

Cooperative Regulation of NOTCH1 Protein-Phosphatidylinositol 3-Kinase (PI3K) Signaling by NOD1, NOD2, and TLR2 Receptors Renders Enhanced Refractoriness to Transforming Growth Factor- β (TGF- β)- or Cytotoxic T-lymphocyte Antigen 4 (CTLA-4)-mediated Impairment of Human Dendritic Cell Maturation^{*[5]}

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Dendritic cells (DCs) as sentinels of the immune system are important for eliciting both primary and secondary immune responses to a plethora of microbial pathogens. Cooperative stimulation of a complex set of pattern-recognition receptors, including TLR2 and nucleotide-binding oligomerization domain (NOD)-like receptors on DCs, acts as a rate-limiting factor in determining the initiation and mounting of the robust immune response. It underscores the need for “decoding” these multiple receptor interactions. In this study, we demonstrate that TLR2 and NOD receptors cooperatively regulate functional maturation of human DCs. Intriguingly, synergistic stimulation of TLR2 and NOD receptors renders enhanced refractoriness to TGF- β - or CTLA-4-mediated impairment of human DC maturation. Signaling perturbation data suggest that NOTCH1-PI3K signaling dynamics assume critical importance in TLR2- and NOD receptor-mediated surmounting of CTLA-4- and TGF- β -suppressed maturation of human DCs. Interestingly, the NOTCH1-PI3K signaling axis holds the capacity to regulate DC functions by virtue of PKC δ -MAPK-dependent activation of NF- κ B. This study provides mechanistic and functional insights into TLR2- and NOD receptor-mediated regulation of DC functions and unravels NOTCH1-PI3K as a signaling cohort for TLR2 and NOD receptors. These findings serve in building a conceptual foundation for the design of improved strate-

gies for adjuvants and immunotherapies against infectious diseases.

Dendritic cells (DCs),⁵ important sentinels of innate immunity, possess an array of pattern recognition receptors (PRRs) that include Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Signaling events associated with innate sensors like TLRs and NLRs often act as regulatory circuits that modulate the overall functions of DCs in terms of maturation process, cytokine or chemokine production, receptor expression, and migration to secondary lymphoid organs for antigen presentation for effectuating T helper (Th) cell polarization (1–7). In these attributes, important directives are often composed of sequential and coordinated activation of TLR- and NLR-driven signal transduction pathways, thus exhibiting foremost influence in determining the overall strength of the innate immune responses. Among TLRs as widely reported, TLR2 exhibits a dominant role in sensing various agonists, including pathogen-associated molecular patterns of microbes at the cell surface, and is generally considered as major effector of proinflammatory responses (8–10). Interestingly, NLRs like NOD1 or NOD2 often play a dual role; they regulate anti-inflammatory responses as well as polarization of T cells toward skewed Th2 phenotype (11). This presents an interesting conundrum to the functionality of DCs in terms of their maturation during rapidly evolving immunological processes, including effects originating from immunosuppressive effectors such as CTLA-4 or TGF- β (12–14).

TLR2 receptors, while acting as sensors for extracellular cues or the endocytic network, drive signaling events in response to recognition of pathogen-associated molecular patterns, including mycobacterial antigens like ESAT-6, PE_PGRS antigens;

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⁵ The abbreviations used are: DC, dendritic cell; PRR, pattern recognition receptor; TLR, Toll-like receptor; NLR, NOD-like receptor; MDP, muramyl dipeptide; Treg, regulatory T cell; mTOR, mammalian target of rapamycin; MDP, muramyl dipeptide; NOD, nucleotide-binding oligomerization domain; NICD, NOTCH intracellular domain.

