CD300a/c regulate type I interferon and TNF-α secretion by human plasmacytoid dendritic cells stimulated with TLR7 and TLR9 ligands

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Activation of human plasmacytoid dendritic cells (pDCs) with ligands for Toll-like receptors (TLRs) 7 and 9 induces the secretion of type I interferons and other inflammatory cytokines as well as pDC differentiation. Transcripts for 2 members of the CD300 gene family, *CD300a* and *CD300c*, were identified on pDCs during gene expression studies to identify new immunoregulatory molecules on pDCs. We therefore investigated the expression of CD300a and CD300c and their potential regulation of pDC function. CD300a/c RNA and surface expression were down-regulated after stimulation of pDCs with TLR7 and TLR9 ligands. Exogenous interferon (IFN)-α down-regulated CD300a/c expression, whereas neutralizing IFN-α abolished TLR ligand–induced CD300a/c down-regulation. This implicates IFN-α in regulating CD300a/c expression in pDCs. In addition, IFN-α favored tumor necrosis factor (TNF)-α secretion by CpG-induced pDCs. CD300a/c triggering by cross-linking antibody reduced TNF-α and increased IFN-α secretion by pDCs. Furthermore, CD300a/c triggering, in the presence of neutralizing IFN-α, further reduced TNF-α secretion. These data indicate that CD300a and CD300c play an important role in the cross-regulation of TNF-α and IFN-α secretion from pDCs. (Blood. 2008;112:1184-1194)

Introduction

Plasmacytoid dendritic cells (pDCs) constitute a distinct population of DCs in the peripheral blood and secondary lymphoid organs. They secrete large amounts of type I interferons (IFNs) and inflammatory cytokines as an immediate response to virus.[1-5] Toll-like receptors (TLRs) are sensors that recognize viral components,[4] and human pDCs express both TLR7 and TLR9. TLR7 and TLR9 signaling in pDCs leads to secretion of IFN-α and tumor necrosis factor (TNF)-α.[4]

A role for pDCs in human diseases is now evident. Several autoimmune diseases, including systemic lupus erythematosus (SLE), Sjögren syndrome, and psoriasis, have been found to have an infiltrate of pDCs in target organs.[6-10] Microarray studies have also identified a type I IFN signature for SLE,[11] and several IFN-α responsive genes were expressed in the involved skin of patients with psoriasis.[12] Thus, the pDC infiltrate in target organs may correlate with the type I IFN signature of these autoimmune diseases. The presence of pDCs was associated with poor outcomes in ovarian and breast cancers.[13,14] Increased pDCs in the transplant were associated with a good prognosis in allogeneic hematopoietic stem cell transplantations, and pDCs were suggested to facilitate engraftment.[15]

Regulation of pDC type I IFN secretion is central to their contribution in autoimmune diseases. Membrane molecules, including BDCA-2, FcεRI, ILT7, NKp44, and Siglec-H, regulate pDC function by inhibiting IFN-α.[16-22] BDCA-2 is a C-type lectin that has been classically associated with potent inhibition of IFN-α.[16] FcεRI is a member of the immunoglobulin (Ig) superfamily, and cross-linking IgE on pDCs reduces TLR9 and inhibits pDC IFN-α production. Thus, FcεRI regulates TLR9 and the reciprocal counterregulation occurs, mediated in part by IFN-α.[17] ILT7 is an Ig superfamily member and uses FcεRI γ chain as an adapter to trigger ITAM signaling. ILT7 cross-linking inhibits both IFN-α and TNF-α production on pDCs.[19] NKp44 is expressed on natural killer (NK) cells, tonsil pDCs, and blood pDCs when cultured with interleukin-3 (IL-3) but not on freshly isolated blood pDCs.[20] Siglec-H, a sialic acid binding Ig-like lectin, has been identified only in mouse pDCs. Cross-linking of Siglec-H inhibits IFN-α production on pDCs in response to TLR9 stimulation.[21,22] Notably, none of these molecules increases IFN-α.

The CD300a and CD300c molecules are the products of independent genes within the CD300 gene complex located on the chromosome 17q22-25.[23-26] CD300a and CD300c share 80% amino acid sequence similarity between their Ig domains, whereas the remainder of the molecules show little amino acid similarity. They are expressed on monocytes, macrophages, granulocytes, DCs, T lymphocytes, and NK cells, and they have regulatory functions in leukocytes.[24,27] Recently, we showed that expression of the CD300a/c molecules identifies a functionally distinctive $CD4^+$ T-lymphocyte subpopulation.[28] This study addresses the expression of CD300a/c molecules on pDCs and their capacity to regulate pDC function.

Gene expression analysis of pDCs identified that expression of *CD300a* and *CD300c* was regulated by CpG. Exogenous TNF-α inhibits pDC IFN-α secretion.[29] Our new data indicate that CD300a and CD300c cross-regulate IFN-α and TNF-α production by pDCs. We also identify CD300a/c as the first modulating membrane molecules to up-regulate type I IFN production and show that they inhibit TNF-α production from activated pDCs, a phenomenon that may have considerable relevance in clinical practice.

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Methods

Samples

Fresh blood samples were obtained with consent from healthy donors. Tonsils were from pediatric patients undergoing tonsillectomy. All samples were collected according to ethical guidelines approved by the Human Research Ethics Committee of the Mater Health Services in accordance with the Declaration of Helsinki.

Reagents

The following reagents were used: CpG-oligodeoxynucleotide type A (CpG ODN 2216; 5 μg/mL; sequence: GGGGGACGATCGTCGGGGGG; Invitrogen, Mount Waverley, Australia)[30]; control oligodeoxynucleotide (Control ODN; 5 μg/mL; sequence: GGGGGAGCATGCTCGGGGGG; Invitrogen); TLR7 ligand R837 (5 μg/mL; InvivoGen, San Diego, CA); TLR9 inhibitor chloroquine (10 μM; InvivoGen); mouse monoclonal antibody (mAb) CMRF-35, control antibody CMRF-81 were made in-house[26,27]; a mixture of neutralizing rabbit anti–human IFN-α Ab (2000 U/mL) and mouse anti–human IFN-α/β receptor (CD118) mAb (10 μg/mL; both from PBL Biomedical Laboratories, Piscataway, NJ) or a mixture of rabbit IgG and mouse IgG_{2b} (Sigma-Aldrich, St Louis, MO); TNF-α antagonist Infliximab (100 μg/mL; Remicade; Schering-Plough, Baulkham Hills, Australia) or an isotype-matched control (human IgG_1; Sigma-Aldrich); recombinant human IL-3 (10 ng/mL; R&D Systems, Minneapolis, MN); TNF-α (5 ng/mL; R&D Systems); recombinant human IFN-α2 (10 ng/mL; PBL Biomedical Laboratories).

Cell preparation and cell culture

Peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation over Ficoll-Paque Plus (GE Healthcare, Little Chalfont, United Kingdom). Tonsil mononuclear cells were prepared by density gradient centrifugation of a cell suspension made by mechanical dissociation of tonsil tissues.[31] pDCs were prepared from mononuclear cells using the BDCA-4 purification kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's recommendations with a final purity of 84% to 95%. Further purification was achieved by sorting for $Lin^-CD4^+CD11c^-$ cells (Lin marker; CD3, CD19, CD20, CD14, CD56, CD34, and CD235a) with purity of 99% by FACSAria (BD Biosciences, San Jose, CA) as described previously.[32] Cells were incubated at 1 to 2×10^5/200 μL in complete RPMI 1640 (RPMI 1640 containing 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, 10 mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; Invitrogen], 50 μM mercaptoethanol [Sigma-Aldrich]) in the presence of 10 ng/mL IL-3.

