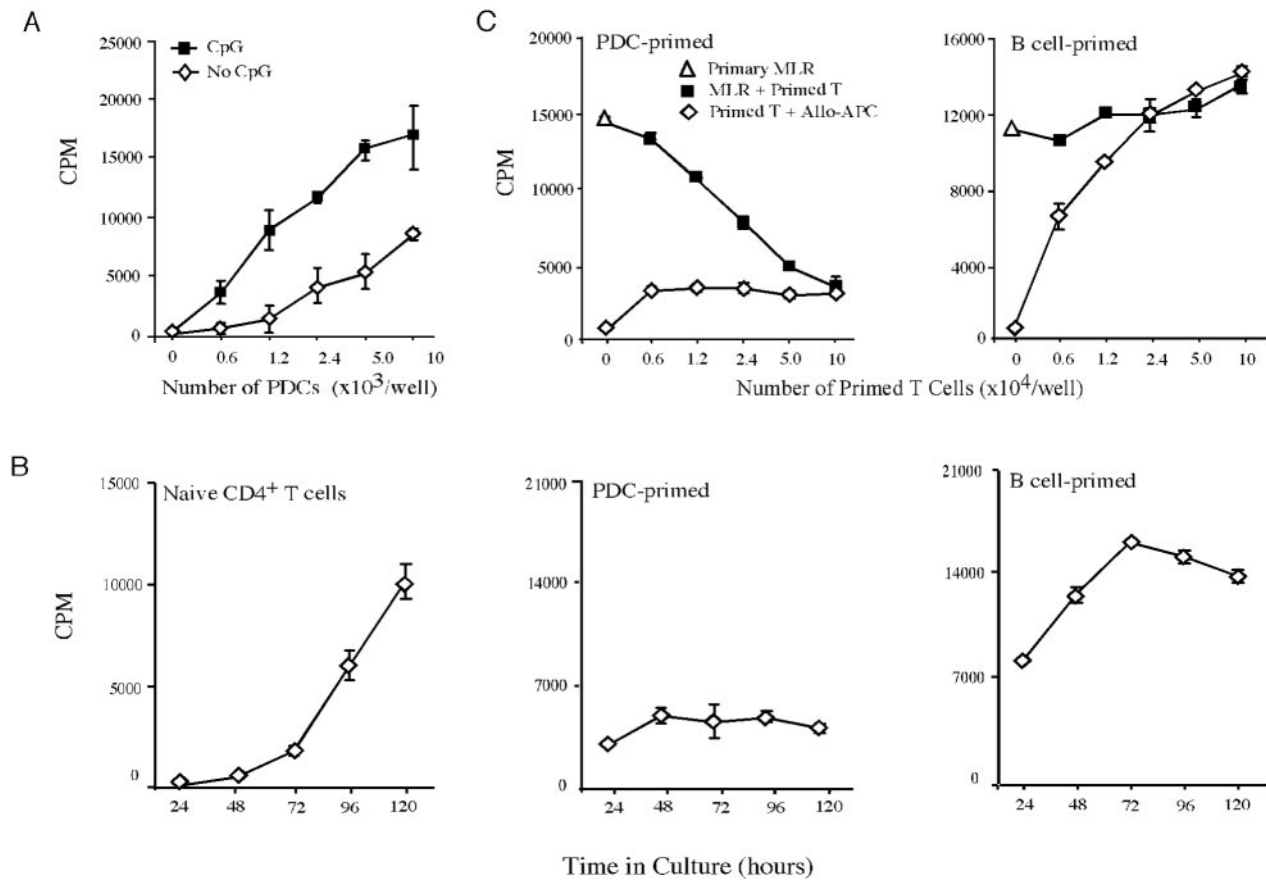


FIGURE 2. CpG ODN 2006-activated PDCs induce a CD4⁺ T cell population that are hypo-responsive and immunosuppressive in MLR. *A*, Purified naive CD4⁺ T cells were cultured in complete medium with graded doses of freshly isolated allogeneic PDCs with or without ODN 2006. T cell proliferation was assessed in a 5-day MLR assay and measured by [³H]thymidine incorporation over the last 18 h. *B*, Naive CD4⁺ T cells primed with allogeneic PDCs or B cells in the presence of ODN 2006 for 7 days were restimulated with primed alloantigens in secondary MLR cultures. Purified autologous naive CD4⁺ T cells were used as a control. T cell proliferation was assessed at days 1, 2, 3, 4, and 5 of MLR cultures. *C*, Naive CD4⁺ T cells primed with either ODN 2006-activated allogeneic PDCs or B cells were plated at graded doses as responders in a secondary MLR culture to the primed alloantigens or added at graded doses into a primary MLR culture in which autologous naive CD4⁺ T cells were stimulated with irradiated alloantigens from the priming donor. All T cell proliferation was assessed in a 5-day MLR assay and measured by [³H]thymidine incorporation for the last 18 h. The data shown are representative results from one of three independent experiments each from different donors.

of TGF- β in the culture supernatants were determined by using TGF- β ELISA kits from R&D Systems.

T cell proliferation assays

Naive CD4⁺ T cells were primed with allogeneic PDCs or B cells for 6–7 days with or without CpG ODN. The function of primed CD4⁺ T cells were determined by plating these primed T cells at graded doses as responders to irradiated allogeneic PBMC in MLR cultures or as third-party T cells into MLR cultures in which freshly purified autologous or allogeneic naive CD4⁺ T cells were stimulated with irradiated allogeneic PBMC. In some experiments, CD25⁺ and CD25⁻ T cell populations in the primed CD4⁺ T cells were separated using CD25 microbeads (Miltenyi Biotec) and used in functional assays. In all T cell proliferation assays, plates were incubated at 37°C for 5 days or the indicated time points and pulsed with 1 μ Ci of [³H]thymidine per well for the last 18 h before harvesting. All determinations were conducted in triplicate and [³H]thymidine incorporation was determined.