TLR2/NOD Regulate DCs Resistance to TGF- β /CTLA-4

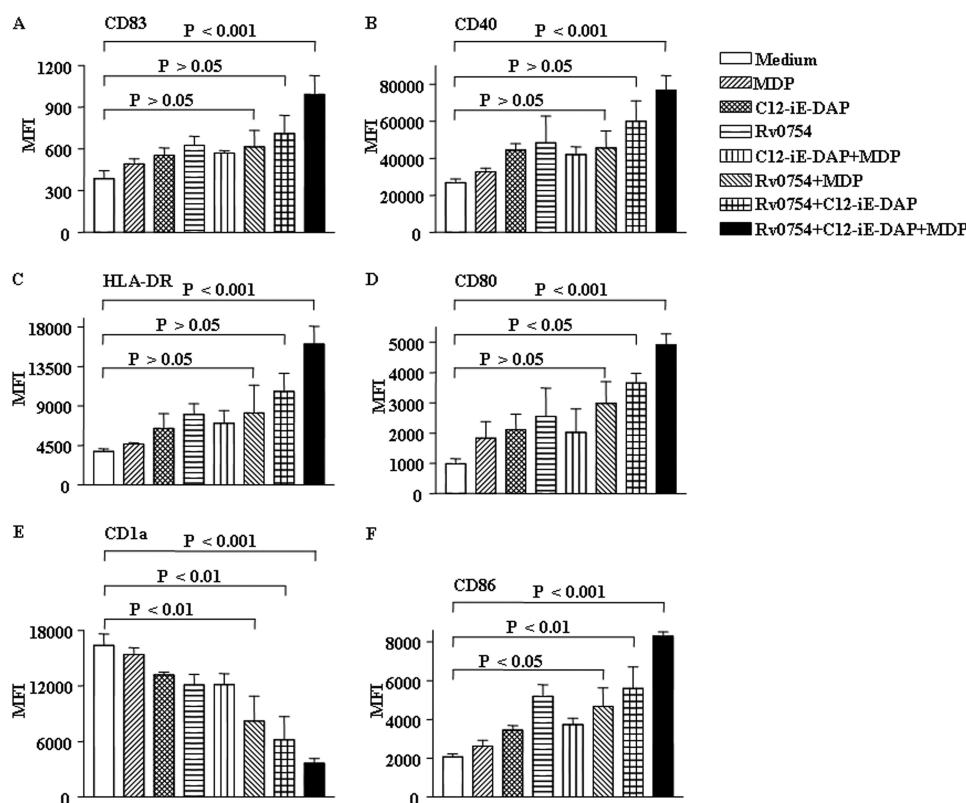


FIGURE 1. **TLR2, NOD1, and NOD2 cooperatively regulate maturation of human DCs.** A–F, immature DCs (0.5×10^6 cells/ml) were cultured with GM-CSF and IL-4 and left untreated (*Medium*) or treated with TLR2 ligand Rv0754 (200 ng/ml) or NOD1 ligand C12-iE-DAP (1 μ g/ml) or NOD2 ligand MDP (1 μ g/ml) as well as with combinations of TLR2, NOD1, and NOD2 ligands for 48 h followed by analysis of the surface expression of maturation markers CD83 (A), CD40 (B), HLA-DR (C), CD80 (D), CD1a (E), and CD86 (F) by flow cytometry. Data are presented as mean fluorescence intensities (MFI) \pm S.E. from six independent donors.

NOD1 and NOD2 operate as cytosolic sensors initiating signaling pathways upon recognition of diaminopimelic acid and muramyl dipeptide (MDP), components of bacterial peptidoglycan (15–20). Although TLR2 or NOD receptor-induced signaling events culminate in activation and nuclear translocation of NF- κ B, transcriptome profiles in response to TLR2 or NOD receptors could markedly diverge. Although TLR2 signaling utilizes MyD88 and TRIF adaptors in executing signaling cascades, NOD receptor oligomerization in conjunction with the adaptor molecule receptor-interacting protein 2 (RIP2), or RICK, triggers signaling assembly, including RIP2 and transforming growth factor- β -activated kinase 1 (TAK1), thus facilitating the activation of NF- κ B (2, 18, 19). Thus, TLR or NOD receptors could trigger similar or contrasting immune responses by cooperative or noncooperative sensing, consequently exhibiting immense complexity during combinatorial triggering of host DC-PRR repertoire (21–24).

