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Adiponectin Induces Dendritic Cell Activation via PLC γ /JNK/NF- κ B Pathways, Leading to Th1 and Th17 Polarization

Mi Young Jung,^{*,1} Han-Soo Kim,^{†,1} Hye-Jin Hong,^{*} Byung-Soo Youn,[‡] and Tae Sung Kim^{*}

Adiponectin (APN) is a crucial regulator for many inflammatory processes, but its effect on Th cell-mediated responses has not been fully understood. Thus, we investigated the immune-modulatory effects of APN on dendritic cells (DCs) controlling Th cell polarization. APN induced maturation and activation of DCs, as demonstrated by the increased expression of MHC class II, costimulatory molecules in both mouse and human DCs, and it significantly enhanced production of proinflammatory cytokines. APN triggered degradation of I κ B proteins, nuclear translocation of NF- κ B p65 subunit, and phosphorylation of MAPKs in DCs. Pretreatment with a phospholipase C (PLC) γ inhibitor and a JNK inhibitor suppressed IL-12 production and NF- κ B binding activity. Additionally, PLC γ inhibitor downregulated phosphorylation of JNK, indicating that PLC γ and JNK may be upstream molecules of NF- κ B. Importantly, APN-treated DCs significantly induced both Th1 and Th17 responses in allogeneic CD4⁺ T cells. The addition of a neutralizing anti-IL-12 mAb to the cocultures abolished the secretion of IFN- γ , whereas the blockage of IL-23 and IL-1 β suppressed APN-induced IL-17 production. Immunization of mice with OVA-pulsed, APN-treated DCs efficiently led to Ag-specific Th1 and Th17 cell responses. Taken together, these results demonstrated that APN effectively induced activation of DCs through PLC γ /JNK/NF- κ B-signaling pathways, leading to enhanced Th1 and Th17 responses. *The Journal of Immunology*, 2012, 188: 2592–2601.

Adipose tissue synthesizes and releases a variety of pro-inflammatory molecules, growth factors, and adhesion molecules, suggesting a novel function of adipocytes in tissue inflammation. Adiponectin (APN), a 30-kDa plasma protein, is the most abundant adipokine, being present at concentrations of ~5–20 μ g/ml. APN exists as multimeric forms in plasma: trimer, hexamer, high m.w. oligomer (HMW), and a free globular domain resulting from the proteolytic cleavage of the C terminus, which may have distinct biologic functions (1, 2). It was reported that the globular, trimer, and HMW forms of APN activate AMPK via AdipoR1, whereas the HMW form also activates NF- κ B (2). Previous reports indicated that APN suppresses inflammatory responses induced by TNF- α (3), whereas others reported that APN by itself activates NF- κ B and promotes inflammatory cytokine production (4, 5). APN is an important regulator of cytokine

responses, and this effect is isoform specific. HWM APN increases IL-6 secretion in human monocytes and human monocytic leukemia cell line cells, but low m.w. APN reduces LPS-mediated IL-6 release and also stimulates IL-10 secretion (2).

APN has received attention because of its insulin-sensitizing effects, and its use as a therapeutic tool for metabolic disorders. Accumulating evidence suggests a relationship between adipocytokines and inflammatory diseases (6–8). Increased plasma APN levels are an indicator of the onset of cardiovascular diseases, such as coronary artery disease and essential hypertension (9, 10). APN is highly expressed in inflamed tissues, such as synovium from patients with rheumatoid arthritis (11–13) and colon tissues from patients with inflammatory bowel disease (14, 15). These observations strongly suggest that APN expression is closely associated with the pathology of chronic inflammatory and autoimmune diseases.

Dendritic cells (DCs) are the most potent APCs in the immune system. They are located in many tissues and organs, and they act as sentinels. When confronted with a pathogen or perceived danger, immature DCs (iDCs) actively capture and process Ags and migrate to the peripheral lymphoid organs where they undergo profound changes in phenotype and function. They recognize Ags through pattern recognition receptors or TLRs, with consequent phosphorylation of various intracellular kinases, including I κ B kinase, ERKs (ERK1/2), p38 MAPK, and JNK1/2 (16, 17). NF- κ B is an important transcription factor regulating innate and adaptive immunity, and it induces proinflammatory cytokines in myeloid populations (18, 19). DC maturation results in the production of functionally different effector DC subsets that release polarizing signals (the most important of which are cytokines), which promote the development of Th1, Th2, or Th17 cell responses (20–22). T lymphocytes and immunoregulatory cytokines are critical determinants of disease outcomes. Th1 and the recently described inflammatory Th17 cell subset emerged as crucial mediators in many inflammatory disorders. Th1 responses predominate in

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Abbreviations used in this article: APN, adiponectin; BM, bone marrow; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; HMW, high m.w. oligomer; iDC, immature dendritic cell; LN, lymph node; PLC, phospholipase C; siRNA, small interfering RNA.

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organ-specific autoimmune disorders, acute allograft rejection, and some chronic inflammatory disorders. Th17 cells are the major pathological T cells in a number of autoimmune and inflammatory diseases, such as rheumatoid arthritis (23), Crohn's disease (24), multiple sclerosis, and experimental autoimmune encephalomyelitis (25).

Given the effect of APN on other myelomonocytic cells, it is possible that it could regulate immune responses through modulation of DCs. Because little is known about its effects on DCs, we focused our efforts on a more thorough investigation of the role played by APN in mouse bone marrow (BM)-derived DC (BMDC)-driven Th differentiation and undertook an analysis of the intracellular signaling events involved. We demonstrated in this study that APN induces maturation and activation of DCs through phospholipase C (PLC) γ /JNK/NF- κ B pathways, finally polarizing naive CD4⁺ T cells into Th1 and Th17 phenotypes. These data indicate that APN may contribute to Th1- and Th17-mediated immune disorders; thus, manipulation of APN can be a target for potential therapeutic application.

Materials and Methods

Experimental animals

Female 8–10-wk-old C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from OrientBio (Kapyong, Korea). The mice were maintained under specific viral pathogen-free conditions and were treated according to the Korea University Guidelines for the Care and Use of Laboratory Animals (Approval No. KLG 08-011).

Cytokines, Abs, and chemicals

Recombinant murine GM-CSF was obtained from ProSpec (Rehovot, Israel), and recombinant murine IL-12 was purchased from PeproTech (Rocky Hill, NJ). Recombinant mouse APN (full-length, recombinantly produced in HEK293 cells) and recombinant human APN (full-length, and globular domain) were provided by AdipoGen. Endotoxin contents in all recombinant APN preparations were <10 pg/ μ g protein, as measured using a *Limulus* amoebocyte lysate assay QCL-1000 kit (Cambrex, East Rutherford, NJ). Anti-IL-12p40 (C17.8 and C15.6), anti-IL-6 (20F3.11 and 32C11.4), anti-IFN- γ (HB170 and XMG1.2), and anti-IL-4 (BVD4 and BVD6) mAbs were purified from ascitic fluid by ammonium sulfate precipitation, followed by DEAE-Sephagel chromatography (Sigma-Aldrich, St. Louis, MO). Anti-IL-10 (JES5-2A5 and JES5-16E3) mAbs

