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*J Immunol* published online 22 July 2013
http://www.jimmunol.org/content/early/2013/07/21/jimmunol.1203053

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Protein Kinase C Inhibitor Generates Stable Human Tolerogenic Dendritic Cells

Takuya Matsumoto,* Hitoshi Hasegawa,⁎† Sachiko Onishi,* Jun Ishizaki,* Koichiro Suemori,* and Masaki Yasukawa*†

Tolerogenic dendritic cells (DCs) are a promising tool for a specific form of cellular therapy whereby immunological tolerance can be induced in the context of transplantation and autoimmunity. From libraries of bioactive lipids, nuclear receptor ligands, and kinase inhibitors, we screened conventional protein kinase C inhibitors (PKCIs) bisindolylmaleimide I, Go6983, and Ro32-0432 with strong tolerogenic potential. PKCI-treated human DCs were generated by subjecting them to a maturation process after differentiation of immature DCs. The PKCI-treated DCs had a semimature phenotype, showing high production of IL-10, and efficiently induced IL-10–producing T cells and functional Foxp3+ regulatory T cells from naive CD4+ T cells, thus eliciting a strong immunosuppressive function. They also showed CCR7 expression and sufficient capacity for migration toward CCR7 ligands. Additionally, PKCI-treated DCs were highly stable when exposed to inflammatory stimuli such as proinflammatory cytokines or LPS. Conventional PKCIs inhibited NF-κB activation of both the canonical and noncanonical pathways of DC maturation, thus suppressing the expression of costimulatory molecules and IL-12 production. High production of IL-10 in PKCI-treated DCs was due to not only an increase of intracellular cAMP, but also a synergistic effect of increased cAMP and NF-κB inhibition. Moreover, PKCI-treated mouse DCs that had properties similar to PKCI-treated human DCs prevented graft-versus-host disease in a murine model of acute graft-versus-host disease. Conventional PKCI-treated DCs may be useful for tolerance-inducing therapy, as they satisfy the required functional characteristics for clinical-grade tolerogenic DCs.

The Journal of Immunology, 2013, 191: 000–000.

Dendritic cells (DCs) are a heterogeneous population of APCs that contribute to innate immunity and initiate, coordinate, and regulate adaptive immune responses in infection and inflammation (1). Successful initiation of these adaptive immune responses requires DC maturation. Immature DCs (iDCs) are able to take up soluble Ag but are poorly immunogenic because they express only modest levels of MHC molecules and low levels of costimulatory molecules and proinflammatory cytokines. During maturation, DCs lose their capacity to capture soluble Ags but gain T cell stimulatory capacity due to increased Ag processing and upregulation of MHC, costimulatory molecules, and cytokines (1, 2). Although potently capable of initiating adaptive immune responses, DCs also play an important role in modulating the induction of tolerance (3–5). Tolerogenic DCs (tDCs) are generally characterized by an immature or semimature phenotype, with a capacity for high Ag uptake and low expression of costimulatory molecules (2, 6). Additionally, tDCs produce low amounts of proinflammatory cytokines and high amounts of anti-inflammatory cytokines. This results in anergy, apoptosis of effector T cells, or induction and expansion of regulatory T cells (7).

For patients with autoimmune diseases and graft rejection, any therapy capable of preventing or reducing the extant immune activation is highly desirable. Ex vivo–generated iDCs are considered to have strong potential for use in cellular therapy for these conditions. In fact, injection of ex vivo–generated tDCs has been shown to be beneficial in animal models of graft rejection (8–10) and autoimmune diseases, including collagen-induced arthritis (11, 12), diabetes (13–16), and experimental autoimmune encephalomyelitis (17). Human tDCs can be cultured in vitro from DC precursors using different compounds (7, 18). For this purpose, anti-inflammatory cytokines such as IL-10 (19–22) and TGF-β (22–24), vitamin D3 (Vit D3) (25–28), neuropeptides, including vasoactive intestinal peptide (37–39). Therefore, to obtain compounds that have stronger tolerogenic potential, we screened libraries of bioactive lipids, nuclear receptor ligands, and kinase inhibitors for molecules that would downregulate the costimulatory molecules CD80 and CD86, as well as expression of proinflammatory cytokines and high amounts of anti-inflammatory cytokines. This results in anergy, apoptosis of effector T cells, or induction and expansion of regulatory T cells (7).

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PKC-dependent cell differentiation and cytokine production in many cell types (40). PKC has been reported to play critical roles in mediating progenitor-to-DC differentiation and cytokine production from monocytes/macrophages and DCs (41–46). In the present study, we found that stable iDCs showing high production of IL-10 were generated by addition of PKCI during the process of maturation from iDCs. We also characterized the PKCI-treated iDCs obtained and clarified the mechanism responsible for their induction.