Cytokine analysis

Cell culture supernatants were harvested and stored at −80°C until assayed. TNF-α and IL-6 levels were analyzed using BD Cytometric Bead Arrays (BD Biosciences) according to the manufacturer's protocols. Samples were analyzed on an LSRII cytometer (BD Biosciences), and data were analyzed using BD FCAP Array software. Human IFN-α in diluted supernatants was assayed using an enzyme-linked immunosorbent assay (ELISA) kit with the standard range of 156 to 5000 pg/mL (PBL Biomedical Laboratories).

DNA microarray analysis

Total RNA from pDCs was isolated with RNeasy Kits (Qiagen, Valencia, CA) and used to generate cDNA according to the Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). cRNA was generated with the BioArray High-Yield Transcript Labeling kit (Enzo Diagnostics, New York, NY) and hybridized to Affymetrix human HG-U95A arrays (Affymetrix) at 45°C for 16 hours and then stained, washed, and scanned according to the manufacturer's protocol. Scanned images were aligned and analyzed using the GeneChip software Microarray Suite 5.0 (Affymetrix) according to the manufacturer's instructions. Signal intensities were normalized to the mean intensity of all the genes on the array with global scaling of 1000.[18,33,34]

Reverse transcription-polymerase chain reaction and real-time reverse transcription-polymerase chain reaction analyses

Total RNA was prepared using the RNeasy MicroKit (Qiagen) and transcribed into cDNA using Superscript III Platinum 2-Step qPCR kit (Invitrogen). Polymerase chain reaction (PCR) amplification was performed as described before,[24,28] and PCR products were separated on 1% agarose and photographed. The housekeeping gene, β-actin, was used to monitor PCR. The primer set for β-actin was forward, 5′-GAAGAGCTACGAGCTGCCTGA-3′; reverse, 5′-TGATCTTCATTCTGCTGGGTG-3′. Real-time PCR probes were labeled with 5′-FAM and 3′-BHQ1 (Biosearch Technologies, Novato, CA). Reactions were prepared using Platinum Quantitative PCR SuperMix-UDG (Invitrogen). Data were normalized to the level of ubiquitin converting enzyme (UCE). The primer and probe sequences for real-time PCR were UCE-For, 5′-TGAAGAGAATCCACAAGGAATTGA-3′; UCE-Rev, 5′-CAACAGGACCTGCTGAACACTG-3′; UCE-Probe, 5′-TGATCTGGCACGGGACCCTCCA-3′[35]; CD300a-For, 5′-CCTGCACAACAGTGACCAAC-3′; CD300a-Rev, 5′-CTGATGGCAACAGAGGGAT-3′; CD300a-Probe, 5′-TGGGAAACCCAGCTGCCTGTC-3′; CD300c-For, 5′-TGTCGCTATGAGAAGGA-3′; CD300c-Rev, 5′-TGTCACATCGGAGAATC-3′, CD300c-Probe, 5′-CAGGACCCTCAACAAATTCTGGTGC-3′; IFN-α1-For, 5′-AGCAAGCCCAGAAGTATC-3′; IFN-α1-Rev, 5′-CACCAGGACCATCAGTA-3′; IFN-α1-Probe, 5′-TGCAATATCTACGATGGCCTCGCC-3′; IFN-β-For, 5′-TGAAGGCCAAGGAGTA-3′; IFN-β-Rev, 5′-CGGAGGTAACCTGTAAG-3′; IFN-β-Probe, 5′-CACTGTGCCTGGACCATAGTCA-3′. Reverse transcriptase ^{2}qPCR Primer Assays were used for analyzing SYBR-Green human MyD88 (PPH009HA), IRAK1 (PPH00835A), and IRF7 (PPH02014E) gene expressions according to the manufacturer's instruction (SuperArray Bioscience Corporation, Frederick, MD). Data were normalized to the housekeeping gene GAPDH (PPH00150A). Amplification for all real-time PCR was performed on a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia). Data were analyzed using Rotor-Gene 6.0 software (Corbett Research).

CMRF-35 cross-linking pDCs in culture

Tissue culture plates (96 wells) were coated with 10 μg/mL CMRF-35 or control CMRF-81 mAb for 1 hour at 37°C and then rinsed with PBS. pDCs were incubated in the CMRF-35–precoated plates for 30 minutes then stimulated with or without CpG ODN in the presence of IL-3. BDCA-2 mAbs (2.5 μg/mL; Miltenyi Biotec) were used as a positive control. The culture supernatants were harvested for cytokine secretion analysis, and the cells were either analyzed by flow cytometry or used to extract total RNA for PCR studies.

Flow cytometric analysis

Cells were stained with the following antibodies: mouse anti–human CD300a/c CMRF-35 mAb (clone: CMRF-35),[27] anti–tetanus toxoid mAb (clone: CMRF-81), anti–human BDCA-2-FITC and anti–human BDCA-4-APC (Miltenyi Biotec), anti–human HLA-DR-APC or HLA-DR-PerCP (BD Biosciences), TLR-9-PE (Imgenex, San Diego, CA), CD3-PE (BD Biosciences), CD80-FITC or CD80-PE (BD Biosciences), CD86-PE (BD Biosciences), CD83-FITC or CD83-PE (Immunotech, Vaudreuil-Dorion, QC), NKp44-PE (Miltenyi Biotec), Alexa Fluor 488–goat anti–mouse IgG $F(ab)'_2$ fragment (Invitrogen) and PE-conjugated anti–mouse Ig $F(ab)'_2$ fragment (Chemicon, Temecula, CA). For analysis of intracellular TLR9 expression in pDCs, cells were labeled using the FIX & PERM cell permeabilization kit according to the manufacturer's instructions (Invitrogen). Cells were analyzed on a FACSCalibur or LSRII cytometer (BD Biosciences) with FCS Express III software (Thornhill, ON).

Tetanus toxoid recall response

pDCs were cross-linked with CMRF-35 mAb or control antibody and stimulated with CpG in the presence of IL-3 for 48 hours. $CD4^+$