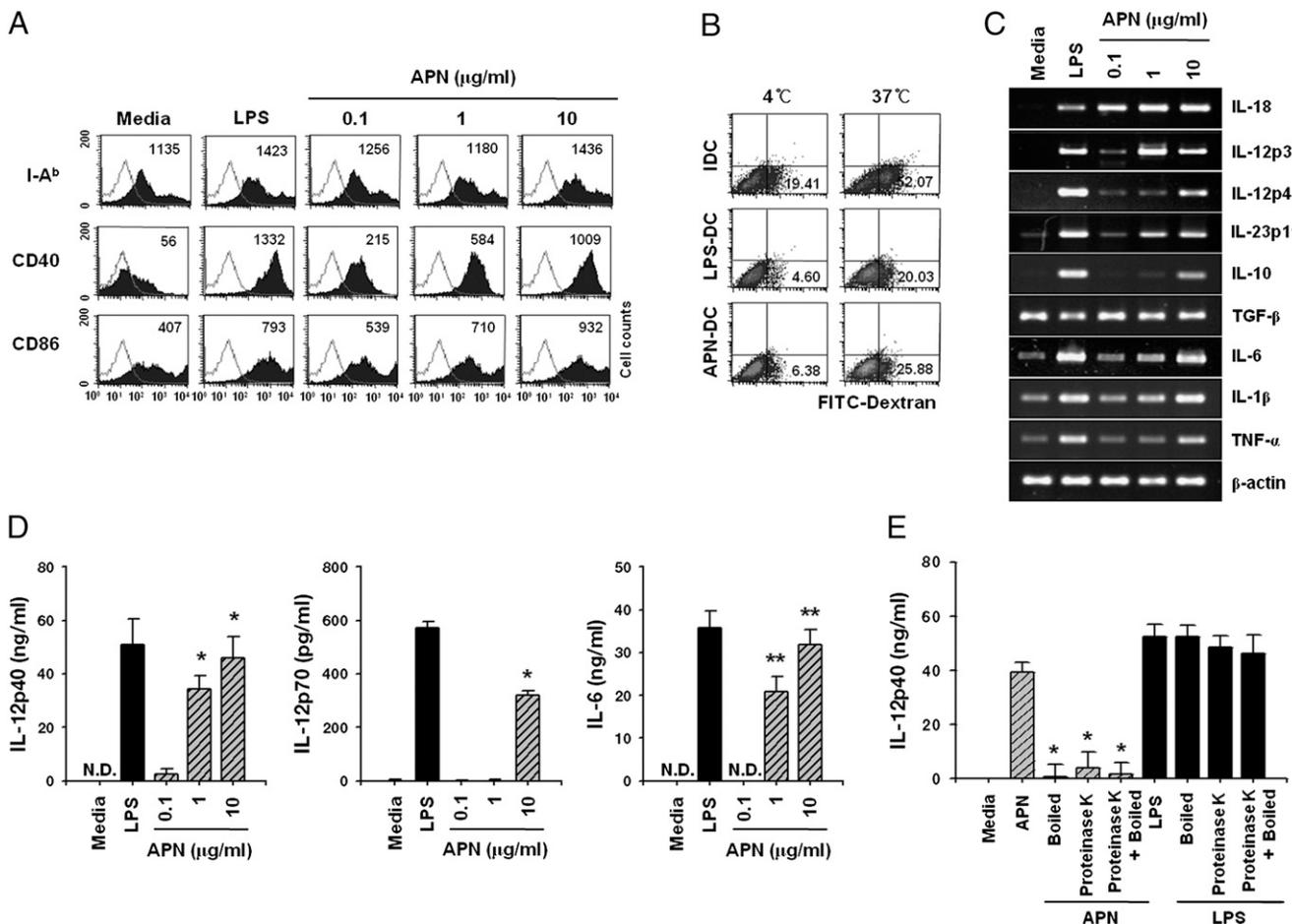


FIGURE 1. APN induces functional maturation of mouse BMDCs. **(A)** iDCs from C57BL/6 mice were incubated for 24 h with 100 ng/ml LPS and various concentrations of APN (0.1–10 μ g/ml). The expression of I-A^b, CD40, and CD86 molecules was analyzed using flow cytometry and is represented by graphs gated on CD11c⁺ cells. Numbers indicate the mean fluorescence intensity. **(B)** iDCs (2×10^5 cells) were incubated for 45 min at 4°C or 37°C, after which the cells were incubated for 1 h at 37°C in the media containing 1 mg/ml of FITC-dextran. The quantitative uptake of FITC-dextran by DCs was analyzed. The numbers represent the percentage of FITC-dextran⁺ cells. **(C)** iDCs were simulated for 6 h with various concentrations of APN or LPS, and total RNA was prepared from the cells. The mRNA levels of the genes were determined via RT-PCR. **(D)** IL-12p40, IL-12p70, and IL-6 secretion levels in the culture supernatants were determined by sandwich ELISAs. Data shown represent mean \pm SD of three independent experiments done in triplicates. * p < 0.05, ** p < 0.005, versus unstimulated DCs. **(E)** iDCs were treated for 24 h with either APN (10 μ g/ml) or LPS, after which the level of IL-12 in the culture supernatants was determined via sandwich ELISA. Recombinant APN was boiled at 95°C for 10 min, digested with 100 μ g/ml of proteinase K for 1 h, or digested with proteinase K and then boiled prior to use. Data shown represent mean \pm SD of three independent experiments. * p < 0.05, versus APN-stimulated DCs. N.D., not detected.

were obtained from BD Pharmingen (San Diego, CA). Anti-IL-12p70 (9A5 and C17.8) and anti-IL-17 (eBio17CK15A5 and eBio17B7) mAbs, allophycocyanin-conjugated anti-mouse IL-17A, and brefeldin A were purchased from eBioscience (San Diego, CA). With the exception of IL-17A, all fluorescent-conjugated Abs were purchased from BD Pharmingen. The MEK1/2 inhibitor U0126 was obtained from Cell Signaling Technologies (Beverly, MA). The protein kinase C inhibitor 1, 2-dimethoxy-12-methyl[1,3]benzoxol[5,6-c]phenanthridin-12-ium (chelerythrine) and 2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-1 (PD 98059) were purchased from Tocris Cookson (Bristol, U.K.). LPS (from *Escherichia coli* 0111:B4), OVA, PMA, ionomycin, p38 inhibitor 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine (SB203580), JNK inhibitor anthrapyrazol-1 (SP 600125), and other reagents were purchased from Sigma-Aldrich.

Preparation of mouse and human DCs

Mouse BMDCs were generated using a modification of the method originally described by Inaba et al. (26). Briefly, BM cells were isolated from femurs and tibiae of mice and cultured in complete RPMI 1640 containing 10% heat-inactivated FBS (WelGENE, Daegu, Korea), 50 μ M 2-ME (Sigma-Aldrich), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies BRL, Rockville, MD), supplemented with 10 ng/ml GM-CSF (ProSpec). The culture medium containing cytokine was replaced every 2 d. At day 7 of the culture, loosely adherent DC aggregates were harvested for use in the experiments.

CD14⁺ human monocytes were positively selected from PBMCs using MACS CD14 Microbeads (Miltenyi Biotec, Auburn, CA). Monocyte-derived DCs were generated with GM-CSF (100 ng/ml) and IL-4 (20 ng/ml). After a 5 d-incubation, iDCs were harvested and cultured with GM-CSF and IL-4 for an additional 48 h in the presence of full-length recombinant APN, globular domain APN, or LPS (from *E. coli* 0111:B4; Sigma-Aldrich). All participants gave written informed consent, and the study protocol was approved by the Institutional Review Board of Severance Hospital (Yonsei University Health System) and met the guidelines for blood donation.

Flow cytometric analysis

DCs (1×10^6 cells) were harvested, washed with PBS, and resuspended in FACS buffer (0.5% FBS and 0.05% sodium azide in PBS). Cells were incubated for 20 min at room temperature with fluorescently conjugated mAbs, washed twice with FACS buffer, and resuspended in FACS buffer for analysis. Abs used (BD Biosciences) were as follows: CD11c (HL3), I-A^b (AF6-120.1), CD40 (3/23), and CD86 (GL1) mAbs for mouse DCs, as well as CD40 (HB15e), CD86 (2331), and HLA-DR (L243) for human DCs. For intracellular staining, the cells were stimulated for 3 h with PMA (50 ng/ml) and ionomycin (1 μ g/ml), followed by additional incubation for 2 h in the presence of brefeldin A (3 μ g/ml). First, cells were stained for CD4 by incubation with anti-CD4-FITC Ab for 20 min at room temperature. Subsequently, the cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. Cells were then stained with PE-conjugated anti-mouse IL-2, IFN- γ (BD Biosciences), or allophycocyanin-conjugated anti-mouse IL-17 (eBioscience). The stained cells were analyzed in a FACSCalibur (BD Biosciences). The data were analyzed using Cell Quest software (BD Biosciences).