RT-PCR for TLR9 and Foxp3 expression

Total RNA was extracted from 2×10^6 freshly purified PDCs, CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells purified from PBMC or from CD4⁺ T cells primed with PDCs or PDCs plus CpG ODN. The RNA was reverse transcribed to cDNA, and analyzed for TLR9 and Foxp3 expression. PCR parameters: 94°C 5 min; 35 cycles: 94°C 30 s, 55°C 30 s, 72°C 1 min; and 72°C 7 min. The sequences of PCR primers for TLR9 (5'-TTATGGACTTCTGCTGGAGGTGC-3' and 5'-CTGCGTTTTGTGCGAAGACCA-3') and for Foxp3 (5'-ATGCCCAACCCAGGCTGGC-3'

and 5'-CTCCAGAGACTGTACCATCTC-3') were as reported (19, 37). β -actin (5'-GCTCGTCGTCGACAACGGCT-3' and 5'-CAAACATGATCTGGGTCATCTTCTC-3') was used as internal control. All RT-PCR reagents were from Invitrogen (Carlsbad, CA). For real-time quantitative RT-PCR, cDNA of each cell population was analyzed for the expression of Foxp3 gene by SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) using a PerkinElmer ABI Prism 7700 Sequence Detection System (Applied Biosystems). PCR parameters: 50°C 2 min, 95°C 10 min; 40 cycles: 60°C 1 min, 95°C 15 s. The sequences of PCR primers for Foxp3 (5'-CAC TGC CCC TAG TCA TGG T-3' and 5'-GGA GGA GTG CCT GTA AGT GG-3') and β -actin (5'-TAC CTC ATG AAG ATC CTCA-3' and 5'-TTC GTG GAT GCC ACA GGAC-3') were designed as shown.

Data analysis

Data from experiments are expressed as the mean \pm SD. Statistical analysis of the results between groups was performed by Student's *t* test. Values of *p* < 0.05 were considered significant.

Results

CpG-B ODN-activated PDCs induce the generation of CD4⁺CD25⁺ T cells that are hypo-responsive and strongly suppress naive CD4⁺ T cell proliferation in MLR assays

Freshly isolated human PDCs from peripheral blood are weak APCs associated with low expression of costimulatory molecules such as CD40, CD80, and CD86. Triggering TLR9 by CpG-B

ODN (2006) rapidly activated PDCs to up-regulate surface expression of CD40, CD80, and CD86 (Fig. 1A) and to produce IFN- α , TNF- α , IL-6, but not IL-10, TGF- β , or IL-12 (Fig. 1B). None of these cytokines were detected in the supernatants from PDCs cultured in medium alone (data not shown). Although freshly isolated PDCs induced a low proliferation of allogeneic naive CD4⁺ T cells, the presence of ODN 2006 increased PDC-induced proliferation of allogeneic naive CD4⁺ T cells by 2.0- to 6.4-fold (Fig. 2A). The function of naive CD4⁺ T cells primed with ODN 2006-activated PDCs (ODN 2006-PDC) was assessed in secondary MLR cultures. CD4⁺ T cells primed with irradiated allogeneic PBMC or B cells exhibited a secondary proliferative response to the primed alloantigens during the 5-day MLR cultures. In contrast, ODN 2006-PDC primed CD4⁺ T cells fail to mount a secondary proliferative response to the primed alloantigens during the 5-day MLR cultures (Fig. 2B and data not shown). Moreover, when the ODN 2006-PDC primed CD4⁺ T cells were added at graded doses into primary MLR cultures in which autologous naive CD4⁺ T cells were stimulated with irradiated alloantigens from the same priming donor, the proliferation of naive CD4⁺ T cells to alloantigens was suppressed in a primed T cell dose-dependent manner (Fig. 2C). In contrast, CD4⁺ T cells primed with ODN 2006-activated B cells that proliferated in response to secondary alloantigen stimulation did not suppress the proliferation of autologous naive CD4⁺ T cells to the same alloantigens (Fig. 2C). These findings indicate that CpG-B ODN-activated PDCs induce the generation of a CD4⁺ T cell population that is hyporesponsive to alloantigens and capable of suppressing naive T cell proliferation in MLR.

We next determined whether CpG-B ODN-PDCs induce the generation of CD4⁺CD25⁺ Treg cells from CD4⁺CD25⁻ naive T cells. Freshly isolated naive CD4⁺CD45RA⁺ T cells did not express CD25, CTLA-4, or CD45RO Ags (Fig. 3A). Naive CD4⁺ T cells cultured in media with or without ODN 2006 showed no phenotypic difference. Culturing naive CD4⁺ T cells with allogeneic PDCs induced CD4⁺CD25⁻ T cells to differentiate into CD4⁺CD25⁺ T cells (Fig. 3B). The frequency of CD4⁺CD25⁺ T cells generated in the PDC-primed T cells was significantly increased from 0.4% before culture to 9.5 \pm 4.5% at day 7 of culture. The addition of ODN 2006 significantly promoted the PDC-induced generation of CD4⁺CD25⁺ T cells and increased the frequency of CD4⁺CD25⁺ T cells to 31.1 \pm 9.7% at day 7 of culture (Fig. 3, B and D). Separating PDCs and naive CD4⁺ T cells in culture by transwells abrogated the capability of PDCs to induce CD4⁺CD25⁺ T cells with or without ODN 2006 (Fig. 3B). This finding suggests that PDC-induced generation of CD4⁺CD25⁺ T cells requires direct contact between PDCs and T cells. CD4⁺CD25⁺ T cells induced by ODN 2006-activated PDCs expressed CD45RO and CTLA-4 Ags (Fig. 3C). Recent studies have shown that naturally arising mouse CD4⁺ Treg cells express certain TLRs, including TLR4, 5, 7, and 8 (38). It is not known whether human naturally arising or ex vivo induced CD4⁺CD25⁺ Treg cells express TLR9. RT-PCR experiments showed that PDCs expressed high levels of TLR9, whereas neither of the purified T cell populations (CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells purified from PBMC and PDC-primed CD4⁺ T cells with or without ODN 2006) expressed TLR9 (Fig. 4A). This finding, together with the results that incubation of naive CD4⁺ T cells with ODN 2006 alone failed to induce CD4⁺CD25⁺ T cells excludes the possibility of direct stimulatory effect of CpG ODN on naive CD4⁺ T cells or PDC-primed CD4⁺ T cells, and suggests that ODN 2006 activates PDCs to promote the generation of CD4⁺CD25⁺ T cells.