Materials and Methods

Abs and reagents

FITC-conjugated mouse anti-human CD1a (HI149), PE-conjugated mouse anti-human CD11c (B-ly6), allophycocyanin-conjugated mouse anti-human CD4 (RPA-T4), allophycocyanin-conjugated mouse anti-human CD14 (M6p9), and PE-conjugated rat anti-human IL-10 (JE83-19F1) mAbs were purchased from BD Biosciences (San Diego, CA). PE-conjugated mouse anti-human CD40 (MAB89), FITC-conjugated mouse anti-human CD80 (MAB104), FITC-conjugated mouse anti-human CD86 (HB15a), PE-conjugated mouse anti-human CD86 (HA5.2B7), FITC-conjugated mouse anti-human HLA-ABC (B9.12.1), and PE-conjugated mouse anti-human HLA-DR (E9.12.2) mAbs were obtained from Beckman Coulter (Brea, CA). Conjugated mouse anti-human Foxp3 (PC10H1) and mouse anti-human CCRR (CCRR6.83) (47) mAbs were obtained from BioLegend (San Diego, CA). FITC-conjugated mouse anti-human CD25 (BC96) mAb was purchased from BioLegend (San Diego, CA). Recombinant human (rh) GM-CSF, rhIL-4, rhIL-1β, rhIL-6, rhCCL19, mouse anti-human IL-10 (25209), and mouse anti-human TGF-β1 (9016) mAbs were purchased from R&D Systems (Minneapolis, MN). IFN-γ was purchased from PeproTech (Rocky Hill, NJ). FITC-conjugated mouse anti-human HLA-ABC (B9.12.1), and PE-conjugated mouse anti-human CD11c (HL3), FITC-conjugated rat anti-mouse CD40 (3/23), FITC-conjugated hamster anti-mouse CD80 (16-10A1), PE-conjugated rat anti-mouse CD83 (Michel-19), FITC-conjugated rat anti-mouse CD86 (GL1), PE-conjugated mouse anti-human H-2Kd (SF1-1.1), and PE-conjugated rat anti-mouse I-A/E (2G9) mAbs were purchased from BD Biosciences. Carboxyfluorescein-conjugated rat anti-mouse CCR7 (4B12) mAb, recombinant mouse (rm) GM-CSF, rmTNF-α, rmIL-1β, and rmCCL19 were obtained from R&D Systems.

Generation of human DCs

Human peripheral blood was obtained from healthy donors after obtaining informed consent in accordance with procedures approved by the Human Ethics Committee, Ehime University. CD14+ cells from PBMCs were isolated using a CD14 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the isolated CD14+ monocytes was >95% upon flow cytometric analysis. iDCs were generated from the CD14+ monocytes by culturing them in RPMI 1640 medium supplemented with 10% FBS (Life Technologies, Rockville, MD), 75 ng/ml rhGM-CSF, and 10 ng/ml rhIL-4 for 5 d. To induce maturation (mature DCs (mDCs)), iDCs were incubated with a maturation mixture containing rhTNF-α (10 ng/ml), rhIL-1β (10 ng/ml), and PGE2 (1 μg/ml) for a further 48 h. PKCI-treated DCs were generated by culturing iDCs with a maturation mixture in the presence of PKCIs such as Bis I (10 μM), G66983 (10 μM), or Ro32-0432 (10 μM) for 48 h.

Flow cytometric analysis

DC cell surface expression and the expression of Foxp3 and IL-10 in CD4+ T cells were analyzed using each of the mAbs described in the Abs and reagents section labeled with FITC or PE on a FACS Calibur flow cytometer (BD Biosciences), as described previously (51). For intracellular Foxp3 and IL-10 staining, permeabilization was performed in accordance with the manufacturer’s protocol.

Assay for cytokine production from DCs

iDCs, mDCs, and PKCI-treated DCs were adjusted to 1 × 10^6 per well in 1 ml and cultured in RPMI 1640 medium supplemented with 10% FBS for assay of IL-6, IL-10, IL-12, and IFN-γ, or in X-VIVO medium only for assay of TGF-β. The cell supernatants were harvested after 96 h, centrifuged, and the supernatants were frozen at −70°C until used. The supernatants were then assayed using a sandwich ELISA (R&D Systems) (IL-6, IL-10, IL-12, IFN-γ, and TGF-β), in accordance with the manufacturer’s instructions.

Phagocytic ability

Phagocytic ability was measured as the cellular uptake of FITC/labeled beads/rabbit IgG using a phagocytosis assay kit (Cayman Chemical, Ann Arbor, MI). DCs were incubated with FITC/labeled beads/rabbit IgG for 24 h in a CO2 incubator and then analyzed by flow cytometry in accordance with the manufacturer’s instructions.