Semiquantitative RT-PCR

Total RNA that was obtained from the cells was reverse transcribed into cDNA, and PCR amplification of the cDNA was performed using a thermal cycler from MJ Research (Watertown, MA). After amplification, the products were separated on 1.5–2% (w/v) agarose gels and stained with ethidium bromide. The primer sequences used in this study were described previously (27). Sequences of mouse AdipoR1 and AdipoR2 were as follows: mouse AdipoR1 (forward, 5'-ATCTCCGCATCCACACAGA-3'; reverse, 5'-CAGAGATGCCAGGACACAG-3'), mouse AdipoR2 (forward, 5'-GCCTGAGTGAATCATCCCT-3'; reverse, 5'-GCACCGACAGCCAGTAAGAA-3').

Quantitation of Ag uptake

To measure Ag uptake ability, CD11c⁺ DCs from BM cultures were resuspended in 100 ml PBS containing 1% FBS and incubated with 1 mg/ml FITC-dextran (Sigma-Aldrich) at either 37°C or 4°C for 45 min. The process was stopped by adding ice-cold PBS, and the cells were washed three times to remove excess dextran. The quantitative uptake of FITC-dextran by DCs was determined by FACS analysis.

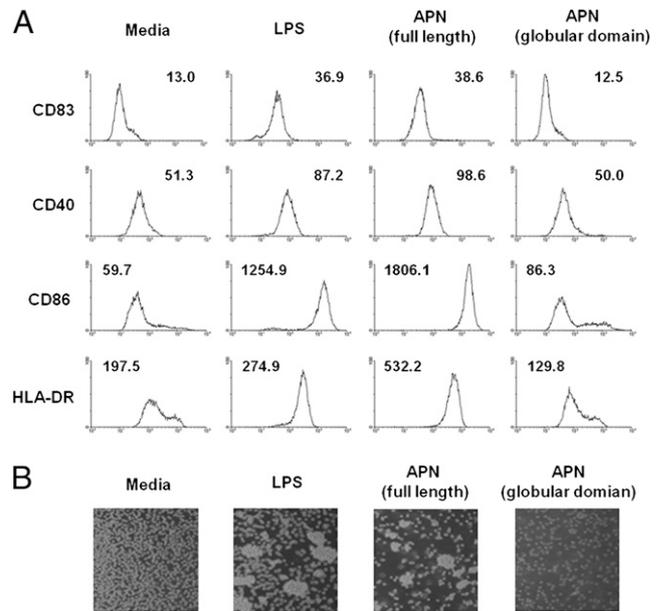


FIGURE 2. Human DCs generated in the presence of APN acquire mature DC features. Human iDCs generated in GM-CSF and IL-4 for 5 d were harvested and cultured for an additional 48 h with 100 ng/ml LPS or each form of APN (10 μ g/ml). **(A)** The expression of HLA class II, CD40, CD83, and CD86 on DCs was analyzed via flow cytometry. Data are representative of three independent experiments. **(B)** The maturation-induced clustering of DCs and morphologic changes were analyzed using phase-contrast microscopy (original magnification $\times 100$).

Cytokine assays

The quantities of IFN- γ , IL-4, IL-17, IL-6, IL-10, IL-12p40, and IL-12p70 in the culture supernatants were determined at the indicated times by sandwich ELISA using mAbs specific for each cytokine.

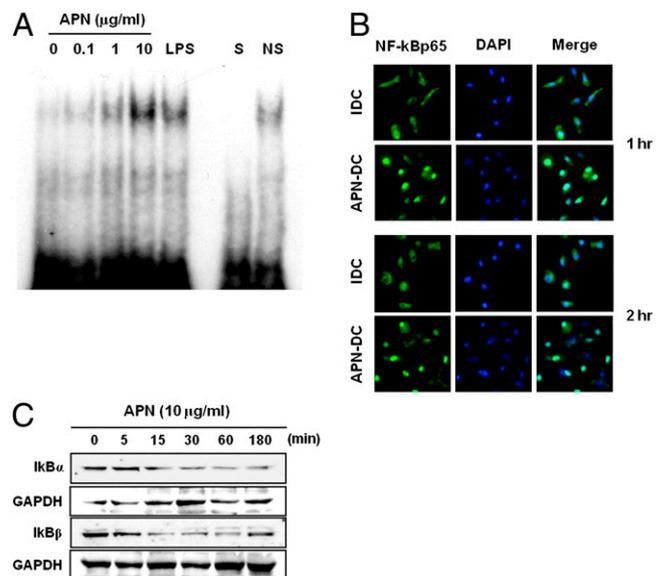


FIGURE 3. APN increases the nuclear localization of p65 and promotes I κ B α and I κ B β degradation. **(A)** Nuclear extracts from APN-stimulated DCs were assessed for NF- κ B DNA-binding activity in the gel-shift assay. **(B)** Cells were treated with 10 μ g/ml of APN for the indicated times. Nuclear translocation of NF- κ B-p65 was detected using an Olympus IX71 fluorescence microscope (original magnification $\times 200$). **(C)** iDCs were treated with APN for 5–180 min, after which the cells were harvested for Western blotting to detect I κ B α , I κ B β , and GAPDH. Data are representative of three independent experiments. NS, unlabeled nonspecific oligonucleotide; S, unlabeled identical oligonucleotide.

Small interfering RNA transfection

Two pairs of small interfering RNAs (siRNAs) were synthesized by Bioneer (Daejeon, Korea). The sequences of the sense siRNAs are as follows: mouse AdipoR1, 5'-GAGACUGGCAACAUCUGGACATT-3'; mouse AdipoR2, 5'-GCUUAGAGACACCUGUUUGUUTT-3'. Cells were transfected with siRNAs (40 pmol) using Lipofectamine 2000 (Invitrogen Life Technology), according to the manufacturer's instructions.

Western blot analysis

Cells were lysed at the indicated time points in immunoprecipitation buffer, as previously described (27). Proteins were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. Immunoreactive proteins were detected by incubating blots with specific Abs and were imaged on a Fuji LAS-3000 system.

Determination of NF-κB activity

Nuclear extracts were prepared from DCs, and EMSA was performed, as previously described (27). An oligonucleotide containing an NF-κB binding site in the Iγγ-chain (5'-CCGGTTAACAGAGGGGCTTCCGAG-3') served as a probe. Labeled oligonucleotides (10,000 cpm) were incubated with 10 μg nuclear extracts in 20 μl binding buffer (10 mM Tris-HCl [pH 7.6], 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly(dI-dC), and 1 mM DTT) for 30 min at room temperature. DNA-protein complexes were resolved by electrophoresis on a 4% native polyacrylamide gel and exposed to x-ray film.

Confocal microscopy

Cells were cultured on coverslips (MultiCell). At the indicated times, cells were fixed and permeabilized for 10 min with a Cytofix/Cytoperm kit. Subsequently, cells were incubated with a rabbit anti-NF-κB p65 Ab (1:200; Santa Cruz Biotech, Santa Cruz, CA), followed by Alexa Fluor 488-labeled anti-rabbit IgG Ab (Molecular Probes, Eugene, OR). Nuclei were counterstained with DAPI (0.1 μM; Invitrogen). The slides were

mounted using DABCO anti-fade mounting medium and then examined under an Olympus IX71 fluorescence microscope.

Mixed lymphocyte response

APN-stimulated or nonstimulated DCs (I-A^b) were harvested and used as stimulators. Allogeneic CD4⁺ T lymphocytes (I-A^d) were isolated by positive immunomagnetic selection using MACS with CD4 MicroBeads (Miltenyi Biotec). For proliferation assays, 1 × 10⁵ CD4⁺ T cells were cultured with irradiated (2000 rad) DCs that were matured under different culture conditions for 3 d. A total of 0.5 μCi [³H]thymidine was added during the last 18 h of coculture, and the level of T cell proliferation was expressed as cpm. For detection of cytokine production, CD4⁺ T cells isolated from BALB/c mice (I-A^d) were cultured with APN-stimulated DCs (I-A^b). IL-2-, IFN-γ-, or IL-17-expressing cells were analyzed by cytofluorometric analysis at specific times. Supernatants were collected, and the secretion levels of IFN-γ, IL-4, and IL-17 were measured by ELISA.