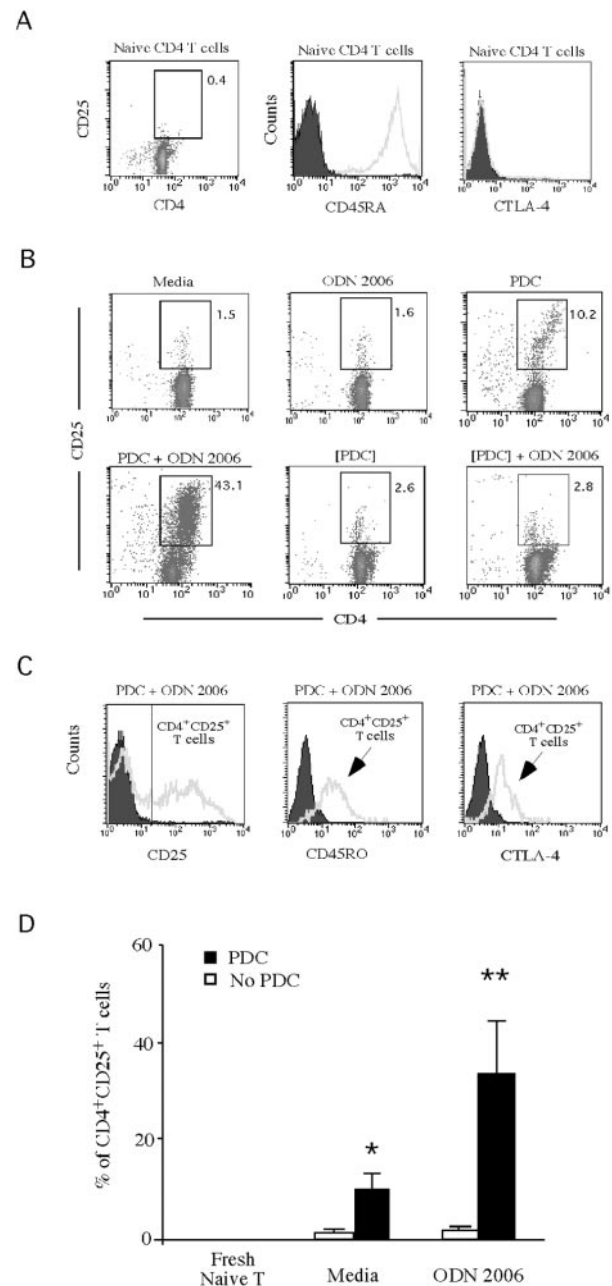


FIGURE 3. CpG-B ODN promotes PDC-mediated priming of allogeneic naive CD4⁺CD25⁻ T cells to differentiate into CD4⁺CD25⁺ T cells. **A**, Surface expression of CD25, CD45RA, and CTLA-4 on purified naive CD4⁺ T cells was determined by staining with fluorescence-conjugated specific Abs. **B**, Purified naive CD4⁺ T cells were cultured in complete medium or complete medium containing: ODN 2006, sorted allogeneic PDCs with or without ODN 2006, or PDCs in transwells with or without ODN 2006. After 6–7 day, T cells were harvested and stained with fluorescence-conjugated Abs against CD4 and CD25. The percentage of CD4⁺CD25⁺ T cells were determined and compared by flow cytometry. **C**, Surface expression of CD45RO and CTLA-4 on CpG ODN PDC-induced CD4⁺CD25⁺ T cells were determined by staining primed T cells with fluorescence-conjugated Abs against CD4, CD25, CD45RO, and CTLA-4 and gated on CD4⁺CD25⁺ T cells to determine their surface expression of CD45RO and CTLA-4 (open histogram) as compared with the fluorescence-conjugated isotype-matched control Abs (filled histogram). **D**, The data presented are aggregated results from five staining experiments each from different individual donors and are expressed as the mean \pm SD. *, $p < 0.05$ (compared the percentage of CD4⁺CD25⁺ T cells induced by PDCs with fresh naive T cells and no PDC cultures). **, $p < 0.05$ (compared the percentage of CD4⁺CD25⁺ T cells induced by CpG ODN-activated PDCs with that induced by PDCs without CpG ODN).

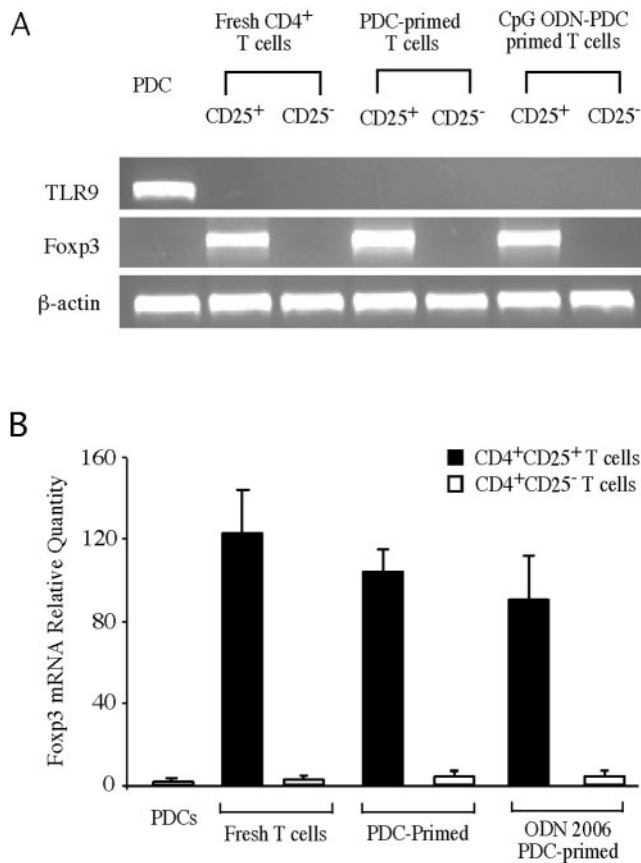


FIGURE 4. $CD4^+CD25^+$ T cells induced by CpG ODN-activated PDCs express Foxp3. *A*, Total RNA was extracted from freshly purified PDCs, naturally occurring $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ naive T cells from PBMC as well as the $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells purified from T cells primed with PDCs with or without ODN 2006. The expressions of TLR9 and Foxp3 mRNA in each cell population were determined by RT-PCR. The expression of a housekeeping gene β -actin mRNA was used as an internal control. The data shown are representative results of three experiments. *B*, Real-time RT-PCR determination of Foxp3 expressions in naturally occurring $CD4^+CD25^+$ Treg cells, naive $CD4^+CD25^-$ T cells, and purified PDCs from PBMC as well as the $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells purified from ODN 2006-PDC primed T cells. The data shown are aggregated results of Foxp3 mRNA relative quantity from three experiments and presented in the mean \pm SD.