In view of these observations, this study comprehensively demonstrates that the maturation processes of human DCs are cooperatively regulated by signaling cascades initiated by engagements of TLR2, NOD1, and NOD2 receptors. Importantly, combined triggering of TLR2 and NOD receptors abolished the TGF- β or CTLA-4-mediated impairment of human DC maturation, which required critical participation of NOTCH1-PI3K signaling cohorts. Using signaling perturbations, we have delineated a unique role for NOTCH1-PI3K-PKC δ -dependent activation of ERK1/2, p38 MAPK, and NF- κ B during TLR2 and NOD receptor-driven maturation of human

DCs. Thus, our data may represent mechanisms by which maturation processes integrate multiple signals from PRRs required for functional maturation of human DCs as well as to impart refractoriness to DCs against various immunosuppressive stimuli.

EXPERIMENTAL PROCEDURES

Generation and Culture of Human DCs—CD14⁺ monocyte-derived human DCs were obtained from healthy donors as described previously (15). Briefly, human peripheral blood mononuclear cells were isolated from buffy coats of healthy donors obtained from Hôpital Hôtel Dieu, Etablissement Français du Sang, Paris, France, upon ethical approval for the use of such materials. Monocytes were isolated from peripheral blood mononuclear cells by immunomagnetic separation with CD14 microbeads (Miltenyi Biotec, France). The purity of the monocytes was >98%. Monocytes were differentiated into immature DCs by culturing them for 7 days in RPMI 1640 medium containing 10% FCS, 50 units/ml penicillin, 50 μ g/ml streptomycin, IL-4 (500 IU/ 10^6 cells), and GM-CSF (1000 IU/ 10^6 cells). Immature DCs were treated with TGF- β (10 ng/ml) or CTLA-4 (1 μ g/ml) along with replenishment of GM-CSF and IL-4 for 6 h followed by culturing them with TLR2 (Rv0754) or NOD ligands (MDP and C12-iE-DAP) for 48 h.

Reagents and Antibodies—Recombinant human IL-4, GM-CSF, and IFN- γ were purchased from ImmunoTools (Friesoythe, Germany). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) to HLA-DR, CD80,