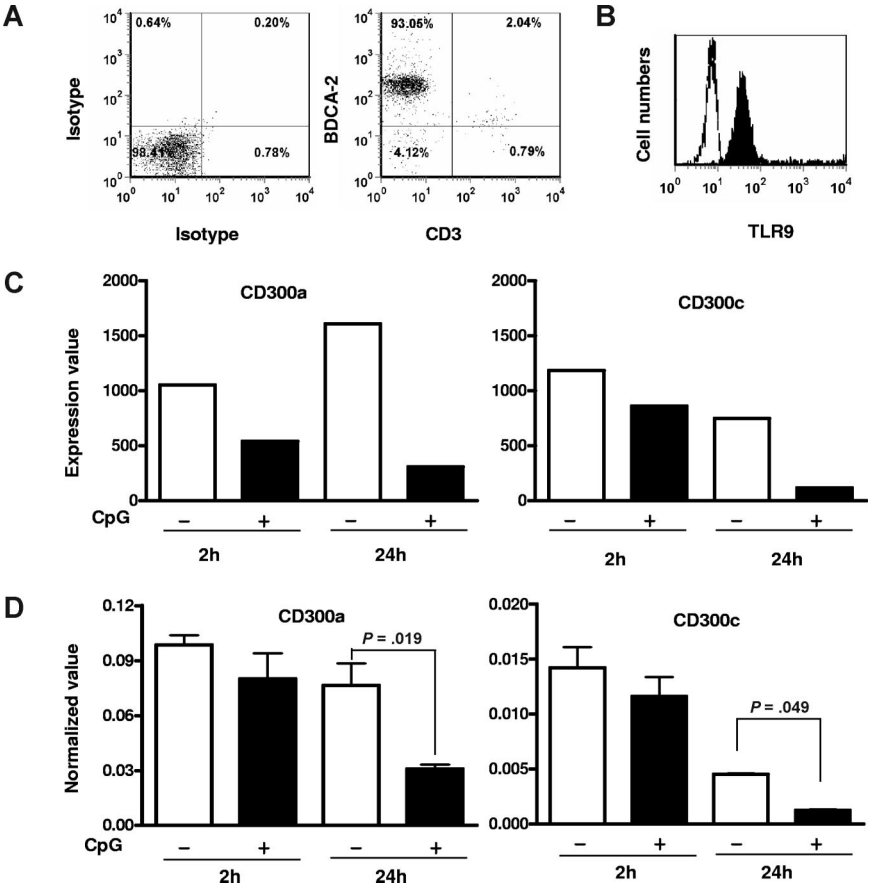

Figure 1. CpG down-regulates CD300a and CD300c transcripts in pDCs after CpG treatment. (A) pDCs isolated with the BDCA-4 kit were analyzed for purity by staining with BDCA-2 mAbs. Data were from one representative experiment (n = 10). Numbers on plots are percentages of purified BDCA-4$^+$ cells. (B) Isolated pDCs were fixed and permeabilized and further stained intracellularly with TLR9-PE antibody and analyzed by flow cytometry. Data were from 1 of the 4 experiments. (C) pDCs were cultured with CpG ODN or control ODN for 2 and 24 hours; RNA were extracted and subjected to DNA microarray analysis. Normalized relative expression values of CD300a and CD300c mRNA in pDCs are shown (from left to right). Data were from one DNA microarray experiment. (D) Expression of CD300a and CD300c mRNA in pDCs (from left to right) cultured with or without CpG for 2 and 24 hours were analyzed using quantitative real-time PCR. Data were normalized according to level of the housekeeping gene UCE. Error bars represent SEM.

T lymphocytes were purified as before.[29] Purified CD4$^+$ T lymphocytes (10^4/well) from the same donor were incubated with pDCs at a ratio of 4:1 (responder/stimulator) ratio in complete RPMI 1640 media at 37°C in 5% CO_2. Tetanus toxoid (TT; gift from Commonwealth Serum Laboratories, Parkville, Australia) was added at a concentration of 20 μg/mL. After 5 days of culture, lymphocyte proliferation was assessed by the addition of [^{3}H]-thymidine (1 μ Ci [0.037 MBq]/well, 6.7 Ci [10.4 $\times 10^{10}$ Bq]/mM; GE Healthcare) 16 hours before harvesting.[36]

Allogeneic mixed leukocyte reactions

Allogeneic mixed leukocyte reactions (MLRs) were established by culturing pDCs that had been cross-linked with CMRF-35 mAb and stimulated with CpG for 48 hours, with 10^5 allogeneic CD4$^+$ T cells in complete RPMI 1640 media at 37°C in 5% CO_2 for 5 days. T-cell proliferation was measured by [^{3}H]-thymidine uptake (1 μCi [0.037 MBq]/well; GE Healthcare). Responses are reported as mean cpm plus or minus standard error of the mean (SEM) for triplicate wells.

Statistics

The cytokine level and real-time PCR data were depicted by mean and SEM. Statistical significance was evaluated using Student *t* test with Prism software (San Diego, CA). *P* value or no significance (ns) was shown.

Results

Down-regulation of CD300a and CD300c transcripts in pDCs after CpG treatment

As the first step in studying the expression of CD300 molecules on pDCs and their effect on pDC biology, we validated our pDC preparation (Figure 1A). The purified pDCs expressed TLR9 (Figure 1B) and produced large amounts of IFN-α and other inflammatory cytokines (such as TNF-α and IL-6) in response to TLR9 ligand CpG ODN stimulation (described later). The gene expression profiles of pDCs treated with CpG at different time points (2 hours and 24 hours) were then analyzed by genome-wide microarray.[18] We searched for immunoregulatory molecules that were differentially regulated by CpG treatment. This identified both *CD300a* and *CD300c* transcripts as being differentially expressed in control versus CpG-treated pDCs (Figure 1C). CD300a and CD300c are 2 immunoregulatory molecules that we have shown to be differentially regulated on leukocytes, including DCs.[27,28]

Treatment of pDCs with CpG resulted in a marked decrease in *CD300a* expression after 2 hours with further down-regulation at 24 hours (Figure 1C). CD300c expression decreased after 2 hours of culture, and, after 24 hours of CpG stimulation, CD300c mRNA was difficult to detect. We confirmed these results by analyzing pDCs cultured in the presence of CpG using real-time PCR. First, approximately 10-fold more CD300a mRNA was expressed than CD300c mRNA. Second, both CD300a and CD300c mRNA decreased during culture of pDCs. This analysis corroborated the CpG-induced down-regulation of pDC CD300a and CD300c mRNA after 24 hours of culture (P = .019 and .049, respectively; Figure 1D). After an extended 48-hour CpG exposure, both CD300a and CD300c were significantly reduced (Materials S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Thus, *CD300a* and *CD300c* transcripts were down-regulated in pDCs activated with CpG. Because the more

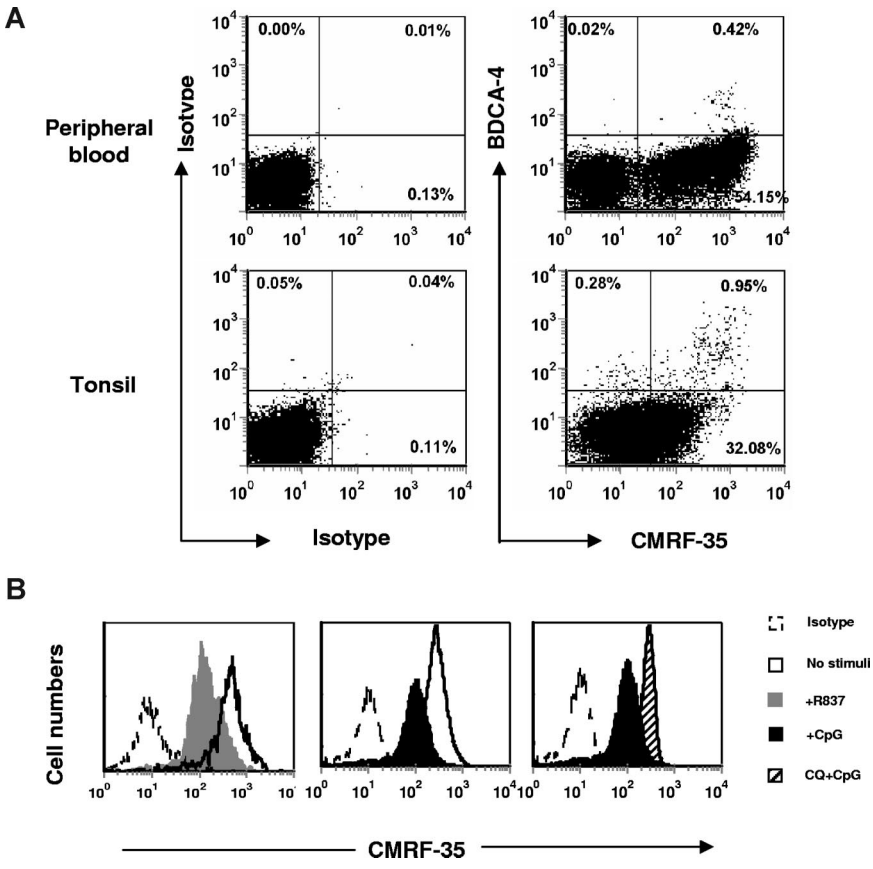

Figure 2. Surface expression of CD300a/c on pDCs and their down-regulation by R837 and CpG. (A) Mononuclear cells from peripheral blood and tonsil tissue were isolated using Ficoll-Paque Plus density centrifugation. Cells were stained with CMRF-35 mAb, anti–mouse Ig F(ab$_2$)′-PE, and BDCA-4-APC, or appropriate isotype controls, and analyzed by flow cytometry. Data are representative of 8 blood and 4 tonsil samples. (B) Blood pDCs were stimulated for 48 hours with R837 or CpG. pDCs were treated with chloroquine (CQ; 10 μM) for 30 minutes before stimulation with CpG. The histograms show cells stained with CMRF-35 or isotype control, to monitor the expression of CD300a/c by flow cytometry. Open dashed line indicate isotype control; open solid line, no stimuli; gray filled histogram, with R837; black filled histogram, with CpG; filled with slash line, CQ + CpG. Data are representative of 3 experiments.

prominent regulation occurred after 24 hours, further studies on the regulation of CD300a/c on pDCs were performed at 24 hours but also 48 hours after CpG exposure.