Stability of PKCI-treated DCs

Phenotypic stability of PKCI-treated DCs was determined in response to inflammatory stimuli, as described by Harry et al. (37). Briefly, PKCI-treated DCs, iDCs, or mDCs were washed, adjusted to 5 × 10^6 per well in 1 ml, and cultured in RPMI 1640 medium supplemented with 10% FBS or in X-VIVO medium only for assay of TGF-β in the absence or presence of a mixture of proinflammatory cytokines containing IFN-γ at 1000 U/ml, IL-1β, IL-6, and TNF-α each at 10 ng/ml or LPS at 0.1 μg/ml. After 1 and 5 d, mDCs were analyzed for cell surface phenotype by flow cytometry, and production of IL-10 and TGF-β in the culture supernatants was determined by ELISA. To examine the functional properties of the DCs, they were washed after stimulation with a mixture of proinflammatory cytokines or LPS and recultured in RPMI 1640 medium supplemented with 10% FBS for a further 24 h. Then, an MLR was performed as described in the in vitro T cell proliferation assay section.

In vitro T cell proliferation assay

Human CD4+ T cells were isolated from PBMCs using a CD4+ T cell isolation kit II (Miltenyi Biotec). Freshly isolated CD4+ T cells (1 × 10^6) were cocultured with 1 × 10^6 (10:1 T cell/DC ratio) or 2 × 10^6 (10:2) mitomycin C (MMC)-treated allogeneic DCs in a final volume of 200 μl X-VIVO medium supplemented with 10% FBS in 96-well round-bottom plates for 4 d. The wells were pulsed with 1 μCi [3H]thymidine (Amersham Biosciences, Piscataway, NJ) 18 h before harvesting.

For mouse in vitro T cell proliferation assays, mouse CD4+ T cells were isolated from spleens of C57BL/6 mice using a CD4+ T cell isolation kit (Miltenyi Biotec). MMC-treated allogeneic DCs were prepared from BALB/c mice.

Induction of regulatory T cells by PKCI-treated DCs

Induction of human regulatory T cells by PKCI-treated DCs was performed as described by Sato et al. (21). Briefly, human naive CD4+CD45RA+ T cells were purified from PBMCs with a CD4+ cell isolation kit II (Miltenyi Biotec) and subsequent negative selection with anti-CD45RO mAb-conjugated immunomagnetic beads (Miltenyi Biotec). Human naive CD4+CD45RA+ T cells (5 × 10^5) were cultured with 5 × 10^5 MMC-treated allogeneic iDCs, mDCs, or PKCI-treated DCs in X-VIVO medium with 10% FBS for 5 d. Then, Foxp3+CD4+CD25+ T cells and IL-10+CD4+ T cells were analyzed by flow cytometry.

In vitro T regulatory activity

In vitro T regulatory activity was modified as described previously (51) and as reported by Gonzalez-Rey et al. (30). Human Th1 cells were generated from naive CD4+CD45RA+ T cells. Briefly, naive CD4+CD45RA+ T cells (1 × 10^6) were cultured with 1 × 10^5 MMC-treated allogeneic mDCs in X-VIVO medium with 10% FBS. Alloreactive T cells were expanded from day 6 in the presence of 100 U/ml rhIL-2. Two weeks after priming, T cells were restimulated with mDCs from the same donor under identical culture conditions. CD4+ T cells were isolated 1 wk after restimulation and used as alloreactive Th1 cells.

Human naive CD4+CD45RA+ T cells (5 × 10^5) were cultured with 5 × 10^5 MMC-treated allogeneic DCs treated with PKCI in X-VIVO medium with 10% FBS for 5 d. Then, CD4+ T cells were purified from the above cocultures with a CD4+ T cell isolation kit II (Miltenyi Biotec). These purified CD4+ T cells (1 × 10^5) were incubated with syngeneic Th1 cells (responder T cells, 1 × 10^5) in the presence of MMC-treated allogeneic mDCs (1 × 10^5) in a final volume of 200 μl X-VIVO medium supplemented with 10% FBS in 96-well round-bottom plates for 4 d. The proliferation of...
responder T cells was determined by [3H]thymidine incorporation. In some experiments, cocultures were performed in the presence of blocking mouse anti-IL-10 (40 μg/ml) and/or anti-TGF-β1 (40 μg/ml) mAbs. The anti-IL-10 and anti-TGF-β1 mAbs had been titrated previously. Concentrations resulting in undetectable levels of IL-10 or TGF-β1 following Ab addition were used in the blocking experiments.