In vitro and in vivo polarization of CD4⁺ T cells with DCs

C57BL/6 mice (I-A^b) were sensitized by s.c. injection of 100 μg OVA protein. After 7 d, CD4⁺ T cells were isolated from OVA-primed mice and then used as responder T cells. iDCs from C57BL/6 mice (I-A^b) were pulsed for 2 h with 10 μg/ml OVA, after which the cells were incubated, or not, for 6 h with APN. Responder CD4⁺ T cells were mixed with OVA-pulsed unstimulated or OVA-pulsed, APN-stimulated DCs at a 10:1 ratio. Supernatants were collected, and cytokine production was measured by ELISA. Some experiments were performed in the absence or presence of neutralizing mAbs against IL-1β, IL-6, IL-12p40, IL-21, and IL-23p19 (5 μg/ml; eBioscience) or isotype Abs.

For in vivo polarization, either OVA-pulsed unstimulated DCs or OVA-pulsed, APN-treated DCs were adoptively transferred s.c. into footpad/haunch of OVA-immunized mice at 1 × 10⁵ cells/mouse. Cells obtained from draining lymph nodes (LNs) were collected on day 7 and cultured for the indicated times with 100 μg/ml OVA. Intracellular and secreted levels of cytokines (IFN-γ, IL-17, and IL-4) were measured by flow cytometry and ELISA.

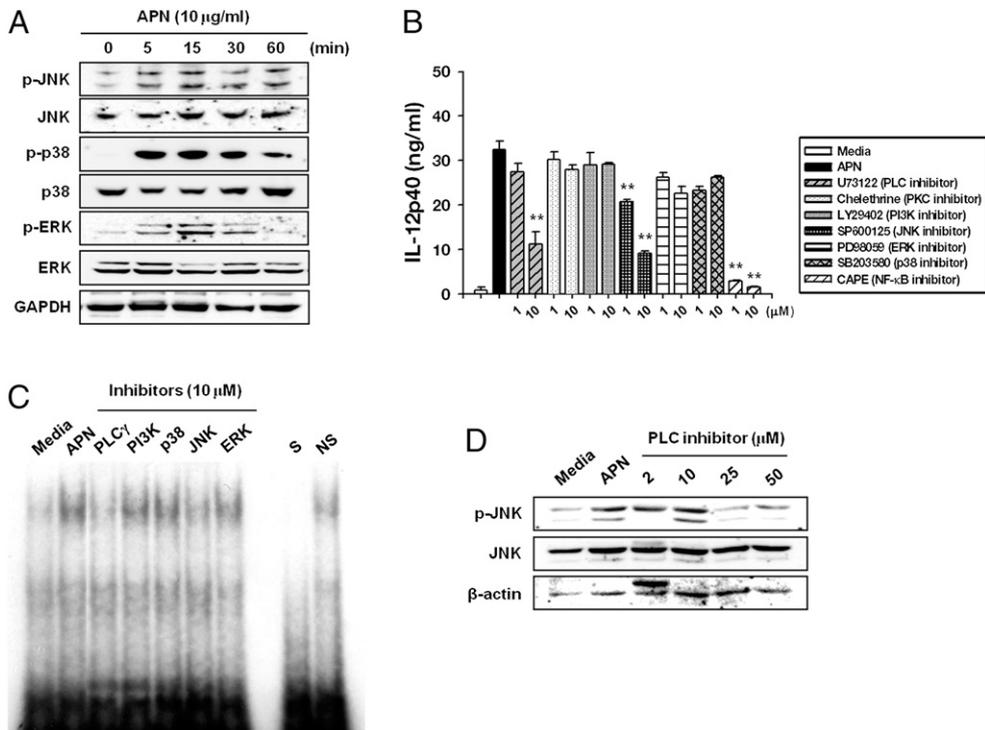


FIGURE 4. PLCγ, JNK, and NF-κB are involved in IL-12 production from the APN-treated DCs. **(A)** iDCs were treated with APN for 5–60 min, after which the cells were harvested for Western blotting to detect JNK, p-JNK, p38, p-p38, ERK, p-ERK, and GAPDH. **(B)** iDCs were pretreated or not for 1 h with the indicated inhibitors and then exposed to 10 μg/ml of APN for 24 h. The levels of IL-12p40 were analyzed via ELISA. The data represent mean ± SD of three independent experiments. ***p* < 0.005, versus APN-stimulated DCs. **(C)** iDCs were pretreated for 30 min with 10 μM of PLCγ inhibitor (U73122), PI3K inhibitor (LY294002), JNK inhibitor (SP600125), p38 inhibitor (SB203580), and ERK inhibitor (PD98059), after which the cells were incubated for 1 h with APN. Nuclear extracts were assessed for NF-κB DNA-binding activity in the gel-shift assay. **(D)** iDCs were pretreated for 1 h with PLCγ inhibitor (2–50 μM), the cells were stimulated for 20 min with 10 μg/ml APN, and the levels of JNK, p-JNK, and β-actin were determined via Western blot analysis. All data are representative of at least three independent experiments.

Statistical analysis

All data are expressed as mean \pm SD or mean \pm SEM. Statistical analysis was performed using the paired Student *t* test. Differences were considered significant at $p < 0.05$.

Results

APN induces maturation and activation of both mouse and human DCs

To demonstrate whether full-length APN (hereafter referred to as "APN") induces maturation and activation of DCs, immature mouse DCs were incubated with various concentrations of APN, and the expression of cell surface molecules was determined using flow cytometry. As depicted in Fig. 1A, APN significantly upregulated the expression of I-A^b (MHC class II), CD40, and CD86 on mouse DCs in a dose-dependent manner. The phenotypic changes induced by APN were comparable to those elicited by LPS (100 ng/ml), suggesting that APN induces DC maturation. To confirm that APN induced the maturation of BMDCs, we measured Ag uptake ability, which diminishes during DC maturation, using FITC-dextran. Particle uptake was also reduced in APN-stimulated DCs compared with unstimulated DCs (Fig. 1B), further supporting that APN provokes DC maturation. DC maturation and activation are known to influence the expression of a series of inflammatory-related genes, thereby modulating subsequent immune responses. Therefore, we assessed the expression levels of cytokine genes that are associated with DC maturation and activation. The expression of most cytokines was dramatically increased (Fig. 1C). APN strongly enhanced the production of IL-12p40, IL-12p70, and IL-6 (Fig. 1D). Although the full-length APN efficiently induced DC maturation, globular APN did not induce the expression of cell surface molecules or the production of IL-12 and IL-6 (Supplemental Fig. 1). Boiled or/and proteinase K-treated APN did not induce IL-12 production, indi-

cating that the effects of APN were not attributable to endotoxin contamination (Fig. 1E). To ascertain that the results obtained with murine BMDCs were applicable to human DCs, the effect of APN on human monocyte-derived DCs was examined. Consistent with the activity observed in murine BMDCs, phenotypic analysis revealed that the full-length APN gave rise to a substantial induction of CD40, CD83, CD86, and HLA-DR expression on DCs, whereas the globular domain of APN did not (Fig. 2A). When iDCs were treated with the full-length APN protein, a significant homotypic cell clustering was observed; this was not observed with the globular APN (Fig. 2B). Collectively, our data demonstrate that only the full-length APN can induce maturation and activation of both mouse and human DCs.

APN induces NF- κ B activation, I κ B degradation, and MAPK phosphorylation

NF- κ B is a key transcription factor that regulates DC maturation and cytokine production. As indicated in Fig. 3A, there was a clear induction of binding activity in response to APN. We also monitored APN-induced nuclear translocation of p65/RelA (Fig. 3B), an NF- κ B family member known to be relevant in DC differentiation. To further support the results from immunohistochemistry, DCs were treated with 10 μ g/ml of APN, and the levels of I κ B proteins were determined by Western blot. As shown in Fig. 3C, APN induced a rapid and transient reduction of I κ B α and I κ B β proteins.

To determine the molecular mechanisms underlying the APN-induced maturation of DCs, we evaluated the signaling pathways of three major MAPKs. It has become clear that LPS or IFN- γ induces a large number of genes through activation of MAPKs (29). Fig. 4A shows that APN remarkably enhanced phosphorylation of p38, JNK, and ERK. iDCs were pretreated with various inhibitors (10 μ M), after which the cells were incubated for 24 h in the presence of APN. PLC γ -, JNK-, and NF- κ B-specific inhibitors remarkably attenuated the APN-induced IL-12 production (Fig.