TLR7 or TLR9 signaling down-regulates CD300a/c surface expression on pDCs

The CMRF-35 mAb identifies both CD300a and CD300c on the surface of leukocytes.[27] The majority (≈92%) of peripheral blood BDCA-4$^+$ pDCs bound CMRF-35. In tonsil, a relatively large population of BDCA-4$^+$ pDCs (≈77%) were CMRF-35$^+$; however, there was a lesser population of BDCA-4$^+$ cells (≈23%) that were CMRF-35$^-$ (Figure 2A). The CMRF-35$^+$ tonsil pDCs expressed NKp44 (28%-70%), CD56 (21%-71%), HLA-DR (≈98%), CD80 (25%-40.6%), CD83 (9%-19.1%), and CD86 (62.3%-76.1%); the CMRF-35$^-$ BDCA-4$^+$ cells were NKp44$^-$, CD56$^-$, HLA-DR$^+$ (27.7%-1.8%), CD80$^-$, CD83$^-$, and CD86$^+$ (20%-31%) (Figure S2). To investigate whether the down-regulation of *CD300a* and *CD300c* transcripts in pDCs exposed to TLR9 ligand was reflected on the surface, we examined CD300a/c membrane labeling by flow cytometry. Consistent with the mRNA analysis, pDC stimulation with either the TLR7 or TLR9 ligands decreased the binding of CMRF-35 at 48 hours of culture (Figure 2B). Chloroquine, which inhibits the TLR endosomal acidification, was then added to the cultures to confirm the role of TLR signaling on CD300a/c expression. Chloroquine alone had no effect on pDC CD300a/c expression, whereas incubation of pDCs with chloroquine before adding CpG completely abolished the CpG-induced down-regulation of CD300a/c at both the mRNA (data not shown) and protein level detected by CMRF-35 (Figure 2B right histogram). Therefore, activation of TLR7 or TLR9 signaling leads to the down-regulation of pDC CD300a/c surface expression.

TLR ligands induce down-regulation of CD300a and CD300c in pDCs by IFN-α

We showed previously that CD300a/c surface expression could be down-regulated on PBMCs by IFN-α.[27] Because pDCs respond to TLR7 and TLR9 ligands by secreting a large amount of IFN-α, we tested whether the CD300a/c down-regulation in response to TLR7 and TLR9 ligands was due to the effect of autologous pDC IFN-α secretion. After 24 hours of culture with 10 ng/mL IFN-α, pDCs expressed reduced levels of surface CD300a/c, which was further decreased at 48 hours. The addition of CpG to the culture further down-regulated CD300a/c expression (mean fluorescence intensity [MFI] of 24 and 48 hours of culture with no IFN/ + IFN/ + CpG: 848, 535, 411 and 126, 62, 42, respectively) (Figure 3A). The intensity of CMRF-35 binding to cultured pDCs divided the pDCs into 2 populations: CMRF-35^{low} (M1) and CMRF-35^{high} (M2) subpopulations (Figure 3B). CpG decreased the CMRF-35^{high} population but increased the CMRF-35^{low} population. The CMRF-35^{low} subpopulation in pDCs expressed higher levels of HLA-DR and CD86 than did the CMRF-35^{high} subpopulation (Figure S3). The addition of neutralizing IFN-α antibody and anti–human IFN-α/β receptor (CD118) mAb to the cultures prevented in part the CpG-induced reduction in CMRF-35 binding (Figure 3B). Likewise, the down-regulation of CD300a and CD300c mRNA was partially prevented by the IFN-α neutralizing antibodies (Figure 3C). Therefore, the down-regulation of pDC CD300a/c induced by TLR7/9 ligand treatment results at least partially from the pDCs secreted IFN-α.

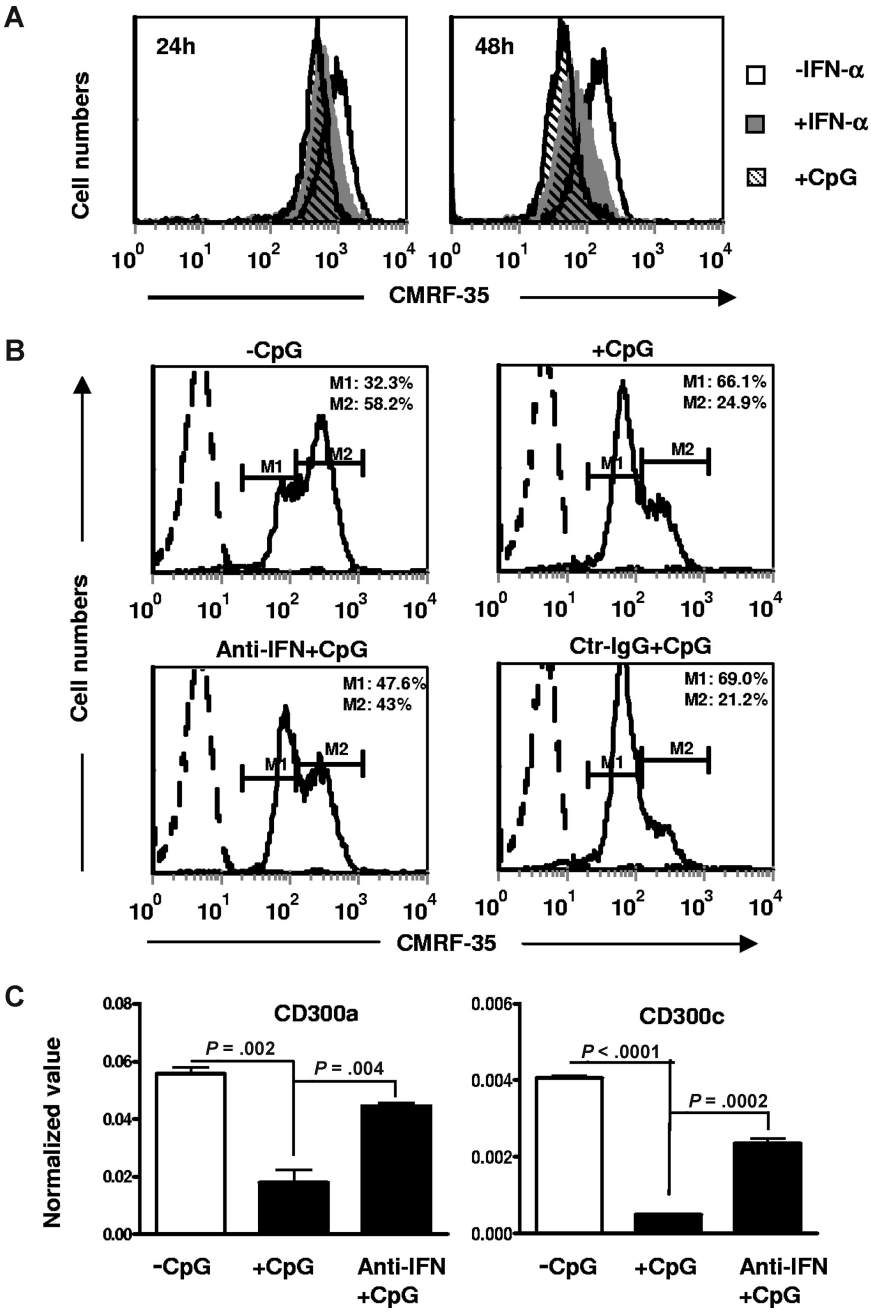

Figure 3. CpG induced down-regulation of CMRF-35 binding is IFN-α dependent. (A) pDCs cultured with CpG or IFN-α (10 ng/mL) for 24 and 48 hours were analyzed for CD300a/c expression by flow cytometry using CMRF-35 mAb. Open histogram indicates without IFN-α; filled with gray histogram, with IFN-α; filled with slash line, with CpG. (B) Histograms displaying the binding of CMRF-35 mAb to pDCs that were incubated with either control ODN (− CpG), CpG (+ CpG), anti–IFN-α + CpG or control IgG + CpG for 48 hours. Open histogram with dashed line indicates isotype control. (C) Real-time PCR analysis of CD300a and CD300c mRNA in pDCs cultured with control ODN, CpG, or anti–IFN-α + CpG for 24 hours. Data are representative of 3 experiments. Error bars represent SEM.