Transwell experiments were performed using 24-well plates (Millicell, 0.4 μm; Millipore, Bedford, MA). Th1 cells (5 × 10^5) together with MMC- treated allogeneic mDCs (5 × 10^5) were placed in the bottom wells. CD4+ T cells (5 × 10^5) generated in the presence of PKCI-treated DCs were placed in the upper wells. The cultures were performed in the absence or presence of anti-IL-10 and/or anti–TGF-β1 mAbs for 4 d. Then, the basket was removed and the proliferation of the responder T cells was measured.

**ELISA for measurement of RelA and RelB**

Human iDCs (1 × 10^6/well) were incubated with PKCI for 2 h and then stimulated with TNF-α (10 ng/ml), IL-1β (10 ng/ml), and/or PGE_2 (1 μg/ml). After 4 h of stimulation, nuclear and cytoplasmic extracts from DCs were separated using a nuclear extraction kit (Imgenex, San Diego, CA). Activated NF-κB (RelA or RelB) in the nuclear extracts were measured by oligonucleotide-based ELISA as described by Bhattacharya et al. (52). Briefly, the extracts from DCs were incubated for 1 h at room temperature with an oligonucleotide in wells of a 96-well microtiter plate that had been coated with the NF-κB consensus nucleotide sequence 5′-GGGAGCTTTCCC-3′. NF-κB from the cells sampled to the wells was captured by an Ab specific to either RelA or RelB (Santa Cruz Biotechnology, Santa Cruz, CA). Binding of the specific NF-κB family member was then detected using an anti-rabbit HRP-conjugated IgG. Color was developed with hydrogen peroxide/tetramethylbenzidine chromogenic substrate, and intensity of development was proportional to the quantity of RelA and RelB in each sample. The sample values were normalized against total cell protein determined using a protein assay kit (Pierce, Rockford, IL).

**cAMP assay**

A cAMP assay was performed as described previously (53). After human iDCs (2 × 10^5/well) had been cultured in a 96-well plate for 12 h, the cells were incubated with PKCIs for 1 h. The cells were then washed twice with Krebs–Ringer HEPES (KRH) buffer (1.24 mM NaCl, 5 mM KCl, 1.25 mM MgSO_4, 1.45 mM CaCl_2, 1.25 mM KH_2PO_4, 25 mM HEPES [pH 7.4], and 8 mM glucose), 90 μl KRH buffer containing 10 μM forskolin (Sigma-Aldrich) with or without PKCI was added, and then the cells were stimulated with TNF-α, IL-1β, and/or PGE_2 for 1 h. The cells were lysed by adding 200 μl lysis buffer 1A (a component of the cAMP enzyme immunoassay system; Amershams Biosciences), and 100 μl cell lysate was used for measurement of cAMP produced during incubation with the kit, as recommended by the manufacturer.

**Chemotaxis assay**

Chemotaxis assays for iDCs, mDCs, and PKCI-treated DCs were performed in polycarbonate-membrane, 6.5-mm-diameter, 5-μm-pore size Transwell cell culture chambers (Costar, Cambridge, MA), as described previously (54). Aliquots (100 μl) of cells (5 × 10^6/ml) suspended in RPMI 1640/0.5% BSA were added to the upper chambers. The CCL19 was added to the lower wells at a concentration of 1 μg/ml. The cells were allowed to migrate for 2 h at 37°C in a 5% CO_2 incubator, after which the filters were fixed with 1% glutaraldehyde in PBS for 30 min and stained with 0.5% toluidine blue overnight. Cell migration was quantified by counting the cells in each lower chamber and cells adhering to the bottom of the polycarbonate filter.

**PKC kinase activity**

iDCs, mDCs, and PKCI-treated DCs (1 × 10^6) at various PKC concentrations were lysed with lysis buffer (20 mM MOPS, 50 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EGTA, 2 mM EDTA, 1% Nonidet P-40, 1 mM DTT, 1 mM benzamidine, 1 mM PMSE, and 10 μg/ml leupeptin and aprotinin). After centrifugation, each supernatant was analyzed for PKC activity using a PKC kinase activity kit (Enzo Life Sciences), in accordance with the manufacturer’s protocol.

**Results**

**Characterization of PKCI-treated human DCs**

PKCI-treated human DCs were prepared by culturing iDCs with a maturation mixture (TNF-α, rhIL-1β, and PGE_2) for 48 h in the presence of a conventional PKCI such as Bis I, G66983, or Ro32-0432. In the preliminary experiment, the optimal concentration of three PKCI compounds, Bis I, G66983, and Ro 32-0432, was decided from downregulation of costimulatory molecules (CD40, CD80, and CD86), viability, and inhibition of PKC activity. Among the concentrations of 1, 10, and 30 μM, 10 μM was the optimal one for all 3 PKCIs, resulting in strong downregulation of costimulatory molecules, a viability of >95%, and inhibition of PKC activity that reached a plateau (Supplemental Fig. 1). Therefore, we used 10 μM for all PKCIs in this study.