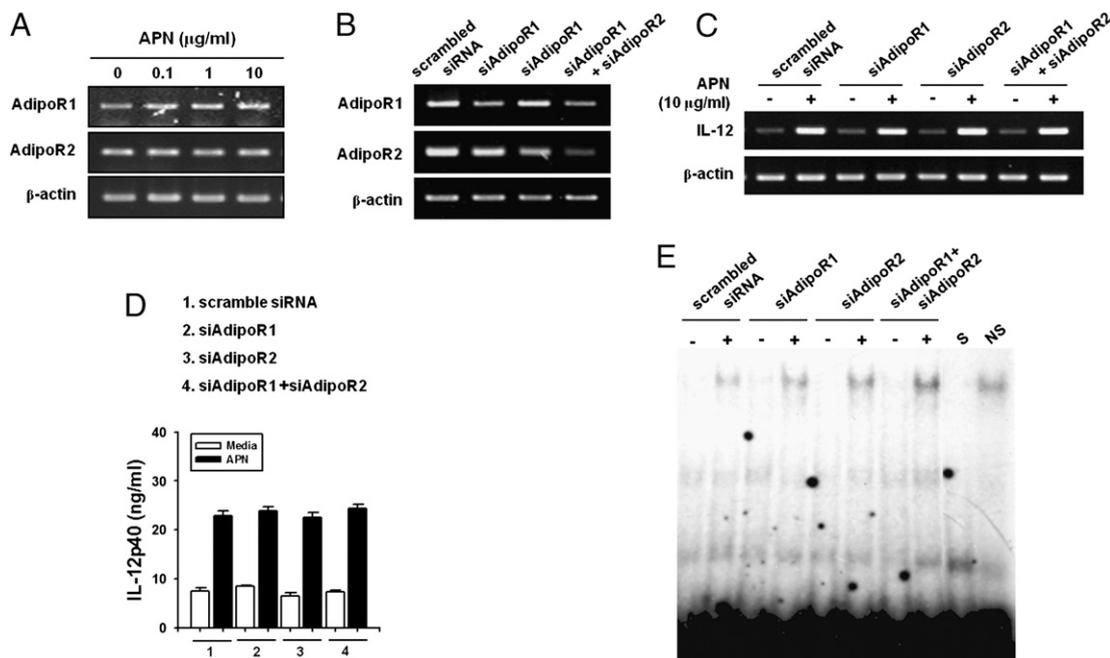


FIGURE 5. APN-mediated production of IL-12 and NF- κ B activation are independent of AdipoR1/AdipoR2. **(A)** Total RNA was extracted from DCs and subjected to RT-PCR for AdipoR1 and AdipoR2 using the respective primers. **(B and C)** iDCs were transfected with scrambled siRNAs as control or siRNAs specific for AdipoR1 and/or AdipoR2 for 30 h, followed by incubation with 10 μ g/ml APN for 6 h to analyze mRNA expression. Total RNA were collected, and the expression of IL-12 was analyzed by RT-PCR. **(D)** iDCs were transfected with siRNAs specific for AdipoR1 and/or AdipoR2 for 24 h, followed by incubation with 10 μ g/ml APN for 24 h to measure IL-12 production. Values are mean \pm SEM. **(E)** iDCs were transfected with siRNAs specific for AdipoR1 and/or AdipoR2 for 30 h, followed by incubation with 10 μ g/ml APN for 1 h to analyze NF- κ B-binding activity. Nuclear extracts were assessed for NF- κ B DNA-binding activity in the gel-shift assay. Data are representative of at least two independent experiments.

4B). The specific inhibitors for PI3K, protein kinase C, and ERK had a slight effect on IL-12 induced by APN. To clarify the upstream signaling mechanism responsible for APN-induced NF- κ B activation, iDCs were pretreated with the inhibitors for PLC γ , PI3K, JNK, p38, and ERK. The cells were incubated for 24 h with 10 μ g/ml of APN, and the NF- κ B-binding activity was analyzed. PLC γ and JNK inhibitors suppressed the NF- κ B-binding activity induced by APN, whereas the inhibitors for ERK, p38, and PI3K had no effect (Fig. 4C). To reveal the relationship between PLC γ and JNK, DCs were pretreated with a PLC γ inhibitor, after which the cells were incubated with APN. As shown, JNK phosphorylation was attenuated in a dose-dependent fashion (Fig. 4D), thereby demonstrating that PLC γ is an upstream molecule of JNK and NF- κ B activation in the APN-treated DCs. Taken together, APN induced DC maturation and IL-12 production mainly through PLC γ /JNK/NF- κ B-signaling pathways.

APN-induced IL-12 production via NF- κ B pathway is independent of AdipoR1/AdipoR2

The APN receptors AdipoR1 and AdipoR2, which mediate the antidiabetic metabolic actions of APN, have been cloned (28). However, little is known about their expression and/or function in primary DCs. To investigate the role of AdipoR1 and AdipoR2 subtype receptors in the APN-mediated increase in cytokine pro-

duction, we assessed the distribution of these APN receptors in DCs by RT-PCR analysis. The mRNA levels of AdipoR1 and AdipoR2 could be detected. Upon APN treatment for 6 h, the transcriptional levels of IL-12 and the AdipoR1 subtype were increased, whereas the other subtype (AdipoR2) remained unchanged (Figs. 1C, 5A). Next, to examine whether APN subtype receptors are involved in the APN-mediated production of IL-12, the expression of AdipoR1 and/or AdipoR2 in DCs was knocked down with siRNAs (Fig. 5B). APN-treated DCs that had been knocked down with AdipoR1 and/or AdipoR2 siRNAs still showed robust upregulation of IL-12 expression (Fig. 5C, 5D). Furthermore, both AdipoR1 and AdipoR2 receptor-specific siRNAs did not block APN-induced NF- κ B-binding activity (Fig. 5E). These results suggest that there may be an unidentified molecule in DCs functioning as the receptor for APN.

APN-stimulated DCs enhance allostimulatory activity

To determine whether APN-stimulated DCs are sufficient to promote the activation of naive T cells, allogeneic CD4⁺ T cells were cocultured with DCs from C57BL/6 mice. As shown in Fig. 6A, a noticeable T cell proliferation resulted from treating DCs with APNs to a similar extent as was observed for LPS-treated DCs. The inductive effect of T cell proliferation by APN was also observed using human DCs (Supplemental Fig. 2). Collectively, we