Triggering of CD300a/c on pDC inhibits HLA-DR expression and reduces TNF-α and IL-6 production

Because CD300a and CD300c are immunoregulatory molecules, we assessed their capacity to regulate pDC function. pDC CD300a/c were cross-linked using the CMRF-35 mAb. There was no observed effect on pDC after CMRF-35 cross-linking in the absence of CpG. Activation of pDCs with TLR7/9 ligands or virus increased the expression of HLA-DR, CD80, CD83, and CD86 and produced type I IFN, TNF-α, and IL-6.[37,38] Cross-linking pDCs with CMRF-35, during CpG activation, reduced HLA-DR expression significantly; however, it had no effect on CD80, CD83, and CD86 expression on pDCs (Figure 4A; data not shown). CMRF-35 cross-linking inhibited TNF-α and IL-6 secretion significantly ($P = .047$ and .031, respectively; Figure 4B,C).

Because cross-linking with CMRF-35 inhibited inflammatory cytokine secretion by pDCs, we tested whether the HLA-DR down-regulation seen was related to the reduced level of TNF-α after CMRF-35 cross-linking. In line with another report,[29] we observed that the pDCs cultured with an anti–TNF-α antibody had reduced HLA-DR, CD80, and CD86 expression, but exogenous IFN-α alone had no effect on the expression of costimulatory molecules (Figure 4D; data not shown). These data suggest that TNF-α is involved in the regulation of HLA-DR on pDCs after CMRF-35 cross-linking.

To examine whether the observed CMRF-35 induced down-regulation of HLA-DR had any effect on T-cell stimulatory activity, we performed TT recall assays and MLR analysis. Cross-linking CMRF-35 on pDCs had no obvious effect on the antigen-dependent autologous and allogeneic $CD4^+$ T-cell proliferation, which was

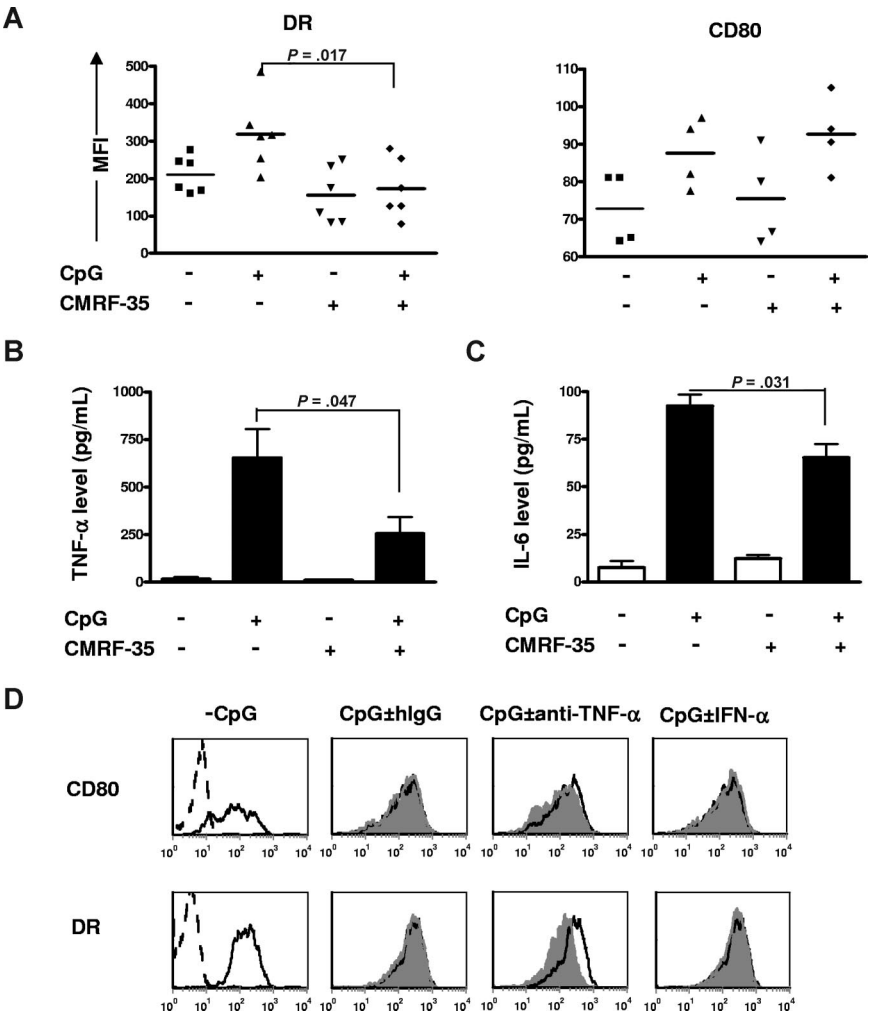

Figure 4. CMRF-35 cross-linking of pDCs on surface molecule expression. (A) Flow cytometric analysis of HLA-DR and CD80 expression in pDCs cultured with CpG or control ODN after cross-linking with CMRF-35 mAb or left not cross-linked. Each dot represents one analysis sample. MFI is shown. (B) pDCs were cultured with or without CMRF-35 cross-linking in the presence of CpG or control ODN for 24 hours. TNF-α secretion level was measured from the cell culture supernatant. Data are representative of 4 experiments. (C) IL-6 was measured in the cell culture supernatant from pDCs cultured with or without CMRF-35 cross-linking in the presence of CpG or control ODN for 24 hours. Data are representative of 4 experiments. Error bars represent SEM. (D) Flow cytometric analysis of HLA-DR and CD80 expression in pDCs cultured with or without anti–TNF-α antibody and IFN-α for 48 hours. Human IgG_1 (hIgG) was used as control antibody for anti–TNF-α. Open histogram with dashed line indicates isotype control; open histogram with solid line (from left to right), no CpG, CpG-hIgG, CpG-anti–TNF-α, CpG-IFN-α; filled histogram (from left to right), CpG + hIgG, CpG + anti–TNF-α, and CpG + IFN-α. Data are from one representative of 3 experiments.

similar to that of control pDCs or control-IgG cross-linked pDCs (Figure S4).

Triggering of CD300a/c on pDCs increases IFN-α production

After cross-linking, pDCs were stimulated with CpG for 24 and 48 hours. There was some increase in IFN-α mRNA levels at 24 hours (≈1.2-fold; $P = .06$; data not shown), but a greater increase (≈5-fold) was observed at 48 hours after CMRF-35 cross-linking (Figure 5A). IFN-β mRNA expression was also increased significantly (≈6.4-fold; data not shown). Accordingly, IFN-α secretion by CMRF-35 cross-linked pDCs after 48 hours culture was significantly increased ($P = .01$), although there was no significant change at 24 hours of culture ($P = .262$; Figure 5B; data not shown). In contrast, incubation of BDCA-2 antibody dramatically reduced IFN-α production as described previously[16,19] (Figure 5B).