First, we compared the surface phenotypes of iDCs, mDCs, and PKCI-treated DCs. As shown in Fig. 1, iDCs showed moderate levels of expression of CD40 and MHC class I, low expression levels of CD11c, CD80, CD86, and MHC class II, and extremely low levels of CD1a and CD83. After inducing maturation with a maturation mixture (mDCs), the expression levels of CD40, CD80, CD86, MHC class I, and MHC class II were increased significantly compared with those of iDCs. DCs treated with PKCIs such as Bis I, G66983, and Ro32-0432 showed significantly decreased levels of expression of CD40, CD80, CD86, and MHC class I, but not CD1a, CD11c, and MHC class II, in comparison with mDCs. CD14, a monocyte marker, was expressed slightly on iDCs but not on mDCs or PKCI-treated DCs (Fig. 1B).

Because migration of DCs to the T cell zone of secondary lymphoid organs is guided by the homing chemokines CCL19 and CCL21 (56), we examined the expression of CCR7, their receptor
was performed in triplicate. Lower chamber and cells adhering to the bottom part of the filter. Each assay were added to the upper chambers, and CCL19 (1 μM) was added to culture supernatants were harvested after 96 h and assayed. Values are the means ± SD from three experiments. *p < 0.01. (C) Chemotactic activity of iDCs, mDCs, and PKCI-treated DCs to CCL19. Aliquots (100 μl) of cells (5 × 10^6/ml) were added to the upper chambers, and CCL19 (1 μg/ml) was added to the lower wells. After 2 h, cell migration was quantified by counting cells in each lower chamber and cells adhering to the bottom part of the filter. Each assay was performed in triplicate. *p < 0.01.

on iDCs, mDCs, and PKCI-treated DCs. As shown in Fig. 1A, barely any expression of CCR7 was evident on iDCs. In contrast, upregulation of CCR7 expression was recognized on mDCs and PKCI-treated DCs, although the expression level of CCR7 on PKCI-treated DCs was lower than that on mDCs. Functionally, the capacity of PKCI-treated DCs to migrate toward CCL19 was ~70–80% of that of mDCs (no significant difference versus mDCs), whereas iDCs showed little or no migration (Fig. 1C).

Next, we examined cytokine production of iDCs, mDCs, and PKCI-treated DCs. Cytokines in the 96-h culture supernatants were harvested after 96 h and assayed. Values are the means ± SD from three experiments. *p < 0.01. (B) Comparison of suppressive properties among iDCs, mDCs, and PKCI-treated DCs. Freshly isolated CD4+ T cells (1 × 10^5) were cocultured with 1 × 10^6 (10:1 T cell/DC ratio) or 2 × 10^6 (10:2) MMC-treated allogeneic iDCs, mDCs, and PKCI-treated DCs. After 4 d in culture, T cell proliferation was determined using [3H]thymidine incorporation. T cell proliferation by iDCs and PKCI-treated DCs was expressed relative to that by mDCs. Values are the means ± SD (n = 6/experiment). Representative data from three experiments are shown. *p < 0.05, **p < 0.01. (C) Phagocytic ability of iDCs, mDCs, and PKCI-treated DCs. DCs were incubated with FITC/latex beads/rabbit IgG for 24 h and then analyzed by flow cytometry.
PKCI-treated human DCs induce IL-10–producing T cells and functional regulatory T cells

Because PKCI-treated DCs produced IL-10 and TGF-β, we examined whether they would induce IL-10–producing T cells and Foxp3+CD4+CD25+ T cells. Naïve CD4+CD45RA+ CD25− T cells were cocultured with iDCs, mDCs, or PKCI-treated DCs for 5 d, and then intracellular IL-10+CD4+ T cells and Foxp3+CD4+CD25+ T cells were analyzed by flow cytometry. As shown in Fig. 3A, PKCI-treated DCs generated >4-fold the number of both IL-10+ CD4+ T cells and Foxp3+CD4+CD25+ T cells than did those induced by mDCs. CD4+ T cells cocultured with PKCI-treated DCs suppressed the proliferation of effector Th1 cells most strongly (Bis I, 48 ± 4%; Gö6983, 41 ± 14%; and Ro32-0432, 36 ± 6%), and CD4+ T cells cocultured with iDCs did so to a lesser degree (26 ± 7%), compared with control (Fig. 3B).