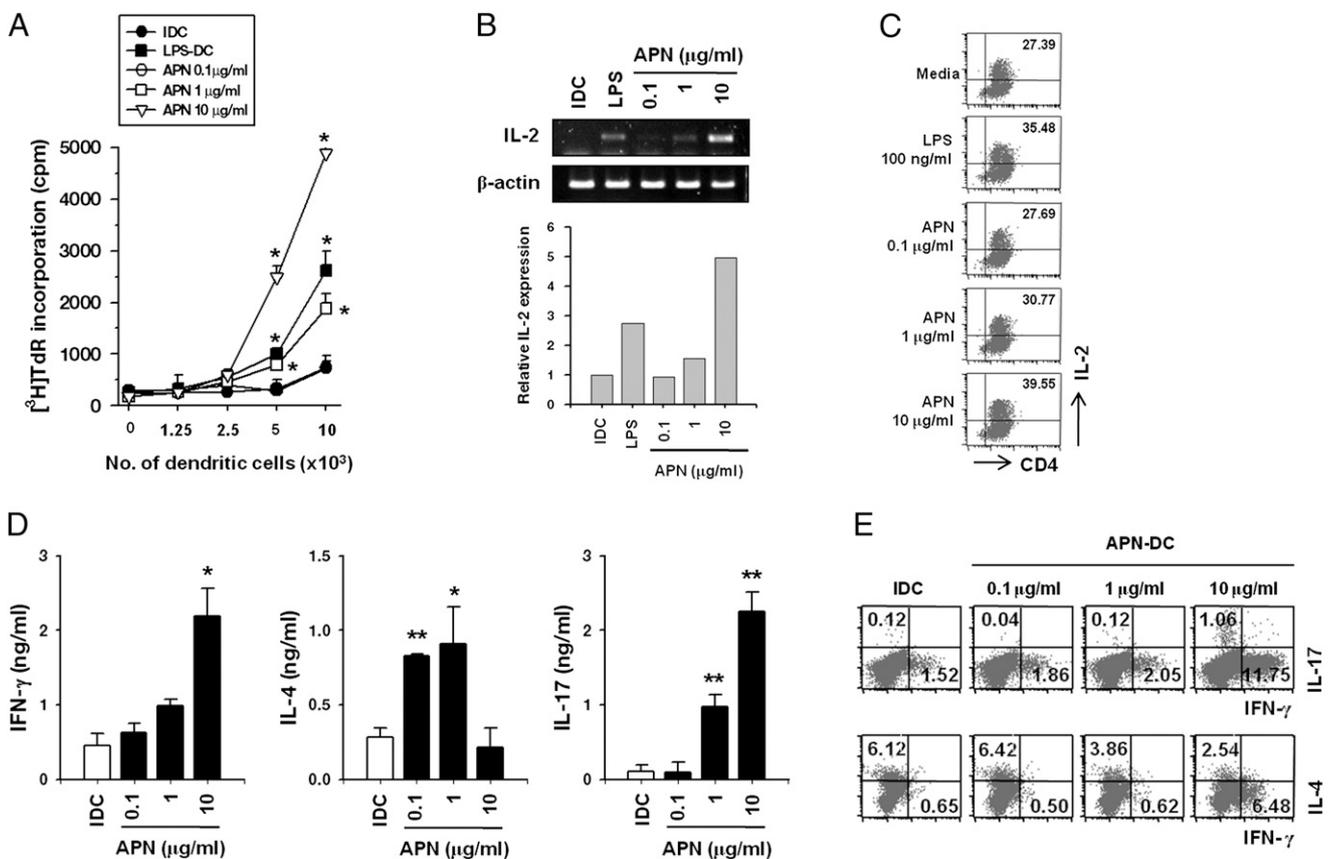


FIGURE 6. APN-treated DCs induce allostimulatory capacity and production of IFN- γ and IL-17 in allogeneic CD4⁺ T cells. (A) iDCs (H-2K^b) were treated for 24 h with various concentrations of APN or LPS. Different numbers of irradiated DCs were cocultured with CD4⁺ T cells (H-2K^d). Proliferation of the responding CD4⁺ T cells was evaluated by [³H]thymidine incorporation. The experiments were repeated three times with similar results. **p* < 0.05, versus allogeneic CD4⁺ T cells cocultured with unstimulated DCs. (B) Allogeneic CD4⁺ T cells were cocultured for 2 d with unstimulated, LPS-stimulated, or APN-stimulated DCs, and total RNA was prepared from the cells. (C) DCs were cocultured for 3 d with allogeneic CD4⁺ T cells at a 1:3 ratio, and the population of IL-2-expressing CD4⁺ T cells was measured via fluorometric analysis. (D) APN-stimulated DCs were cocultured for 3 d with allogeneic CD4⁺ T cells at a 1:10 ratio. IFN- γ , IL-4, and IL-17 secretion was determined in culture supernatants using ELISA. Data represent mean \pm SD of three independent experiments done in triplicates. **p* < 0.05, ***p* < 0.005, compared with CD4⁺ T cells cocultured with unstimulated DCs. (E) APN-stimulated DCs were cocultured with allogeneic CD4⁺ T cells (1:3 ratio) for 3 d, and intracellular expression of IFN- γ , IL-4, and IL-17 was detected in activated cells. Data are representative of five independent experiments with similar results.

concluded that APN caused T cell proliferation by differentiating iDCs into mature DCs, accompanied by the upregulation of costimulatory molecules and MHC class II. To further confirm the proliferation-promoting activity of APN-treated DCs, the expression level of IL-2 was determined. APN-treated DCs enhanced the mRNA and intracellular protein levels of IL-2 in CD4⁺ T cells (Fig. 6B, 6C).

Next, we investigated CD4⁺ T cell differentiation driven by APN-stimulated DCs. APN-stimulated DCs were cocultured with allogeneic CD4⁺ T cells for 3 d. The secretion of IFN- γ and IL-17 markedly increased in a dose-dependent manner, whereas production of IL-4 was only increased by treatment with low dose APN (Fig. 6D). As indicated in Fig. 6E, APN-stimulated DCs also significantly increased the populations of IFN- γ - and IL-17-secreting cells in cocultured CD4⁺ T cells. These results indicate that APN-treated DCs cue the polarization of the CD4⁺ response into Th1 and Th17 responses.

APN-stimulated DCs promote Th1 and Th17 polarization via IL-12 and IL-1 β /IL-23 β

To clarify whether APN-treated DCs can modulate the Th cell-mediated response in an Ag-dependent manner, we assessed the cytokine profiles that direct Th response. OVA-pulsed, APN-treated

DCs were cocultured with syngeneic CD4⁺ T cells, which had been sensitized by OVA, and then cytokines were detected using ELISA. As shown in Fig. 7A, the Th1-polarizing cytokine (IL-12) increased significantly in the DC-CD4⁺ T cocultures as DC numbers increased, and the levels were amplified in an OVA-restimulated group. IFN- γ and IL-17 production was increased in the cocultures of APN-treated DCs with CD4⁺ T cells (Fig. 7B). Additionally, IFN- γ - and IL-17-producing cell populations were also increased (Fig. 7C), thereby demonstrating that APN contributes to induction of Th1 and Th17 responses. However, IL-4 production and the numbers of Foxp3-expressing T cells were not enhanced by APN treatment (Fig. 7B, 7C). These findings suggest that the cytokine milieu generated by APN-stimulated DCs induces both Th1 and Th17 adaptive immune responses on exposure to Ags.

Next, we investigated the factors that facilitated Th1 and Th17 cell differentiation by neutralizing cytokine candidates that induce IFN- γ and IL-17 production. The addition of anti-IL-12p40-neutralizing Ab effectively blocked IFN- γ production, demonstrating the importance of IL-12 for Th1 development (Fig. 7D). We neutralized candidate Th17 cell-inducing cytokines in APN-treated DCs prior to coculturing with OVA-primed CD4⁺ T cells. Compared with isotype-matched control Ab, anti-IL-12p40 and anti-IL-23p19 Abs also significantly reduced the production of