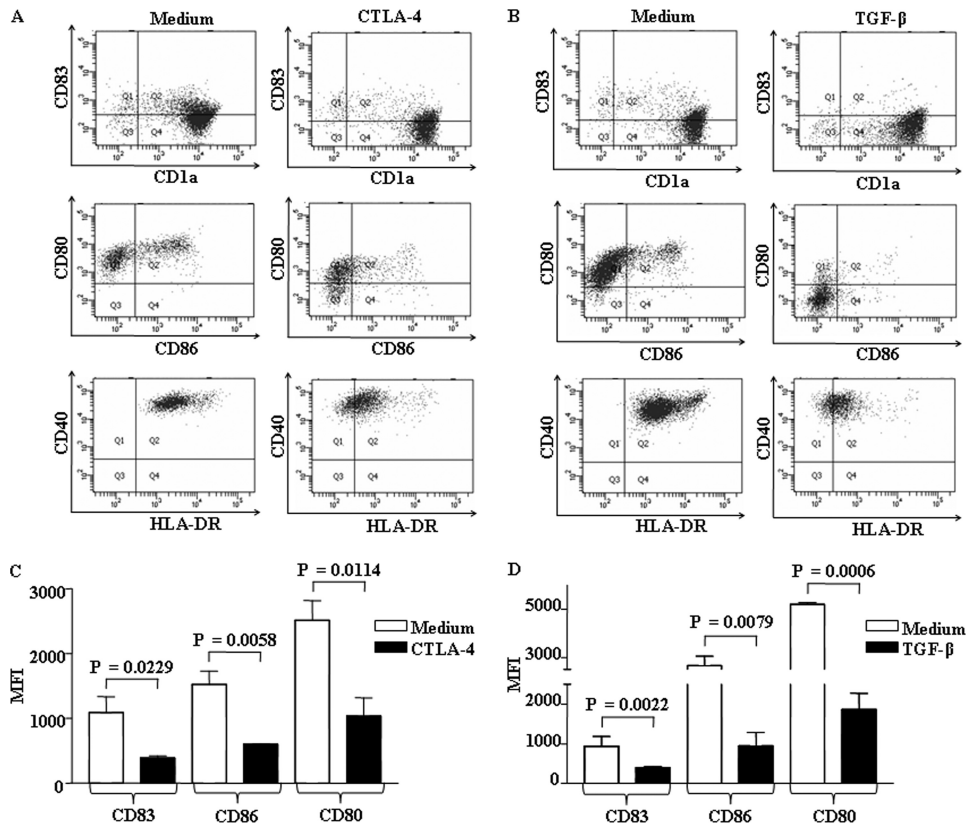


FIGURE 2. **CTLA-4 and TGF-β markedly inhibit human DC maturation.** *A* and *B*, immature DCs were maintained with GM-CSF and IL-4 and were treated with CTLA-4 (1 μg/ml) (*A*), TGF-β (10 ng/ml) (*B*), or left untreated for 48 h, and maturation of human DCs was assayed by flow cytometry for expression of CD83, CD1a, CD80, CD86, CD40, and HLA-DR. Representative dot plots of three independent experiments are shown. *C* and *D*, representative mean fluorescence intensities (MFI) of CD83, CD86, and CD80 expression on the surface of DCs that are treated with either CTLA-4 (1 μg/ml) (*C*) or TGF-β (10 ng/ml) (*D*) are shown. Data in bar diagrams are represented as mean ± S.E. from three independent donors.