Next, we addressed the question of whether this suppression was due to direct cellular contact and/or soluble factors. In the coculture experiments, addition of saturating amounts of anti–IL-10 or anti–TGF-β mAbs reversed the inhibition only moderately (percentage suppression of effector Th1 proliferation without anti–IL-10 and anti–TGF-β mAbs (no Abs-C), with only anti–IL-10 mAb, and with only anti–TGF-β mAb: 48 ± 4, 32 ± 8, and 30 ± 5%, respectively). Addition of both anti–IL-10 and anti–TGF-β mAbs to the cocultures reversed the inhibition more markedly, although the inhibition still persisted (21 ± 9%). When effector Th1 cells and CD4+ T cells cocultured with Bis I–treated DCs were separated in the Transwell experiments, the proliferation of effector Th1 cells was still inhibited to a lesser degree (21 ± 5%). Addition of both anti-IL-10 and anti–TGF-β mAbs in the Transwell experiments reversed the inhibitory effect almost completely (Fig. 3C). Similar results were obtained from the experiments using CD4+ T cells cocultured with DCs treated with two other PKCIs, Gö6983 and Ro32-0432 (data not shown). These findings indicated that both direct contact and soluble factors mediate the inhibitory effect of CD4+ T cells cocultured with PKCI-treated DCs. Additionally, it was shown that the immunosuppressive function of PKCI-treated DCs was elicited by generating IL-10–producing T cells and functional Foxp3+ regulatory T cells from naïve CD4+ T cells.

Tolerogenic properties of PKCI-treated human DCs are highly stable

A potential risk of ex vivo–generated DCs is that they may switch to an activating phenotype after encountering danger signals in vivo. To address this issue, the stability of PKCI-treated DCs was examined by stimulation with proinflammatory mediators such as proinflammatory cytokines and LPS, after removal of PKCI. We compared the surface phenotype of DCs, cytokine production, and T cell proliferation on the first day and the fifth day after stimulation with proinflammatory mediators. As shown in Fig. 4, the expressions of CD80, CD83, CD86, and MHC class II on PKCI-treated DCs were upregulated only minimally and remained low, even on the fifth day after stimulation. Additionally, CCR7 expression on PKCI-treated DCs after stimulation kept the similar level to unstimulated PKCI-treated DCs. Productions of both IL-10 and TGF-β1 by PKCI-treated DCs were >4-fold and >2-fold higher than those of mDCs on the fifth day after stimulation of proinflammatory cytokines or LPS, respectively (Fig. 5A). Consequently, PKCI-treated DCs retained their T cell hyporesponsiveness on the first day and the fifth day after stimulation (Fig. 5B). There were no significant differences of production of both IL-10 and TGF-β1 in PKCI-treated DCs as compared with Bis I–, Gö6983–, and Ro32-0432–treated DCs. In contrast, iDCs matured and showed upregulated expression of costimulatory molecules such as CD80, CD83, and CD86, resulting in loss of T cell hyporesponsiveness upon addition of proinflammatory mediators. These findings indicated that PKCI-treated DCs are refractory to...
PKC inhibitor–induced human tolerogenic DCs

stimulation with proinflammatory mediators and that their tolerogenic properties are highly stable.

**PKCI reduces maturation stimuli–induced increases in NF-κB family members in DCs**

It has been reported that a crucial pathway for the maturation of DCs upon exposure to proinflammatory stimuli involves the transcription factor NF-κB (57–60). iDCs pretreated with DMSO (control) or PKCIs were exposed to TNF-α, IL-1β, IL-6, and PGE2 at 10 ng/ml or LPS (0.1 µg/ml). After 1 and 5 d, DCs were analyzed for cell surface phenotype by flow cytometry. Values are the means ± SD of mean fluorescence intensity (MFI) of each phenotype on DCs from four experiments (n = 2 or 3 samples/experiment). *p < 0.05 versus mDCs, †p < 0.01 versus mDCs.

**PKCI increases intracellular cAMP in DCs upon stimulation with a maturation mixture**

Intracellular cAMP/CREB has been reported to play a critical role in transcriptional regulation of IL-10 in monocytes and DCs (61, 62). iDCs pretreated with DMSO (control) or PKCIs were stimulated with TNF-α, IL-1β, and/or PGE2, and production of intracellular cAMP was determined by ELISA. Values are the means ± SD of four experiments (n = 2 or 3 samples/experiment). *p < 0.01. Representative data from four experiments are shown. *p < 0.05 versus mDCs, **p < 0.01 versus mDCs.
relative to DCs stimulated with PGE2 alone. Upon stimulation with a maturation mixture, intracellular cAMP production was reduced significantly compared with that of DCs stimulated with PGE2 alone. However, upon treatment with PKCI, production of cAMP and IL-10 recovered to a level that was the same as, or higher than, that of DCs treated with PGE2 alone. Values are the means ± SD of three experiments. *p < 0.05, **p < 0.01.