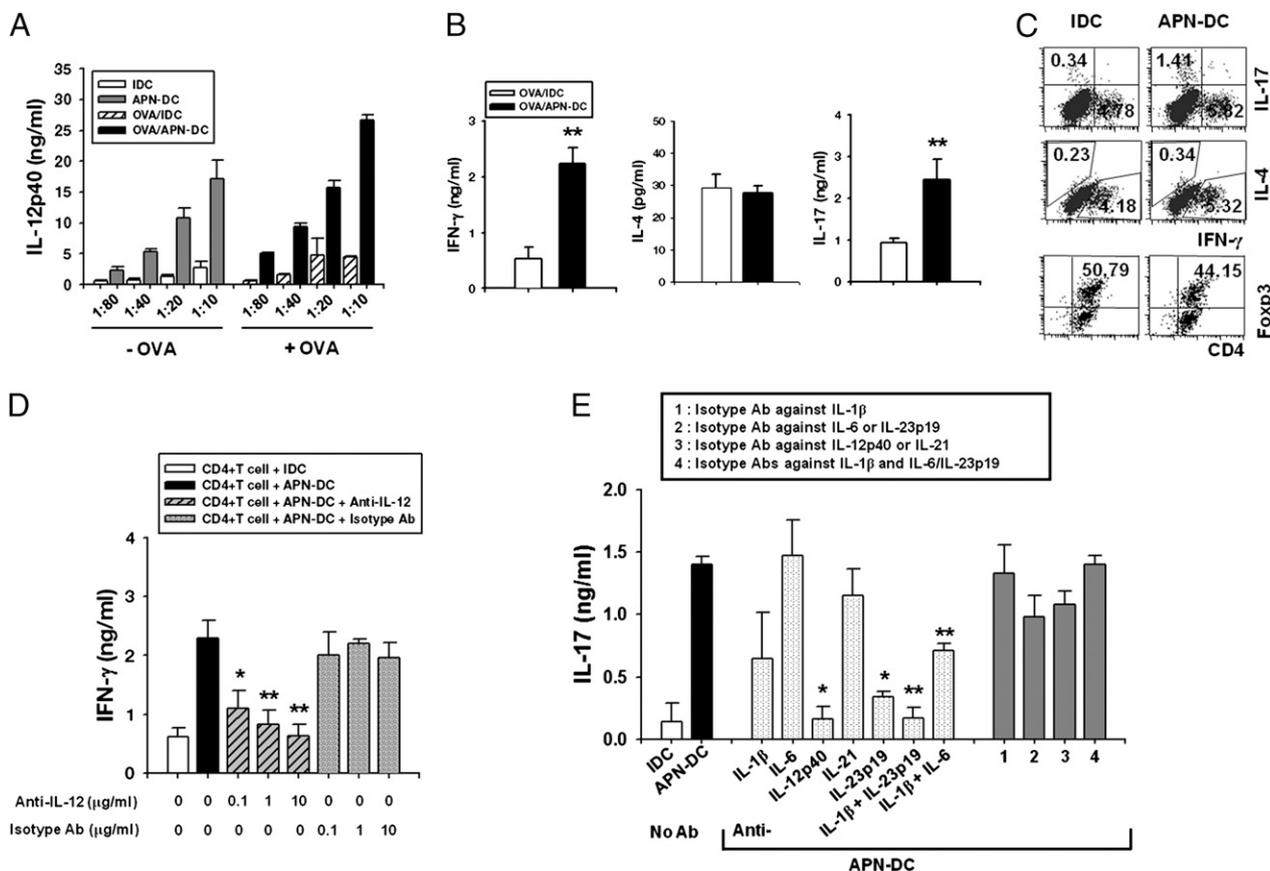


FIGURE 7. APN-stimulated DCs strongly enhance Th1 and Th17 responses in vitro. (A) Various numbers of OVA-pulsed, APN-treated DCs were cocultured with OVA-primed CD4⁺ T cells, and the level of IL-12p40 was measured via ELISA. (B) OVA-pulsed, APN-treated DCs were cocultured for 5 d with OVA-primed CD4⁺ T cells at a 1:10 ratio, and the levels of IFN- γ , IL-4, and IL-17 in the culture supernatants were measured via ELISA. Values and error bars represent mean \pm SD. ** p < 0.005, versus CD4⁺ T cells cocultured with unstimulated DCs. (C) CD4⁺ T cells were cocultured for 3 d with OVA-pulsed, APN-activated DCs or OVA-pulsed, unstimulated DCs, and the IFN- γ -, IL-4-, IL-17-, and Foxp3-expressing populations were detected via flow cytometry. One experiment of three is presented. (D) OVA-pulsed, APN-treated DCs were cocultured with CD4⁺ T cells obtained from OVA-primed mice in the presence of anti-IL-12 Ab (C17.8: 0.1–10 μ g/ml) or isotype Ab (rat IgG2a: 0.1–10 μ g/ml). Data shown represent mean \pm SD (n = 3). (E) OVA-pulsed, APN-activated DCs were treated with Abs blocking IL-1 β , IL-6, IL-21, IL-23p19, and IL-12p40 at 5 μ g/ml, after which the DCs were cocultured for 5 d with CD4⁺ T cells from OVA-sensitized mice. The amount of IL-17 protein in the supernatant was detected via ELISA. Data shown represent mean \pm SD (n = 5). * p < 0.05, ** p < 0.005, versus CD4⁺ T cells cocultured with APN-stimulated, isotype-treated DCs.

IL-17, whereas, surprisingly, anti-IL-6 Ab did not suppress IL-17 production. Anti-IL-1 β Ab combined with anti-IL-23p19 Ab or anti-IL-6 Ab synergistically decreased Th17 responses, although the neutralization with anti-IL-1 β Ab alone had little effect (Fig. 7E). This result is in agreement with the observations from other groups demonstrating the importance of IL-1 β , IL-6, and IL-23 for Th17 development (30–33). These findings provide strong evidence that APN can induce Th1 and Th17 responses in CD4⁺ T cells via elevated IL-12 and IL-1 β /IL-23 production.

APN-stimulated DCs induce both Th1 and Th17 responses *in vivo*

Finally, we evaluated the role of APN-mediated DCs in the regulation of Th polarization *in vivo*. OVA-pulsed, APN-stimulated DCs that had been activated for 3, 8, or 24 h were transferred *s.c.* to sensitized mice. Seven days later, draining LNs were isolated from DC-immunized mice, and Th1, Th2, and Th17 cytokine profiles were measured in the presence of OVA. Immunization of mice with APN-pulsed DCs significantly increased IFN- γ and IL-17 production but not IL-4 production. The increase in IL-17 mediated by APN-stimulated DCs was much stronger than that of IFN- γ , indicating that APN-treated DCs promote development of the Th17 response rather than Th1 response. The inducing effect of APN-activated DCs on the Th17 response *in vivo* might depend on the length of time that DCs were stimulated with APN. Relatively long exposure of DCs to APN still resulted in an increased Th17 response compared with short-term activated DCs (Fig. 8A). Both Th1 and Th17 cell populations were significantly upregulated in mice that received APN-stimulated DCs (Fig. 8B). The Foxp3⁺ regulatory T cell population was not affected by the adoptive transfer of APN-activated DCs in freshly isolated draining LN cells and OVA-restimulated LN cells (data not shown). These findings

indicate that APN plays an important role in DC maturation and activation, resulting in Th1 and Th17 polarization.

Discussion

APN is significantly higher in patients with chronic inflammatory diseases, such as inflammatory bowel disease, osteoarthritis, and rheumatoid arthritis (11–15). It remains unclear whether APN can regulate immunological and inflammatory diseases. Our current study emphasized the role of APN in the immune system and revealed that it has immunoregulatory ability by activating DCs through enhancement of the costimulatory molecules and MHC class II, as well as by release of proinflammatory cytokines, resulting in Th1 and Th17 responses.

APN exerts biological effects through two types of APN receptors: AdipoR1 and AdipoR2. AdipoR1 is a high-affinity receptor for globular APN and a low-affinity receptor for the full-length ligand, whereas AdipoR2 is an intermediate-affinity receptor for both forms of APN. AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver (28). However, expression of AdipoR1 or AdipoR2 receptors in DCs is largely unknown. Using RT-PCR analysis, we found that primary iDCs express both AdipoR1 and AdipoR2 receptor isoforms. In addition, APN slightly increased the expression of AdipoR1 but not AdipoR2. Transfection with AdipoR1 and/or AdipoR2 siRNAs did not antagonize APN-induced IL-12 production and NF- κ B activation. Awazawa et al. (34) recently demonstrated that IL-6 production by macrophages induced by APN through NF- κ B activation is independent of its authentic receptors. These results suggest that another possible receptor for hexameric and HMW forms of APN can exist in DCs and that APN could exert distinct actions through different receptors in a context-dependent manner.