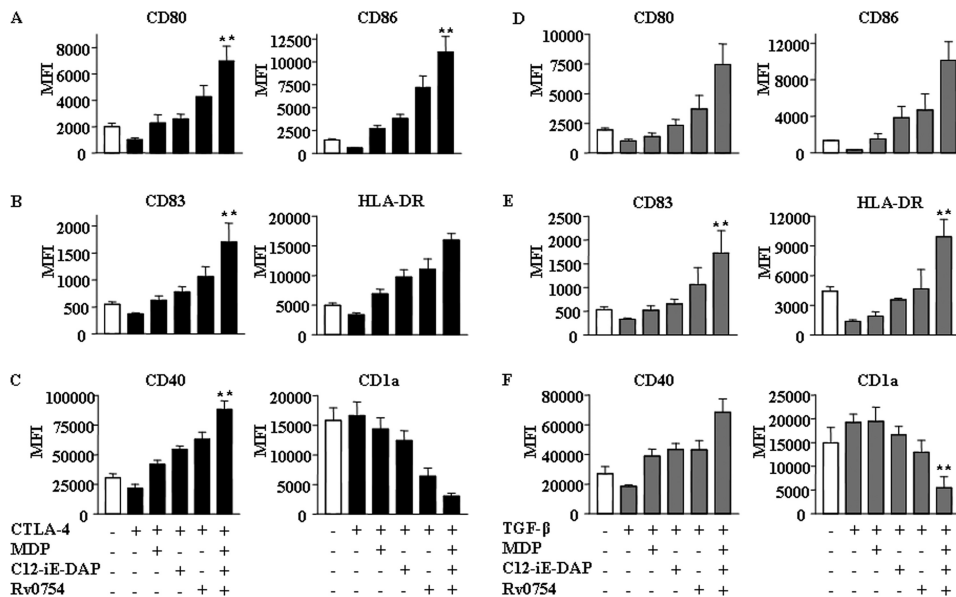


FIGURE 3. **TLR2, NOD1, and NOD2 synergistically surmount CTLA-4- and TGF-β-mediated suppressed maturation of human DCs.** *A–C*, DCs were pretreated with CTLA-4 (1 μg/ml) for 6 h followed by treatment with Rv0754 or C12-iE-DAP or MDP as well as with a combination of Rv0754, C12-iE-DAP, and MDP for an additional 42 h, and expression of maturation markers CD80 and CD86 (*A*), CD83 and HLA-DR (*B*), and CD40 and CD1a (*C*) was analyzed. *D–F*, Rv0754, C12-iE-DAP, and MDP induced synergistic maturation of human DCs under TGF-β-triggered immunosuppressive conditions as analyzed by surface expression of maturation markers CD80 and CD86 (*D*), CD83 and HLA-DR (*E*), and CD40 and CD1a (*F*). Data are presented as mean fluorescence intensities (MFI) ± S.E. from six independent donors. **, $p < 0.05$ versus CTLA-4 or TGF-β.

and CD1a and phycoerythrin-conjugated mAbs to CD40, CD86, and CD83 were from BD Biosciences. The anti-Ser-65 p4EBP1, anti-Thr-180/Tyr-182 pp38 MAPK, anti-Thr-202/

Tyr-204 pERK1/2, anti-NF-κB p65, anti-cleaved NOTCH1 (NOTCH intracellular domain (NICD)), anti-Ser-9 pGSK-3β, anti-Thr-505 pPKCδ, anti-Thr-389 pp70 ribosomal protein S6