Characterization of PKCI-treated mouse DCs

First, to examine the effect of PKCI-treated DCs in vivo in a murine model of acute GVHD, we tested whether it would be possible to generate PKCI-treated mouse DCs using a maturation mixture (TNF-α, IL-1β, and PGE2). Mouse iDCs were generated by culturing BM cells with GM-CSF for 6 d and then matured with LPS or a maturation mixture for a further 48 h. LPS was used as a maturation control. As shown in Fig. 8A, as well as LPS-induced mDCs, mDCs induced by the maturation mixture showed high expression levels of CD40, CD80, CD83, CD86, MHC class I (H-2Kd), MHC class II (I-A/I-E), and CCR7. Functionally, mDCs induced by the maturation mixture showed migration activity toward CCL19 (Fig. 8B), high IL-12 production (Fig. 9A), sufficient proliferation of allogeneic T cells (Fig. 9B), and low phagocytic capacity (Fig. 9C), similar to LPS-induced mDCs.

PKCI-treated DCs were generated by culturing iDCs with a maturation mixture in the presence of Bis I for 48 h. Bis I–treated DCs showed markedly and significantly decreased levels of expression of CD40, CD80, CD83, CD86, and MHC class I, and slightly decreased levels of expression of MHC class II, in comparison with mDCs induced by the maturation mixture (Fig. 8A). Bis I–treated DCs showed CCR7 expression and migration activity toward CCL19, similar to mDCs. Bis I–treated DCs also had high production of IL-10 and TGF-β, T cell hyporesponsiveness, and efficient phagocytic capacity, as was the case for human PKCI-treated DCs (Fig. 9A–9C). Thus, using a maturation mixture, we were able to generate mouse PKCI-treated DCs that had properties similar to those of human PKCI-treated DCs.

PKCI-treated DCs prevent acute GVHD

Next, we examined the therapeutic effect of PKCI-treated DCs on acute GVHD following allogeneic BMT. We transplanted lethally irradiated BALB/c recipients (H-2b) with either T cell–depleted BM cells or with T cell–depleted BM plus spleen T cells (GVHD induction) from C57BL/6 (H-2d) mice. Two days after BMT, we
injected iDCs, mDCs, and Bis I–treated DCs obtained from BALB/c mice. As shown in Fig. 10, mice given only T cell–depleted BM cells appeared healthy, and 100% of the animals survived for at least 50 d. Mice that received BM cells plus spleen T cells developed severe signs of GVHD, including weight loss, reduced mobility, hunched posture, diarrhea, and ruffled fur, and they died within 30 d. There were no significant differences of GVHD lethality among untreated, iDC-treated, and mDC-treated mice. In contrast, administration of Bis I–treated DCs protected the mice from lethal GVHD, and 80% of them survived for 50 d ($p_{0.01}$).

From these findings, we confirmed that PKCI-treated DCs were more efficient than iDCs and mDCs for preventing GVHD in vivo.

**Discussion**

In this study, we demonstrated that human tolerogenic DCs were generated by adding a conventional PKC inhibitor during the maturation process after iDC differentiation, but not during the process of differentiation from progenitors to iDCs. The PKCI-treated DCs had a semimature phenotype with high production of IL-10, and they induced IL-10–producing T cells and functional regulatory T cells from naive CD4+ T cells efficiently, thus eliciting a strong immunosuppressive function. These cells expressed CCR7 and had sufficient capacity to migrate toward CCR7 ligands, especially secondary lymphoid organs. Additionally, PKCI-treated DCs were highly stable upon exposure to inflammatory stimuli. Moreover, PKCI-treated mouse DCs that had properties similar to PKCI-treated human DCs prevented acute GVHD in a murine model.