TLR7/9 induction of type I IFN occurs through the MyD88-dependent signaling pathway, which finally activates IRF7.[39] IRF7 is known to be very unstable with a 1 hour half-life.[40] Our DNA microarray data and real-time PCR analysis showed that CpG increased IRF7 mRNA transiently in pDCs, but after 15 hours IRF7 mRNA decreased (Figure 5C; data not shown). These data are similar to another report.[30] Interestingly, CMRF-35 cross-linking enhanced IRF7 mRNA expression significantly at 24 hours ($P = .007$) and at 48 hours ($P < .001$) (Figure 5C; data not shown), whereas the expression of adaptor molecule MyD88 did not change (Figure 5D). Triggering of CD300a/c had no effect on the IRAK1 mRNA (data not shown). These data indicated that triggering of CD300a/c increased type I IFN secretion by activation of IRF7 transcription.

CD300a/c are involved in the cross-regulation of IFN-α and TNF-α in pDCs

When pDCs are exposed to bacteria or virus, both IFN-α and TNF-α are produced. How the balance of these cytokines is maintained is not completely understood.[29,41] We next investigated whether CD300a/c molecules were involved in the cross-regulation of IFN-α and TNF-α secretion by pDCs. As expected, cultured pDCs in the absence of CpG or in the presence of IFN-α alone did not secrete TNF-α. We did not observe any prominent change of TNF-α secretion in pDCs cultured with CpG and exogenous IFN-α for 24 and 48 hours. However, neutralizing IFN-α by combining IFN-α and IFN-α/β receptor antibodies led to decreased TNF-α production (33.9%; $P = .049$) in pDCs stimulated with CpG at 24 hours, with a further reduction (22%; $P < .001$) at 48 hours (Figure 6A). The reduced pDC TNF-α production was not related to cell death, examined in both instances by staining with 7AAD (data not shown). These data indicated that coincidental IFN-α signaling favored the ongoing secretion of TNF-α by pDCs in response to CpG stimulation. Interestingly, pDCs cross-linked with

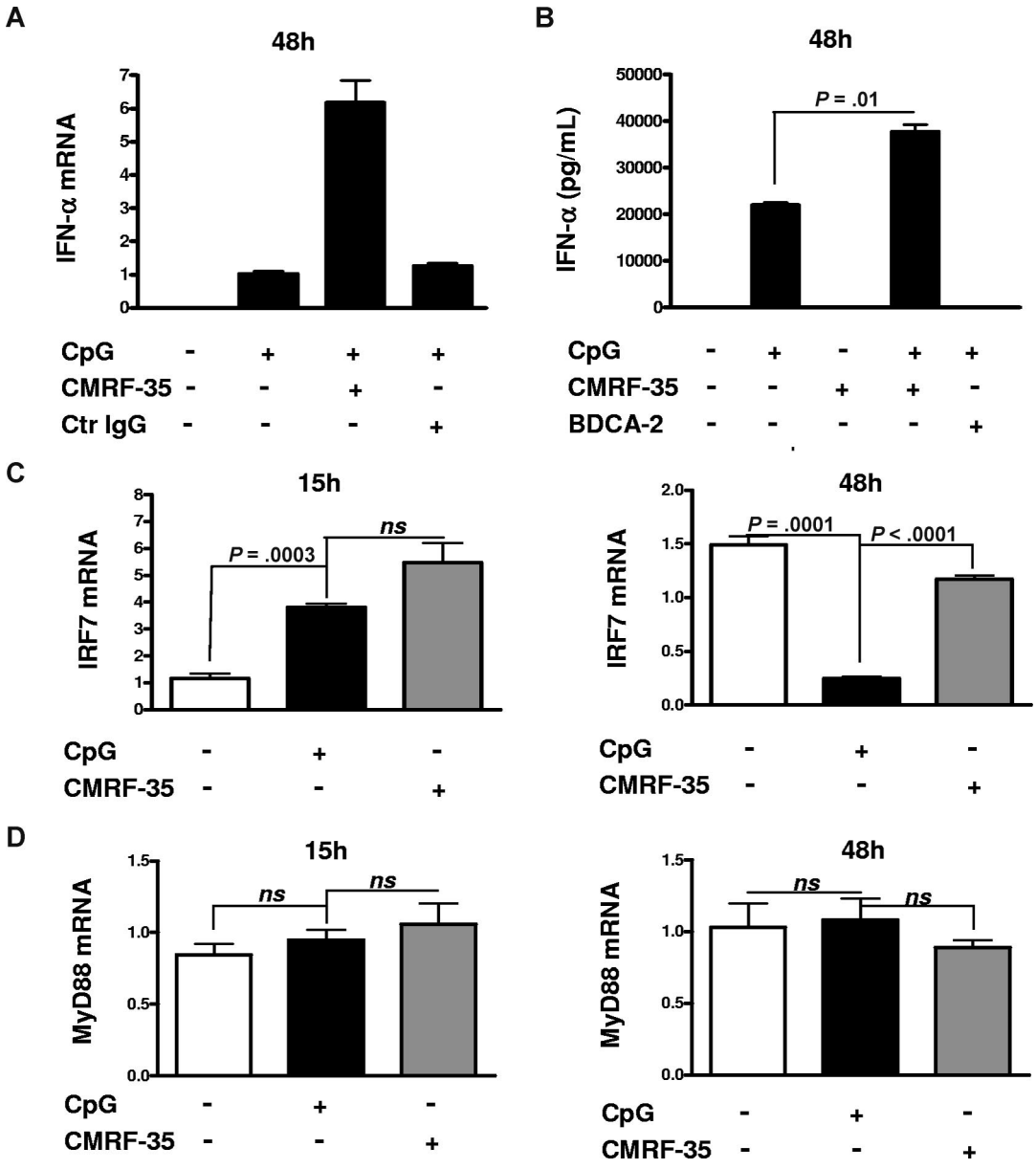

Figure 5. Triggering of CD300a and CD300c increases type I IFN and IRF7 expression by pDCs. (A) pDCs were cross-linked with 10 μg/mL CMRF-35 mAb or CMRF-81 control antibody (10 μg/mL; Ctr IgG) for 30 minutes, then stimulated with CpG. IFN-α mRNA was analyzed by real-time PCR in pDCs cultured for 48 hours. Data are representative of 3 experiments. (B) IFN-α levels in supernatants collected from pDCs after CMRF-35 cross-linking and cultured for 48 hours was measured by ELISA (from left to right). Cross-linking with BDCA-2 mAb or non–cross-linking of pDCs were used as control. Data are from 1 of the 3 experiments. pDCs were cross-linked with 10 μg/mL CMRF-35 mAb for 30 minutes, then stimulated with CpG for 15, 48 hours. mRNA from IRF7 (C) and MyD88 (D) was analyzed by real-time PCR. Data were representative of 3 experiments. Error bars represent SEM.

the CMRF-35 antibody, whereas simultaneously exposed to IFN-α neutralizing antibody produced even less TNF-α ($P = .002$; Figure 6B). This reinforces the capacity of CD300a/c to regulate pDC cytokine release in a manner that is likely to influence immune reactions significantly.

Regulation of pDC IFN-α secretion by TNF-α in the context of CMRF-35 cross-linking was investigated further. Neutralizing TNF-α using anti–TNF-α antibody increased the IFN-α mRNA expression on pDCs by real-time PCR (data not shown). Adding exogenous TNF-α inhibited both IFN-α transcript (Figure 6C) and pDC IFN-α secretion in response to CpG stimulation (Figure 6D). Cross-linking with CMRF-35 mAb increased IFN-α/β production (Figures 5A,B and 6C,D) and exogenous TNF-α only partly abolished the up-regulation of IFN-α production on pDCs after CMRF-35 cross-linking (Figure 6D). Thus, triggering of CMRF-35 also has both direct and indirect effects (by decreasing TNF-α) on the up-regulation of IFN-α.