CD4⁺CD25⁺ T cells induced by ODN 2006-activated PDCs express Foxp3 and produce a Treg cell cytokine profile

Foxp3, a forkhead transcription factor, has been identified as a gene preferentially expressed in $CD4^+CD25^+$ Treg cells. The expression of Foxp3 is associated with the development and function of $CD4^+CD25^+$ Treg cells in mice and humans (39, 40). RT-PCR experiments were performed to determine Foxp3 expression in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells isolated from PBMC or from ODN 2006-PDC primed naive $CD4^+$ T cell cultures. Strong Foxp3 expression was detected in naturally occurring $CD4^+CD25^+$ Treg cells purified from PBMC (Fig. 4A). Negligible Foxp3 expression was detected in PDCs and naive $CD4^+$ T cells purified from PBMC. Intriguingly, $CD4^+CD25^+$ T cells isolated from either PDC-primed or ODN 2006-PDC primed naive $CD4^+CD25^-$ T cell cultures showed strong Foxp3 expression, whereas the $CD4^+CD25^-$ T cells purified from both PDC-primed or ODN 2006-PDC primed naive $CD4^+$ T cell cultures remained negative for Foxp3 expression (Fig. 4A). Real-time quantitative RT-PCR results showed that Foxp3 expression in natural occurring

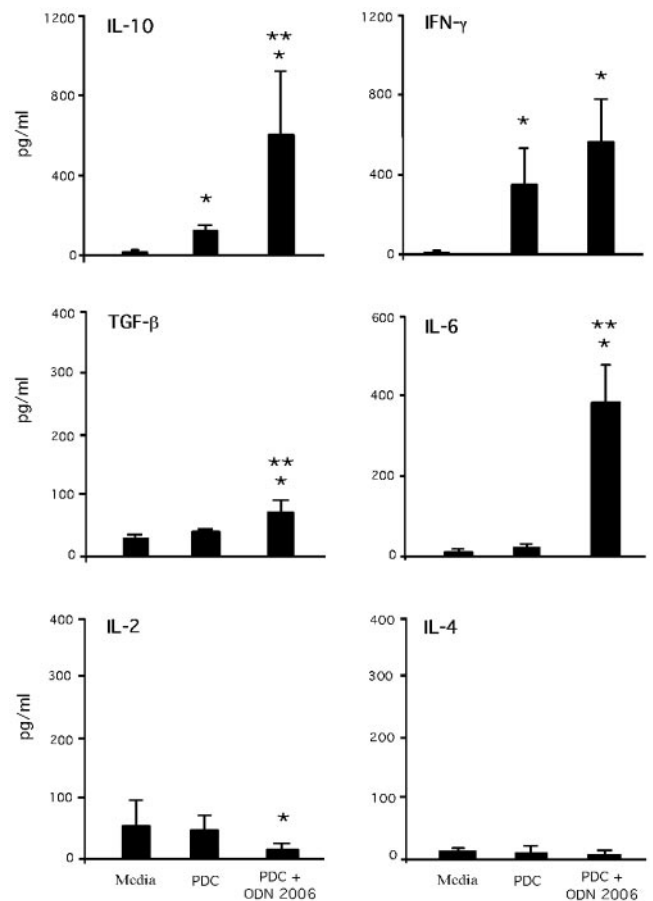


FIGURE 5. CpG ODN-activated PDCs prime allogeneic naive $CD4^+$ T cells to produce a Treg cell cytokine profile. Purified naive $CD4^+$ T cells were cultured with allogeneic PDCs with or without the presence of ODN 2006. Naive $CD4^+$ T cells incubated in media were used as a control. After 6–7 day, cells were harvested, washed, and restimulated with anti-CD3 and anti-CD28 Abs in X-VIVO-15 serum-free medium for 24 h. Culture supernatant was collected and cytokines were analyzed using Fluorokine MAP immunoarray and ELISA. The data shown are results from five experiments each from different donors and presented in the mean \pm SD. *, $p < 0.05$ (compared the cytokine production of naive $CD4^+$ T cells cultured in media alone and with PDCs). **, $p < 0.05$ (compared the cytokine production of $CD4^+$ T cells primed by PDCs with or without CpG ODN).

$CD4^+CD25^+$ Treg cells and the $CD4^+CD25^+$ T cells induced by ODN 2006-activated PDCs were 27.9- and 20.4-fold higher than Foxp3 expression in the naive $CD4^+CD25^-$ T cells, respectively (Fig. 4B). Analysis of cytokine production showed that PDC-primed $CD4^+$ T cells produced higher amounts of IL-10 and IFN- γ than those produced by naive $CD4^+$ T cells cultured in media alone. The addition of ODN 2006 to PDC-T cell priming cultures enhanced T cell production of IL-10, TGF- β , IFN- γ , IL-6 but not IL-2 or IL-4 (Fig. 5). These findings demonstrate that type B CpG ODN enhances PDC-induced differentiation of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ T cells that express Foxp3 and produce a Treg cytokine profile.