DCs are a heterogeneous group of APCs involved in the initiation of both adaptive immunity and tolerance induction. Generally, iDCs are characterized by an immature or semimature phenotype, with low expression of costimulatory molecules, low production of proinflammatory cytokines, including IL-12 and TNF-$\alpha$, and high production of anti-inflammatory cytokines such as IL-10 and TGF-$\beta$. In contrast, mDCs (2–6). Partial maturation resulting in...
intermediate expression of MHC and costimulatory molecules, but little or no production of proinflammatory cytokines, has been termed semimaturity (2). The mechanisms by which tDCs induce tolerence include 1) deletion of T cells, 2) induction of T cell hyporesponsiveness, 3) deviation of the T cell cytokine profile, and 4) induction of regulatory T cells (7). The anti-inflammatory cytokines IL-10 and TGF-β, neuropeptides including vasoactive intestinal peptide and NPY, and their receptors are all involved in the expression of costimulatory molecules (CD40, CD80, and CD86) using the specific PKCIs Go6976, CGP53535 and HBDDE (a PKCα-specific inhibitor), Bis I–, and HBDDE–treated DCs all showed down-regulation of costimulatory molecules (CD40, CD80, and CD86) using the specific PKCIs Go6976 (a PKCα- and βII- specific inhibitor), CGP53533 (a PKCβII-specific inhibitor), and HBDDE (a PKCα- and γ-specific inhibitor). Bis I–, Go6976–, CGP53535–, and HBDDE–treated DCs all showed down-regulation of costimulatory molecules. Of these, Bis I–treated DCs showed the strongest down-regulation, indicating that PKCα signaling is a key driver of monocyte-to-macrophage differentiation (44, 45). Our present study showed that stable, tolerogenic DCs with a semimature phenotype were generated by addition of conventional PKC-I during the maturation process. To examine which of the conventional PKC-I affected DC tolerogenicity, we compared the downregulation of costimulatory molecules (CD40, CD80, and CD86) using the specific PKC-I Go6976 (a PKCα- and βII-specific inhibitor), CGP53533 (a PKCβII-specific inhibitor), and HBDDE–treated DCs all showed down-regulation of costimulatory molecules. Of these, Bis I–treated DCs showed the strongest down-regulation, indicating that PKCα, β, and γ are all involved in the expression of costimulatory molecules on DCs (Supplemental Fig. 4). Taken together, these findings suggest that the conventional PKC subfamily plays a critical role not only in DC differentiation but also maturation in response to exogenous stimuli.

Studies involving culture with cytokines including GM-CSF, IL-4, and TNF-α have indicated that PKCβII plays an essential role in DC differentiation through noncanonical/RelB-mediated NF-κB signaling (44). PKCε is involved in LPS-induced IL-12 production by DCs through IkB-α degradation and subsequent NF-κB activation (42). These findings suggest that NF-κB signaling may be involved in the mechanism by which conventional PKC-I induces tDCs. NF-κB is required for proper development and function of DCs, and its activation is upregulated early during differentiation from precursors such as CD34+ hematopoietic progenitors and monocytes. Additionally, when matured in the presence of TNF-α alone or LPS, NF-κB activation plays an essential role in IL-12 production and expression of MHC class II and costimulatory
molecules such as CD80, CD86, and CD40 in DCs (58). When matured with a mixture of TNF-α, IL-1β, and PGE2, DCs treated with NF-κB inhibitors BAY 11-7082 or aspirin have been shown to express high levels of MHC class II and CD86 but markedly reduced expression of CD40 and generated anergy and regulatory T cell activity (66). NF-κB family members include RelA (p65), RelB, p52/p100, p50/p105, and c-Rel. The heterodimers RelA/p50 and RelB/p52 are required for activation of the canonical and noncanonical pathways, respectively. Inhibitory IκBs can be degraded by the proteasome to allow nuclear translocation of NF-κB and activate transcription in the canonical pathway (60). Among the NF-κB family members, RelB is the most crucial NF-κB subunit for expression of CD40, CD86, and MHC class II on DCs during maturation (57, 67, 68). RelB-deficient mice show impaired APC function and cellular immunity (67). Ag-primed DCs with inhibited RelB function have been shown to lack typical costimulatory molecules (68). In the present study, DC maturation induced by TNF-α or IL-1β was NF-κB–dependent, whereas that induced by PGE2 was NF-κB–independent. Moreover, conventional PKCIs inhibited NF-κB activation of both the canonical and noncanonical pathways of DC maturation, leading to suppression of costimulatory molecule expression and IL-12 production and resulting in a semimature state.

The cAMP/CREB-dependent pathway plays a critical role in IL-10 production by DCs (61, 62). However, an increase of the intracellular level of cAMP by PGE2 reportedly has its own limited effect on IL-10 production (62), and this is reflected in our present data. Additional stimulation is necessary to elicit a synergistic increase of IL-10 production with cAMP. Competition between NF-κB and cAMP/CREB for IL-10 gene transcription has been reported (69, 70), and NF-κB inhibition is associated with an increase of IL-10 production (62, 71, 72). In the present study, upon stimulation with PGE2 alone or a maturation mixture including TNF-α, IL-1β, and PGE2, the amount of intracellular cAMP in PKCI-treated DCs increased only 2- to 3-fold relative to that in PKCI-untreated DCs. However, IL-10 production in PKCI-treated DCs showed a much greater increase (>5-fold) than that in PKCI-untreated DCs. This augmentation of IL-10 production may be a synergistic effect of increased cAMP and NF-κB inhibition elicited by conventional PKCIs.

tDCs are a promising therapeutic tool for specific cellular induction of immunological tolerance in the context of transplantation and autoimmunity. As there will be a demand for clinical-grade tDCs for tolerance-inducing therapy, PKCI-treated tDCs may be suited for this purpose.

Disclosures

The authors have no financial conflicts of interest.

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