Discussion

The activation of TLR7 and TLR9 triggers a cascade of signaling pathways to induce type I IFN production after infection with a wide range of viruses.[4,37,38] The expression of CD300a and CD300c on myeloid cells, T cells, and NK cells has been postulated to regulate immune responses.[27,28] In this study, we showed that approximately 92% of blood pDCs and more than 75% of tonsil pDCs express CD300a/c on the surface. The immunoregulatory molecule NKp44, which is expressed on 10% to 50% tonsil pDCs[21] was coexpressed by 28% to 70% of the CMRF-35$^+$ tonsil pDCs,

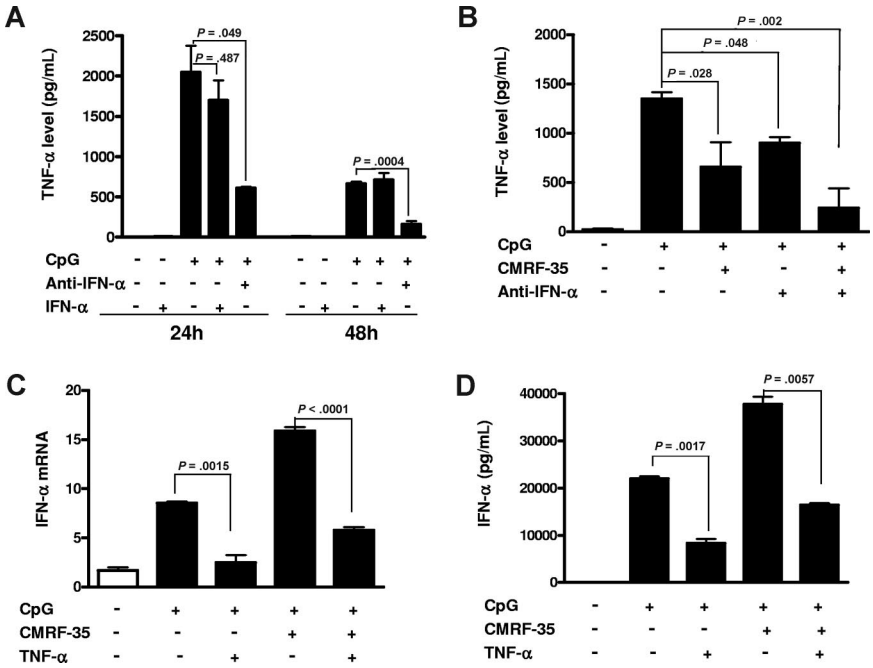

Figure 6. CMRF-35 mAb cross-regulates IFN-α and TNF-α production by pDCs. (A) pDCs are cultured with or without IFN-α neutralizing antibody for 30 minutes, then cells were stimulated with CpG or IFN-α for 24 and 48 hours. TNF-α secretion level was analyzed in the supernatant of cultured pDCs. Data are representative of 3 experiments. (B) pDCs were cross-linked with CMRF-35 antibody for 30 minutes and cultured with or without IFN-α neutralizing antibody for another 30 minutes, then CpG or control ODN was added to the culture. Non–cross-linked pDCs were used as control. TNF-α secretion level was analyzed in the supernatant of cultured pDCs at 48 hours. Data are representative of 3 experiments. (C) pDCs were cultured with or without TNF-α and CpG after cross-linking with CMRF-35 antibody for 48 hours; IFN-α mRNA was measured by real-time PCR. Data are representative of 3 experiments. (D) IFN-α secretion level from pDCs cultured with or without TNF-α and CpG after cross-linking with CMRF-35 antibody for 48 hours was measured by ELISA. Non–cross-linked pDCs were used as control. Data are representative of 3 experiments. Error bars represent SEM.

suggesting distinct CD300a/c and NKp44 functional utilization. The data raise the interesting possibility that tonsil pDCs are a heterogenous population. TLR7 and TLR9 activation led to the down-regulation of CD300a/c and such down-regulation was IFN-α dependent based on 2 observations: first, IFN-α neutralizing antibodies partially prevented CD300a/c down-regulation and, second, exogenous IFN-α decreased CD300a/c expression. Other molecules such as BDCA-2 and ILT7 are also known to be down-regulated in activated pDCs.[16,19]

TLR7/9 ligand-activated pDCs increased their expression of CD80 and CD86. This up-regulation of costimulatory molecules was found to be independent of CD300a/c cross-linking, a result consistent with reports that cross-linking ILT7 or Siglec-H on pDCs plays no role in regulating costimulatory molecule expression.[19,22] Some changes on HLA-DR expression were noted on pDCs after cross-linking of CD300a/c, but such changes were related to reduced TNF-α. When tested in functional assays, CD300a/c cross-linked pDCs had no obvious influence on priming T-cell proliferation. However, our studies made major new findings and establish that the CD300a/c molecules had the capacity to regulate TLR-driven pDC cytokine secretion.

The first significant finding was that CD300a/c cross-linking, in conjunction with the TLR ligands, increased the production and secretion of pDC IFN-α. CD300a and CD300c are the only molecules identified so far to have a positive regulatory function on type I interferon secretion. In contrast, BDCA-2, ILT7, FcεRI, and NKp44 in human pDCs and Siglec-H in mouse pDCs have all been shown to inhibit IFN-α production in pDCs.[16,17,19-21] Furthermore, we found triggering CD300a/c enhanced IRF7 mRNA especially after 15 hours of culture with CpG. However, the transcript for the upstream signaling component IRAK1, which activates IRF7, remained stable after CMRF-35 cross-linking. We do not know whether triggering CD300a/c influences the phosphorylation of IRAK1; thus, we have not excluded the activation of IRAK1 by IRAK4-induced phosphorylation onCD300a/c triggering. Cross-linking of CD300a/c on pDCs also reduced the TNF-α and IL-6 production, similarly to ILT7, emphasizing that their regulatory profile has the potential to produce different immunologic outcomes.

TNF-α plays a role in the regulation of IFN-α secretion in pDCs. Exogenous TNF-α inhibits IFN-α secretion by pDCs.[29] Our data supported and extended previous findings that there is a balance between the IFN-α and TNF-α levels produced by pDCs.[29,41] In this study, we showed that type I IFN favored and sustained the TNF-α production in pDCs as blocking of IFN-α/β signaling, using neutralizing IFN-α antibody and IFN-α/β receptor blocking antibody, inhibited TNF-α production. Similarly, one recent study showed that streptococci induce type I IFN secretion by macrophages and that there is a marked reduction of TNF-α level in macrophages from IFN-α/β receptor-deficient mice after exposure to bacteria.[42] These data indicate type I IFN production as part of the host defense promotes proinflammatory cytokine TNF-α secretion. After pDC activation, TNF-α levels reach a maximum before that of IFN-α, and then the TNF-α levels decline[29] (see also Figure 6A). There is also a reciprocal critical level of TNF-α required to complete pDC final differentiation and maturation that is sustained by IFN-α. On its own, IFN-α is a poor inducer of costimulatory molecules and HLA-DR, but up-regulation of CD80, CD86, and MHC-class II resulted from a combination of IFN-α and TNF-α and produced immunocompetent pDCs[38] (Figure 4D). During this pDC maturation process, some innate receptors, such as BDCA-2 and TLR9, are down-regulated. Here, we show that activated pDCs down-regulate both CD300a and CD300c partially by IFN-α production. However, triggering CD300a/c decreased TNF-α but increased IFN-α production in response to TLR7/9 activation. Interestingly, triggering CD300a/c, whereas simultaneously inhibiting IFN-α/β signaling, further reduced TNF-α levels secreted by activated pDCs. These data suggest the CD300a/c molecules play an important role in balancing pDC IFN-α and TNF-α production in response to TLR activation (Figure 7). It is a complex process in which CD300a/c immunoregulatory molecules are, in turn, regulated by the pDC outputs they themselves regulate.