CD4⁺CD25⁺ T cells induced by ODN 2006-activated PDCs suppress autologous and allogeneic T cell proliferation in an Ag-nonspecific manner

Functional analysis of naive $CD4^+$ T cells (donor A) primed with allogeneic PDCs (donor C), with or without the presence of ODN 2006, showed that $CD4^+$ T cells primed under either culture condition were hyporesponsive to secondary alloantigen stimulation

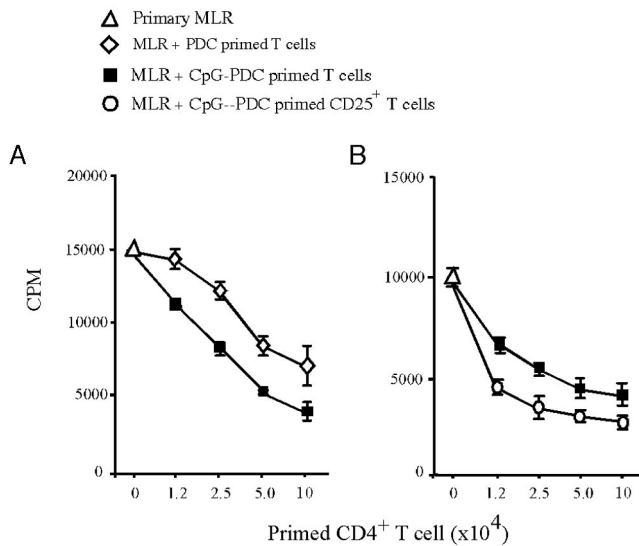


FIGURE 6. Naive CD4⁺ T cells primed with ODN 2006-activated PDCs suppress the proliferative responses of autologous T cells in MLR. **A**, Naive CD4⁺ T cells (donor A) primed by PDCs (donor C) with or without ODN 2006 for 7 day were harvested and plated at graded doses into MLR cultures where purified autologous (donor A) naive CD4⁺ T cells were stimulated with irradiated PBMC from the priming PDC donor (donor C). **B**, Naive CD4⁺ T cells primed by ODN 2006-PDCs (donor A vs C) for 7 days were harvested and CD25⁺ T cells were isolated from the total primed T cell population for functional assays. Graded doses of ODN 2006-PDCs primed total T cell population or purified CD25⁺ T cells were plated into MLR cultures in which purified autologous (donor A) naive CD4⁺ T cells were stimulated with irradiated PBMC from the priming PDC donor (donor C). T cell proliferation was assessed in a 5-day MLR assay and measured by [³H]thymidine incorporation for the last 18 h. The data shown are representative results from one of four independent experiments each from different donors.

and failed to mount a secondary proliferative response to the primed alloantigens (data not shown). When PDC-primed or ODN 2006-PDC primed CD4⁺ T cells (donor A vs C) were added to a primary MLR in which purified autologous (donor A) naive CD4⁺ T cells were stimulated with the primed alloantigens (donor C), they strongly suppressed T cell proliferation in a primed T cell dose-dependent manner (Fig. 6A). ODN 2006-PDC primed CD4⁺ T cells were more effective than PDC-primed CD4⁺ T cells in suppressing naive CD4⁺ T cell proliferation in MLR. To determine whether this difference is attributable to the higher frequency of CD4⁺CD25⁺ Treg cells present in the ODN 2006-PDC primed CD4⁺ T cell population, the CD4⁺CD25⁺ T cells were isolated. Functional analysis showed that purified CD4⁺CD25⁺ T cells were not only immunosuppressive but also were more efficient than the total ODN 2006-PDC primed T cell population in suppressing autologous T cell proliferation to alloantigens in MLR (Fig. 6B). In subsequent experiments, naive CD4⁺ T cells (donor A) primed by ODN 2006-activated PDCs (donor C) were separated into CD4⁺CD25⁺ and CD4⁺CD25⁻ T cell populations. These CD4⁺CD25⁺ T cells (donor A vs C) were hyporesponsive to secondary alloantigen stimulation and failed to mount a secondary proliferative response to either the primed alloantigens (donor C) or third-party alloantigens (donor D) (Fig. 7A). When the purified CD4⁺CD25⁺ T cells were added at graded doses into primary MLR cultures in which purified autologous (donor A) or allogeneic (donor B) naive CD4⁺ T cells were stimulated with irradiated PBMC from donor C or donor D, they strongly suppressed the proliferation of both autologous and allogeneic naive