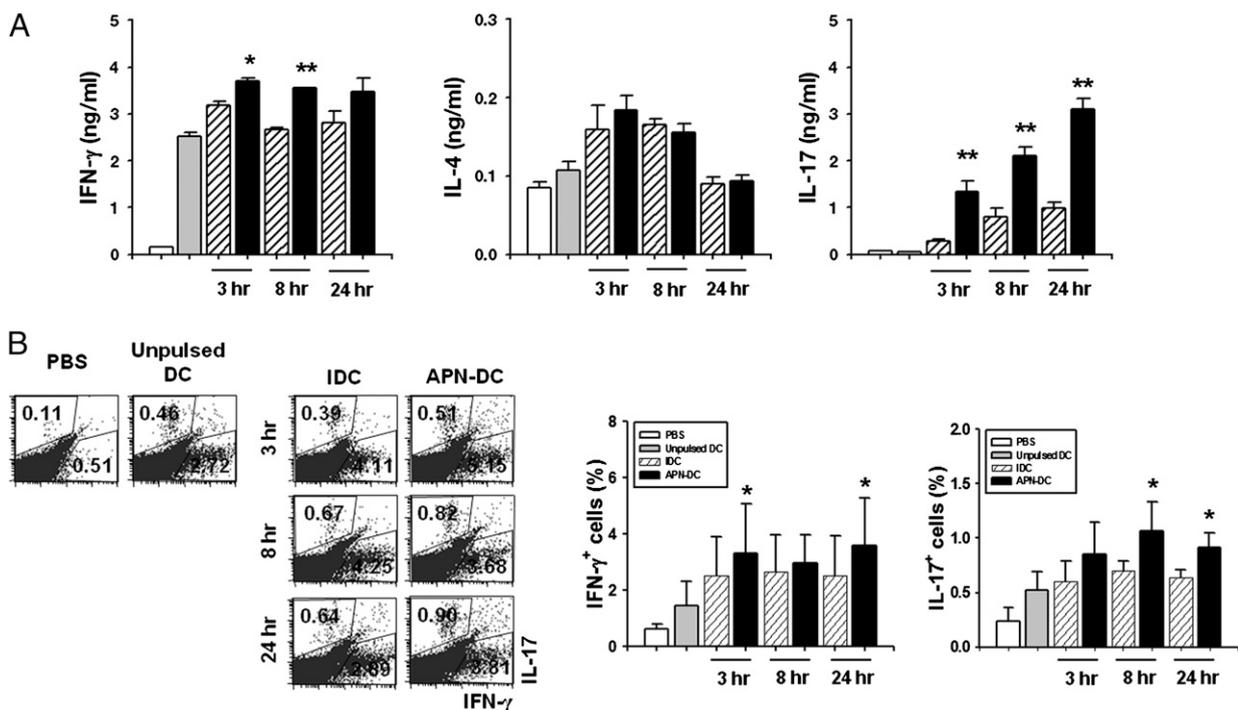


FIGURE 8. APN-stimulated DCs induce both Th1 and Th17 polarization *in vivo*. Mice were sensitized *s.c.* in the footpads with 100 μ g of OVA. Ten days after sensitization, DCs were pulsed with OVA, stimulated with APN, and applied to mice via footpad injections. **(A)** Seven days later, draining LNs were collected and restimulated with OVA for 5 d. The levels of IFN- γ , IL-4, and IL-17 production were measured via ELISA. Values are mean \pm SEM. * p < 0.05, ** p < 0.005, versus mice transferred with OVA-pulsed, unstimulated DCs. **(B)** Intracellular IFN- γ and IL-17 levels were detected using the appropriate fluorescent-labeled Abs. *Left panels*, Data are representative of three independent experiments with similar results. *Right panels*, The bar graphs indicate the percentages of IFN- γ - or IL-17-expressing CD4⁺ T cells. Data are mean \pm SEM of four independent experiments. * p < 0.05, versus mice adoptively transferred with OVA-pulsed iDCs.

There is growing evidence suggesting that DC maturation is dependent on NF- κ B activity. The mechanism through which APN stimulates iDCs was analyzed by determining the signaling molecules. APN enhanced the degradation of I κ B proteins, translocation of the p65 subunit to the nucleus, and the binding activity of NF- κ B (Fig. 3). NF- κ B is the most frequently documented transcriptional factor, and it is known to perform critical roles in DC maturation (35). Pharmacological inhibition of PLC γ , JNK, and NF- κ B significantly suppressed IL-12 production in APN-treated DCs (Fig. 4B). Additionally, treatment with PLC γ inhibitor and JNK inhibitor decreased NF- κ B DNA-binding activity, demonstrating that APN controls cytokine production through the PLC γ /JNK/NF- κ B–signaling pathways (Fig. 4D). PLC γ is a critical player in the pattern recognition receptor-mediated signal-transduction pathway. Xu et al. (36) demonstrated that PLC is required for the activation of ERK and JNK MAPKs and the induction of NF- κ B, AP-1, and NFAT transcription factors. In the absence of PLC, BMDCs have impaired production of IL-2, IL-6, IL-10, IL-23, and TNF. PLC-dependent signaling pathways were implicated in the development of acute and chronic inflammatory responses under in vivo conditions (37). We also demonstrated that APN activates ERK, JNK, and p38 MAPKs. APN activates proinflammatory cytokine release and phospholipid metabolism in human placenta and adipose tissue through ERK1/2 MAPK, peroxisomal proliferator-activated receptor- γ , and NF- κ B (38). Hirsumi et al. (39) reported that JNK1 is indeed a crucial component of the biochemical pathway responsible for obesity-induced insulin resistance in vivo. Our findings suggest that APN acts as an inducer of inflammatory cytokines via the PLC γ /JNK/NF- κ B–signaling pathways.

Although several studies demonstrated that APN is an anti-inflammatory protein (40–42), upregulation of IL-12 and IL-6 by APN reflects the proinflammatory effects of this adipokine. APN is known to exert significant proinflammatory and matrix-degrading effects. APN induces the production of IL-6, matrix metalloproteinase-1, and IL-8 from RA synovial fibroblasts in vitro (6, 43). Fayad et al. (14) reported that APN served as a proinflammatory molecule in vivo in dextran sulfate sodium- and 2,4,6-trinitrobenzene sulfonic acid-induced colitis. APN induced production of proinflammatory cytokines and inhibited bioactivity of protective growth factors in the colon. Enhanced production of proinflammatory cytokines derived from DCs may contribute to the pathogenesis of immune disorders in a number of ways controlling T cell activation/differentiation. As shown in the neutralizing experiments, IL-12p40 is a crucial factor for both Th1 and Th17 development. Our findings indicate that IL-23, IL-1, and IL-6 are capable of inducing the development of Th17 cells. Blockage of IL-23p19 alone significantly suppressed IL-17 production. Neutralization of IL-1 β , together with IL-23p19 or IL-6, synergistically inhibited IL-17 production. IL-1 β , IL-6, and IL-23 were reported to be crucial for Th17 commitment (33). IL-1 and IL-6 are known to play a critical role in the induction of pathogenic Th17 cells, as demonstrated in experimental models of experimental autoimmune encephalomyelitis or rheumatoid arthritis (30, 31, 44). IL-23 has been widely recognized as an important factor in the differentiation and maintenance of Th17 cells (32, 45). Inflammation loop linking IL-1–IL-23–IL-17 axis and further enhancement of IL-23 and IL-1 production through T cell feedback may amplify the APN-mediated differentiation of Th17 cells (31, 46). Therefore, we speculate that APN-mediated Th17 responses can be induced by a variety of cytokine combinations, including IL-1 β , IL-6, and IL-23.

Tsang et al. (47) reported that CD4⁺CD25⁺Foxp3⁺ T cells increased in the cocultures of T cells and APN-stimulated DCs. In

contrast, our results showed that APN treatment had no effect on, or even slightly decreased, Foxp3⁺ T cells (Fig. 7C). These contrasting results may be due to different schemes of APN stimulation. In Tsang et al.'s experiment, BM cells were cultured in the presence of APN during the differentiation into DCs. Thus, we assumed that DCs differentiated with APN acquired tolerogenic properties and showed blunt immune responses following stimulation. Tsatsanis et al. (48) demonstrated that pre-exposure of macrophages to APN rendered them tolerant to further APN treatment or to other proinflammatory stimuli, such as polyinosinic:polycytidylic acid and LPS. In contrast, APN could evoke Th1 and Th17 responses through activation of DCs. The effects of APN on IL-17 production were much more potent than were those on IFN- γ production in vivo, indicating that the Th17 response was predominant in the APN-mediated immune response.

We demonstrated that APN has the ability to activate DCs and release proinflammatory cytokines, leading to Th1 and Th17 phenotypes. Elevated levels of APN released locally in inflamed regions, such as in established colitis and arthritis, contribute to the perpetuation of inflammation, exacerbating the disease process. The development of an APN-specific antagonist may be beneficial for the treatment of Th1- or Th17-related immune disorders. Furthermore, the DC maturation-inducing capability of APN can be extended to an immunotherapy, such as DC-mediated cell therapy.

Disclosures

B.-S.Y. is employed by AdipoGen, Inc. The other authors have no financial conflicts of interest.

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