Virus- or bacteria-induced IFN-α and inflammatory cytokines (such as TNF-α and IL-6) result from different signaling pDC pathways. IFN-α production depends on MyD88-TRAF6-IRAK1-IRF7, but TLR signaling also activates IRF5 and kinase TAK1, inducing the production of TNF-α and IL-6, by the NF-κB signaling pathway.[39] It was shown

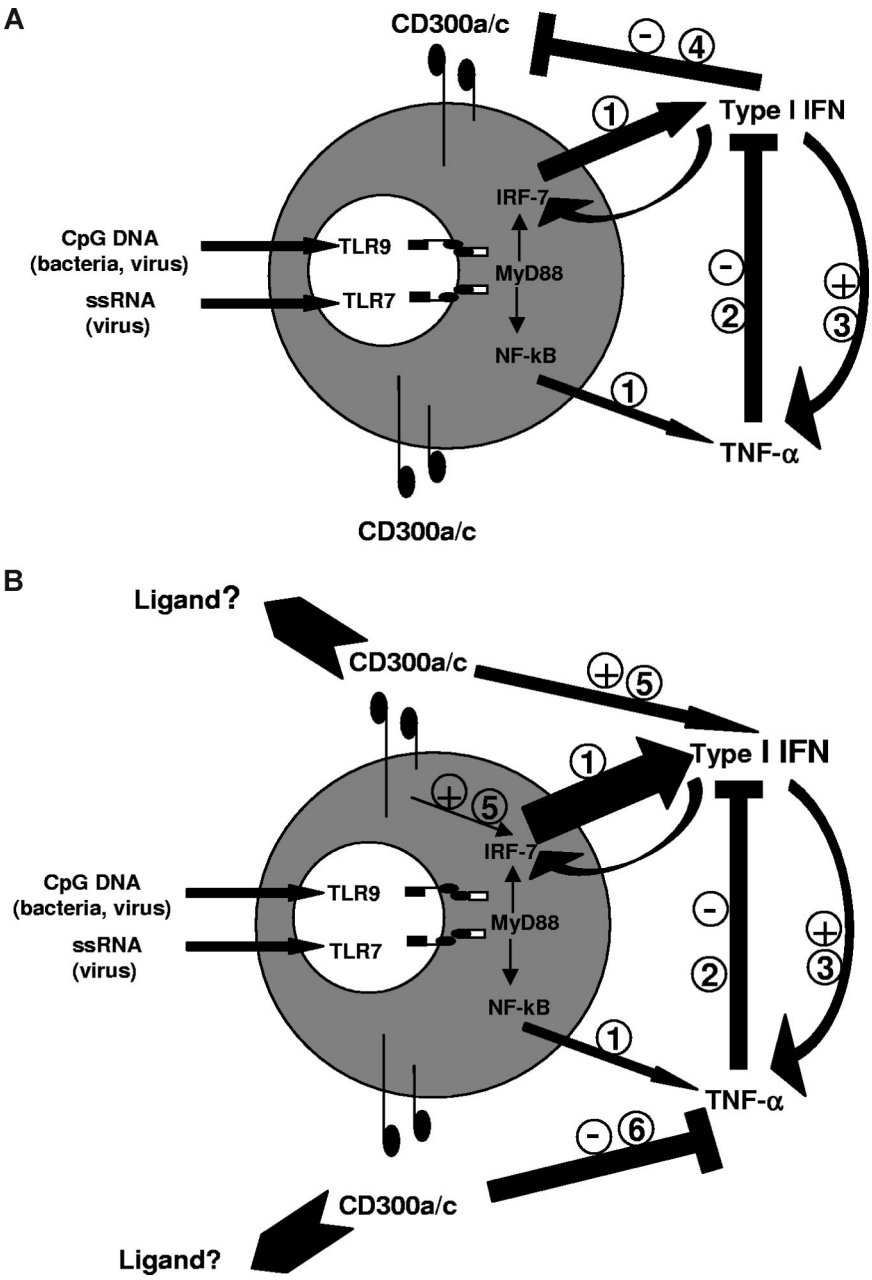

Figure 7. CMRF-35 mAb cross-regulates IFN-α and TNF-α production by pDCs. (A) pDC stimulation with TLR7 ligand ssRNA or TLR9 ligand CpG DNA induces type I interferon and TNF-α secretion (1). Overproduction of TNF-α inhibits secretion of type I interferon (2), whereas a certain level of type I interferon sustains pDC TNF-α production level (3), thus maintaining the balance of type I interferon and TNF-α secretion on pDCs. High levels of type I interferon down-regulate CD300a/c on pDCs (4), indicating regulation of the immunoregulatory molecules in a feedback loop. (B) Cross-linking pDCs with CMRF-35 mAb, which recognizes both CD300a and CD300c, increases IRF7 expression and the secretion of type I interferon (5) and down-regulates TNF-α production (6), indicating the potential of these molecules to be powerful modulators of immune reactions.

that TLR7/9-induced IFN-α production is severely decreased in Iκκα knockout mice[43] and that pDC IFN-α production involves both NF-κB and p38MAPK activities, indicating that cross-talk between these different pathways is likely.[44] Our data showing that IFN-α blockade leads to reduced pDC TNF-α secretion again emphasize this. How CD300a and CD300c regulate the signaling pathway on pDCs after TLR7/9 activation is still unknown. We attempted to use siRNA technology to dissect the individual contributions of CD300a and CD300c; however, to this point, despite our success and that of others with siRNA in down-regulating some gene expression in pDCs,[45] this technology was not selective enough to distinguish between the related CD300 family members.

Elevated IFN-α levels in the peripheral blood of SLE has been found.[41] There is increased expression of IFN-α–responsive genes (*Irf7*, *MxA*) in psoriasis plaque lesions,[12] and local production of TNF-α was identified as crucial for the induction and maintenance phase of psoriatic lesions. This has led to the successful use of TNF-α inhibitors for the treatment of psoriasis.[46] Our findings suggest that disordered function of immune regulator molecules such as CD300a/c may contribute to the down-regulated immune responses producing altered IFN-α/TNF-α balance in these diseases.[1,12,41] Altered regulation of these molecules may also be pertinent to the patients' responses to the conditioning and allogeneic interactions in hematopoietic stem cell transplantations.

Although CD300a and CD300c share 80% sequence similarity across the extracellular Ig domain, these 2 molecules have distinct structures. CD300a contains 3 ITIMs, of which at least one is functional in NK cells, mast cells, and eosinophils.[47-49] CD300c has a short intracellular domain with a charged amino acid in the transmembrane domain, which may be associated with other signaling molecules. Thus, they almost certainly have different intracellular pDC signaling capabilities, although they may share a common ligand. The level of CD300a transcription in pDCs is much higher, so the cross-linking effects described here may reflect

signaling CD300a dominance over CD300c signaling. Although it is clear they can regulate a crucial aspect of pDC function such as cytokine production, the individual contributions of CD300a and CD300c molecules cannot be distinguished with the present reagents. Their future distinction may make the individual CD300a and CD300c regulating roles even more compelling. Thus, 2 challenges remain: first, to define the natural CD300a/c ligands and, second, to generate antibodies or other reagents to specifically trigger or block their signals. Studying CD300a/c influences on the pDCs in psoriasis and SLE will be interesting, but the preclinical models involving human DCs and T cells[1,50] in clinical allogeneic hematopoietic stem cell transplantations are more defined models in which to begin these studies. Long term, targeting CD300a/c may provide opportunities for more subtle therapeutic regulation of the immune responses.

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Authorship

Contribution: X.J. performed most of the technical work and contributed to the experimental design, data interpretation, and paper writing; X.J. and M.Z. performed the microarray studies; D.N.J.H. contributed to the experimental design, data interpretation, and paper writing; and G.J.C. contributed to the technical work and contributed to the experimental design, data interpretation, and paper writing.

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