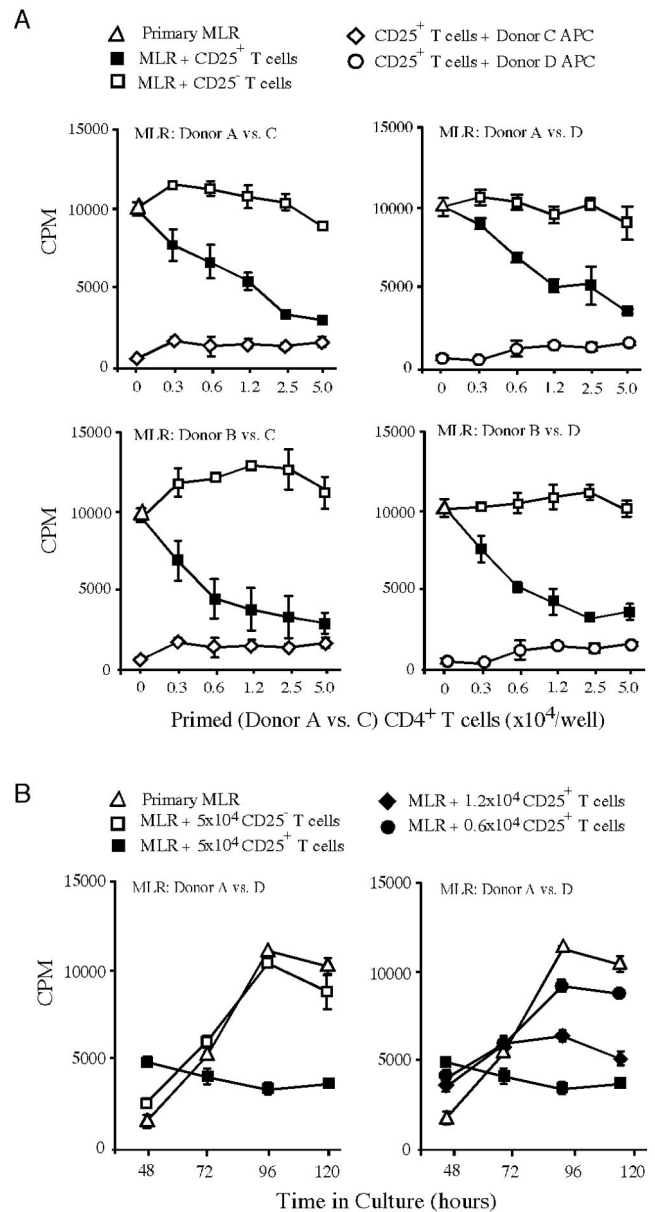


FIGURE 7. CD4⁺CD25⁺ Treg cells induced by ODN 2006-activated PDCs suppress the proliferation of naive CD4⁺ T cells in an Ag-nonspecific manner. Naive CD4⁺ T cells (donor A) were primed with allogeneic PDCs (donor C) in the presence of ODN 2006 for 7 day and then harvested for functional assays. **A**, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells isolated from ODN 2006-PDC primed CD4⁺ T cells (donor A vs C) were plated at graded doses into MLR cultures in which freshly purified autologous (donor A) and allogeneic (donor B) naive CD4⁺ T cells were stimulated with irradiated PBMC from donors C or D. **B**, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells isolated from ODN 2006-PDC primed CD4⁺ T cells (donor A vs C) were plated at graded doses into MLR cultures in which freshly purified autologous naive CD4⁺ T cells (donor A) were stimulated with irradiated PBMCs from donor C. T cell proliferation was assessed at days 2, 3, 4, and 5 of MLR cultures. All T cell proliferation was assessed in a 5-day or at indicated time points of MLR assays and measured by [³H]thymidine incorporation for the last 18 h. The data shown are representative results from one of three independent experiments.

CD4⁺ T cells in a CD4⁺CD25⁺ T cell dose-dependent manner (Fig. 7A). The CD4⁺CD25⁺ T cells effectively inhibited the naive CD4⁺ T cell proliferation to alloantigens at a suppressor to responder ratio lower than 1:33. Depletion of CD4⁺CD25⁺ T cells abrogated the immunosuppressive effect of the ODN 2006-PDC

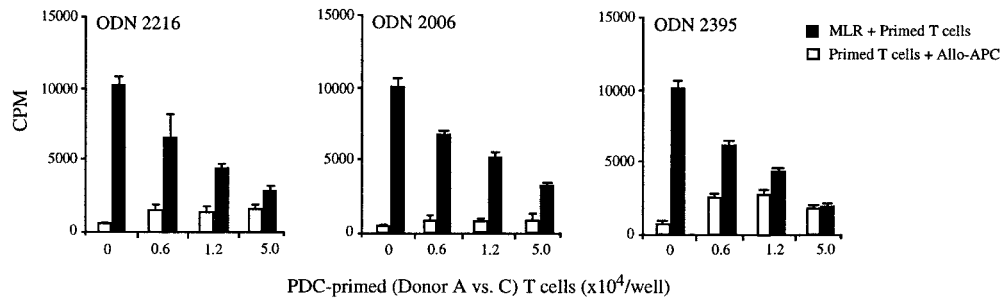


FIGURE 8. CpG-A or CpG-C ODN-activated PDCs also induce CD4⁺CD25⁺ T cells that are hyporesponsive to secondary alloantigen stimulation and strongly inhibit naive CD4⁺ T cells proliferation in MLR. Naive CD4⁺ T cells (donor A) were primed with allogeneic PDCs (donor C) in the presence of ODN 2216, ODN 2006, or ODN 2395 for 7 day and then harvested for functional assays. The CD4⁺CD25⁺ cells isolated from CD4⁺ T cells primed with different CpG ODN activated PDCs (donor A vs C) were plated at graded doses as responder cells in a secondary MLR to the primed alloantigens or into MLR cultures where freshly purified autologous (donor A) naive CD4⁺ T cells were stimulated with irradiated PBMC from donor C. T cell proliferation was assessed in a 5-day MLR assay. The data shown are representative results from one of three independent experiments.

primed CD4⁺ T cells (Fig. 7A). Kinetic analysis of primary MLR cultures containing the CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells purified from ODN 2006-PDC primed CD4⁺ T cell cultures (donor A vs C) showed that CD4⁺CD25⁺ T cells consistently suppressed the proliferation of autologous naive CD4⁺ T cells (donor A) to alloantigen stimulation during the 5-day culture (Fig. 7B). The suppression mediated by ODN 2006-PDC induced CD4⁺CD25⁺ T cells in the MLR system was CD4⁺CD25⁺ T cell dose-dependent. The addition of the CD4⁺CD25⁻ T cells into the primary MLR cultures did not alter the proliferation curves of freshly purified autologous naive CD4⁺ T cells to alloantigens during the same time course. CD4⁺CD25⁺ T cells, but not CD4⁺CD25⁻ T cells, from naive CD4⁺ T cells primed with allogeneic PDC alone, similarly suppress the proliferation of autologous and allogeneic naive CD4⁺ T cell to allogeneic stimulation in an Ag-nonspecific manner (data not shown). These results demonstrate that CD4⁺CD25⁺ T cells induced by PDCs or CpG ODN-activated PDCs suppress autologous and allogeneic T cell proliferation in an Ag-nonspecific manner.

CpG-A and CpG-C ODN activated PDCs also induce the generation of CD4⁺CD25⁺ Treg cells that suppress autologous and allogeneic T cell proliferation in MLR

CpG-A ODN (2216) and CpG-C ODN (2395) are also known to potently activate human PDCs. Unlike CpG-B ODN, both CpG-A and CpG-C ODN can effectively induce high levels of IFN- α production from PDC precursors. To determine whether CpG-A or CpG-C ODN-activated PDCs similarly induce the generation of CD4⁺CD25⁺ Treg cells, naive CD4⁺ T cells were cultured with allogeneic PDCs in the presence or absence of CpG-A, CpG-B, or CpG-C ODN. Interestingly, PDC activation by all three classes of CpG ODN (class A, B, and C) induced the generation of CD4⁺CD25⁺ T cells that strongly suppress autologous and allogeneic T cell proliferation in MLR (Fig. 8 and data not shown). The phenotypic change and cytokine production profile of ODN 2216-activated PDCs was similar to that shown with CpG-B ODN in Fig. 1, except for IFN- α production. IFN- α accumulation in 24–48 h culture supernatants from ODN 2216-activated PDCs was 1532- to 1940-fold (24213–35110 pg/ml vs 13.9–22.4 pg/ml) higher than that induced by ODN 2006-activated PDCs. Similar to the results obtained using ODN 2006 activated PDCs, CD4⁺CD25⁺ T cells from naive CD4⁺ T cells primed with ODN 2216 activated PDCs were also hyporesponsive and failed to mount a secondary proliferative response to either primed (donor C) or third-party alloantigens (donor D) (Fig. 9A). In experiments mirroring those done with ODN 2006 (Fig. 7), ODN 2216-PDC primed CD4⁺CD25⁺

T cells suppressed the proliferation of autologous and allogeneic naive CD4⁺ T cells to alloantigenic stimulation. Kinetic analysis again revealed that the naive CD4⁺ T cell proliferation to alloantigen was consistently suppressed throughout the 5-day culture, and that suppression was CD4⁺CD25⁺ T cell dose dependent. Addition of ODN 2216-PDC primed CD4⁺CD25⁻ T cells failed to suppress naive T cell proliferation to alloantigen at any point during the 5-day culture (Fig. 9B). These results demonstrate that PDCs activated by all three classes of CpG ODN induce the generation of CD4⁺CD25⁺ T cells that strongly inhibit proliferation of naive CD4⁺ T cells in an Ag-nonspecific manner.

Discussion

Recent studies demonstrate that DCs not only play a key role in the induction of immune responses, but also participate in the induction and maintenance of immune tolerance (4). In this study, we demonstrate that human PDCs prime naive CD4⁺CD25⁻ T cells to differentiate into CD4⁺CD25⁺ Treg cells characterized as Foxp3⁺ IL-10-producing immunosuppressive T cells. Treatment of human PDCs with all three classes (type A, B, and C) of CpG ODN-induced the generation of CD4⁺CD25⁺ Treg cells with strong Ag-nonspecific immunosuppressive effects on naive CD4⁺ T cell proliferation. These findings provide new insights to understanding the immunostimulatory effects of CpG ODN on human PDCs and their potential therapeutic application in the induction and maintenance of immune tolerance.

It is known that blood PDCs can differentiate into mature CD11c⁻ DCs following immunostimulatory signals from microbial pathogenic stimuli. These maturation stimuli are recognized by the engagement of specific receptors (e.g., TLR9) on DCs upon microbial infection or by cytokines produced during inflammation or infection (16, 19). PDCs have been shown to induce either a Th2 or a Th1 response in naive CD4⁺ T cells depending upon the stimulatory signals (8, 9, 14). A recent study also showed that CD154-matured PDCs prime naive CD8⁺ T cells to differentiate into IL-10-producing CD8⁺ Treg cells that display poor secondary proliferative and cytolytic responses to alloantigen but strongly inhibit allospecific proliferation of naive CD8⁺ T cells in a primary MLR (10). The cytokine profile of HSV-infected PDCs primed T cells is characterized by production of IFN- γ and IL-10, low IL-4 and no IL-5. CD154-matured PDCs induced a Th2 type response that resulted in significant production of IL-4, IL-5, IL-10 but some IFN- γ in PDC-primed naive CD4⁺ T cells. Unlike the cytokine profile reported in these prior studies, our results show that CpG ODN-activated PDCs prime allogeneic naive CD4⁺ T cells to produce IL-10, TGF- β , IFN- γ but negligible levels of IL-2

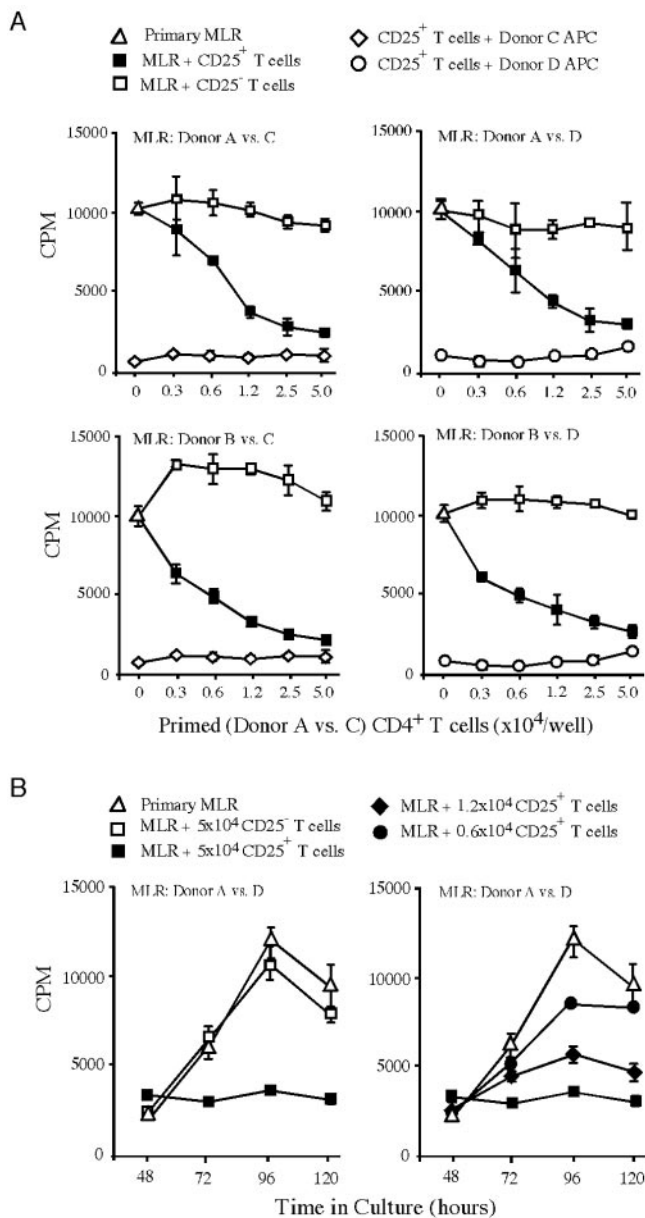


FIGURE 9. CD4⁺CD25⁺ Treg cells induced by ODN 2216-activated PDCs suppress the proliferation of naive CD4⁺ T cells in an Ag-nonspecific manner. Naive CD4⁺ T cells (donor A) were primed with allogeneic PDCs (donor C) in the presence of ODN 2216 for 7 day and then harvested for functional assays. **A**, The CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells isolated from ODN 2216-PDC primed CD4⁺ T cells (donor A vs C) were plated at graded doses into MLR cultures in which freshly purified autologous (donor A) and allogeneic (donor B) naive CD4⁺ T cells were stimulated with irradiated PBMC from donors C or donor D. T cell proliferation was assessed in a 5-day MLR assay. **B**, The CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells isolated from ODN 2216-PDC primed CD4⁺ T cells (donor A vs C) were plated at graded doses into MLR cultures in which freshly purified autologous naive CD4⁺ T cells (donor A) were stimulated with irradiated PBMCs from donor C. T cell proliferation was assessed at days 2, 3, 4, and 5 of MLR cultures. The data shown are representative results from one of three independent experiments.

and IL-4, a typical Treg cell type cytokine profile (41–44). Our findings demonstrate that CpG ODN preferentially polarize PDC-primed CD4⁺ naive T cells to differentiate into CD4⁺CD25⁺ Treg cells.

Little is known about human CD4⁺CD25⁺ Treg cell interactions with other immune-regulatory cells such as subsets of DCs.

Studies by Jonuleit et al. (33) showed that in vitro repetitive weekly stimulations of human naive CD4⁺ T cells with allogeneic monocyte-derived immature DCs induced an IL-10-producing, nonproliferating CD4⁺CD25⁺ Treg cell population. It has been suggested that immature DCs play an important role in maintaining immune tolerance and prime T cells to differentiate into regulatory/suppressor T cells whereas mature DCs prime T cells to induce strong immune responses. In mice, Treg cell development and/or function controlled by immature DCs can be reversed by CD154 ligation or TLR stimulation (45–47). However, this paradigm does not apply to the previous finding that CD154-activated human PDCs are tolerogenic (10). Our results further demonstrate that human PDCs activated by CpG ODN not only induce but enhance the generation of CD4⁺CD25⁺ Treg cells with strong immunosuppressive function. We show that blood PDC precursors can prime allogeneic naive CD4⁺ T cells to generate CD4⁺CD25⁺ Treg cells in a 7-day culture. The addition of CpG ODN effectively promoted PDC-induced generation of CD4⁺CD25⁺ Treg cells with phenotypic and functional properties similar to the reported CD4⁺CD25⁺ Treg cells isolated from human peripheral blood (42, 48–50). These findings suggest that the capacity of DCs in maintaining immune tolerance may not be simply attributed to their immature stage (low levels of expression of MHC molecules, CD80, and CD86), it may well depend upon the subtypes of DCs and the signals that induce their activation. The induction of CD4⁺CD25⁺ Treg cells by CpG ODN-activated PDCs requires PDC-T cell direct contact. There is a possibility that although CpG ODN-treated PDCs up-regulate costimulatory molecules, there may be a concomitant up-regulation of suppressive surface molecules such as Ig-like transcripts 3 and 4 (51, 52) and Notch receptor ligands (50, 53, 54) as well as down-regulation of surface markers such as glucocorticoid-induced TNF receptor ligand (55–57). Interestingly, we did not detect IL-10 and TGF- β , known to induce CD4⁺CD25⁺ T cells to acquire regulatory function (30–32), in supernatants derived from CpG ODN-stimulated PDC cultures. There is also a possibility that PDCs may produce other cytokines that serve to polarize naive CD4⁺ T cells to differentiate into CD4⁺CD25⁺ Treg cells. Although the molecular mechanisms by which PDCs induce CD4⁺CD25⁺ Treg cells remain to be investigated, the findings from this study demonstrate that human PDCs play a role in CD4⁺CD25⁺ Treg cell generation and that CpG ODN can efficiently promote the PDC-induced generation of CD4⁺CD25⁺ Treg cells. In vivo, the effects of CpG ODN on PDC-mediated T cell priming will be complicated by the presence of mixed cell populations. Proinflammatory cytokines produced by CpG ODN-activated PDCs will lead to subsequent activation of CD11c⁺ immature DCs and NK cells, which produce other cytokines that may affect the microenvironment and outcome of naive T cell priming. The role of CpG ODN in promoting PDC-mediated generation of Treg cells is likely balanced by other immune effects in vivo.

In experimental bone marrow transplantation models, donor CD4⁺CD25⁺ Treg cells have been shown to play a pivotal role in preventing graft-versus-host disease (GVHD) and in tolerance induction to allogeneic hemopoietic cell transplants (HCT) (58–62). CD4⁺CD25⁺ T cells isolated from donor mice are potent inhibitors of alloresponses in vitro and induce marked protection from lethal GVHD in vivo (58, 60–62). Removal of CD4⁺CD25⁺ T cells present in the graft dramatically accelerates GVHD, whereas the addition of either freshly isolated or in vitro expanded CD4⁺CD25⁺ donor Treg cells significantly delays or prevents GVHD (58, 60–62). Adoptive transfer of ex vivo activated, cultured CD4⁺CD25⁺ T cells resulted in significant inhibition of rapidly lethal GVHD (58, 61, 62). These findings show the great

potential of Treg cells as new therapeutics for controlling GVHD in allogeneic HCT (63). Several clinical studies indicated that PDCs may play an important role in modulating immune responses after HCT to facilitate engraftment and prevent GVHD reactions (64, 65). Our finding that human PDCs prime allogeneic naive CD4⁺ T cells to generate CD4⁺CD25⁺ Treg cells with strong immunosuppressive effects of alloresponses in vitro represent potential therapeutic uses of PDCs as cellular therapies to modulate immune responses post-HCT or the use of PDC-induced CD4⁺CD25⁺ Treg cells to control GVHD in allogeneic HCT. We have recently developed a novel hemopoietic progenitor cell culture system that allows in vitro generation of large numbers of human PDCs from CD34⁺ hemopoietic progenitor cells to facilitate future studies of PDC development, their immune function and potential clinical application in immune-based therapies (66). The use of CpG ODN to modulate PDC function and to specifically regulate immune responses in the recipient may provide novel immune-based therapies to control GVHD and viral diseases in post-HCT patients.

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