TLR2/NOD Regulate DCs Resistance to TGF- β /CTLA-4

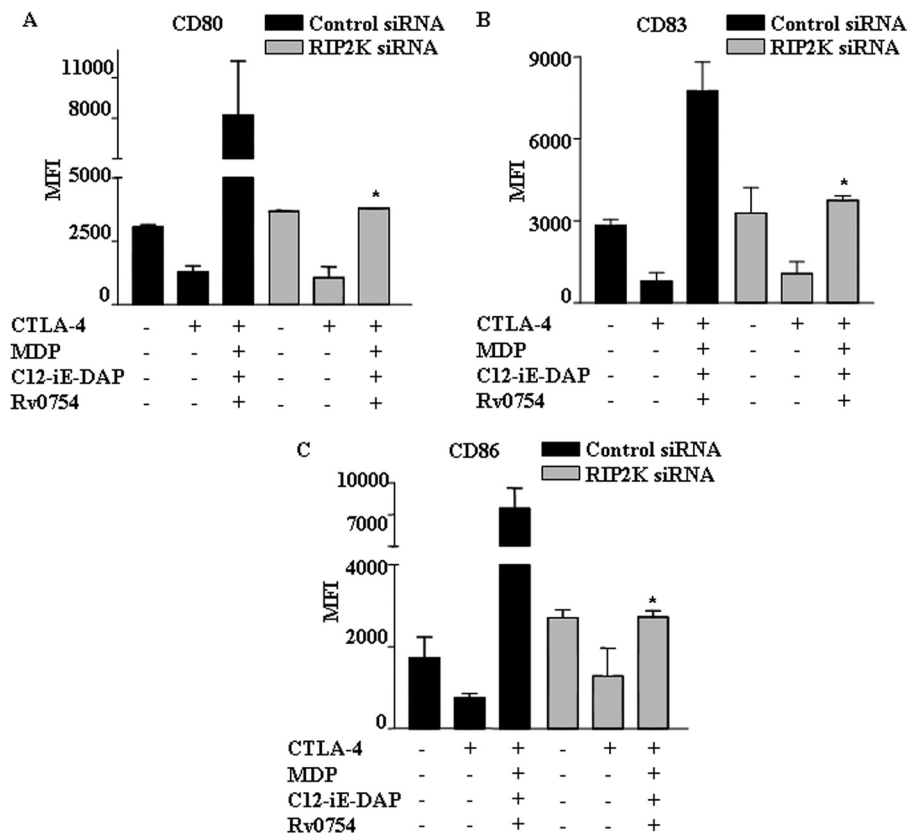


FIGURE 4. **NOD signaling collaborates with TLR2 to subvert CTLA-4- and TGF- β -induced suppression of human DC maturation.** A–C, human DCs were transfected with RIP2K siRNA or control siRNA at a final concentration of 100 nm. After 72 h, DCs were treated with CTLA-4 for 6 h, and human DC maturation was assayed by flow cytometry by monitoring human DC maturation markers as follows: CD80 (A), CD83 (B), and CD86 (C). Data represent mean \pm S.E. from three independent donors. *, $p < 0.05$ versus CTLA-4 and MDP-C12-iE-DAP-Rv0754.

kinase (pp70 S6K), and anti-Ser-2448 pmTOR were purchased from Cell Signaling Technology, and anti- β -actin antibody (AC-15) was procured from Sigma. NOTCH1, RIP2K, and siGLO Lamin A/C control siRNAs were purchased from Dharmacon as siGENOMETM SMARTpool reagent, which contains a pool of four different double-stranded RNA oligonucleotides (siRNA). Oligofectamine transfection reagent was obtained from Invitrogen.

Expression and Purification of Rv0754—Rv0754 was PCR amplified from *Mycobacterium tuberculosis* H37Rv genomic DNA using the gene-specific primers 5'-CGGGATCCATGT-CATTTGTGATCGTGGCG-3' (forward) and 5'-CCCA-AGCTTTCATGGGATCAGGCTGGGCG-3' (reverse). The amplified PCR product was cloned into the pGEMT-Easy vector (Promega), and the recombinant clones carrying the appropriate gene insert were confirmed by DNA sequencing. The Rv0754 gene insert was subcloned into pRSET series of vectors for protein expression and purification. *Escherichia coli* BL21 cells carrying recombinant plasmids were induced with isopropyl β -D-thiogalactopyranoside, and His-tagged recombinant Rv0754 was purified with nickel-nitrilotriacetic acid columns (Qiagen).

Flow Cytometric Analysis of DC Maturation Markers—Cell surface staining for maturation markers of DCs was performed with specifically labeled mAbs, and samples were analyzed by processed flow cytometry (LSR II, BD Biosciences). For each sample, five thousand events were recorded. Data were analyzed using FACSDIVA software (BD Biosciences).

Mixed Lymphocyte Reaction—CD4⁺ T cells used in allogeneic mixed lymphocyte reactions were isolated from peripheral blood mononuclear cells of healthy donors by immunomagnetic separation using CD4-conjugated microbeads (Miltenyi Biotec). After 48 h of treatment, DCs were washed extensively and were co-cultured with 1×10^5 responder allogeneic CD4⁺ T cells at DC/T cell ratios of 1:20, 1:40, and 1:80. After 4 days of co-culture, cells were pulsed with 0.5 μ Ci of [³H]thymidine for 16 h. The proliferation of T cells was analyzed by radioactive incorporation using standard liquid scintillation counting. The proliferation of cells was measured as counts/min (cpm) (mean \pm S.E. of quadruplicate values) after subtracting values of responder T cell cultures alone.

Analysis of Cytokines—Cytokines were quantified in cell-free culture supernatants using CBA human inflammation kit (BD Biosciences).

Treatment of DCs with Pharmacological Inhibitors of Signaling Pathways—The pharmacological inhibitors used in the study were purchased from Calbiochem and were reconstituted in sterile cell-culture grade DMSO (Sigma). DMSO was used as vehicle control in experiments involving utilization of pharmacological inhibitors. The following concentrations of each inhibitor were used after determining the viability of DCs in titration experiments using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay: GSI-I (1 μ M), LY294002 (50 μ M), rapamycin (100 nM), PKC α inhibitor (Safingol) (50 μ M), PKC β inhibitor (50 μ M), PKC δ inhibitor (Rottlerin) (10

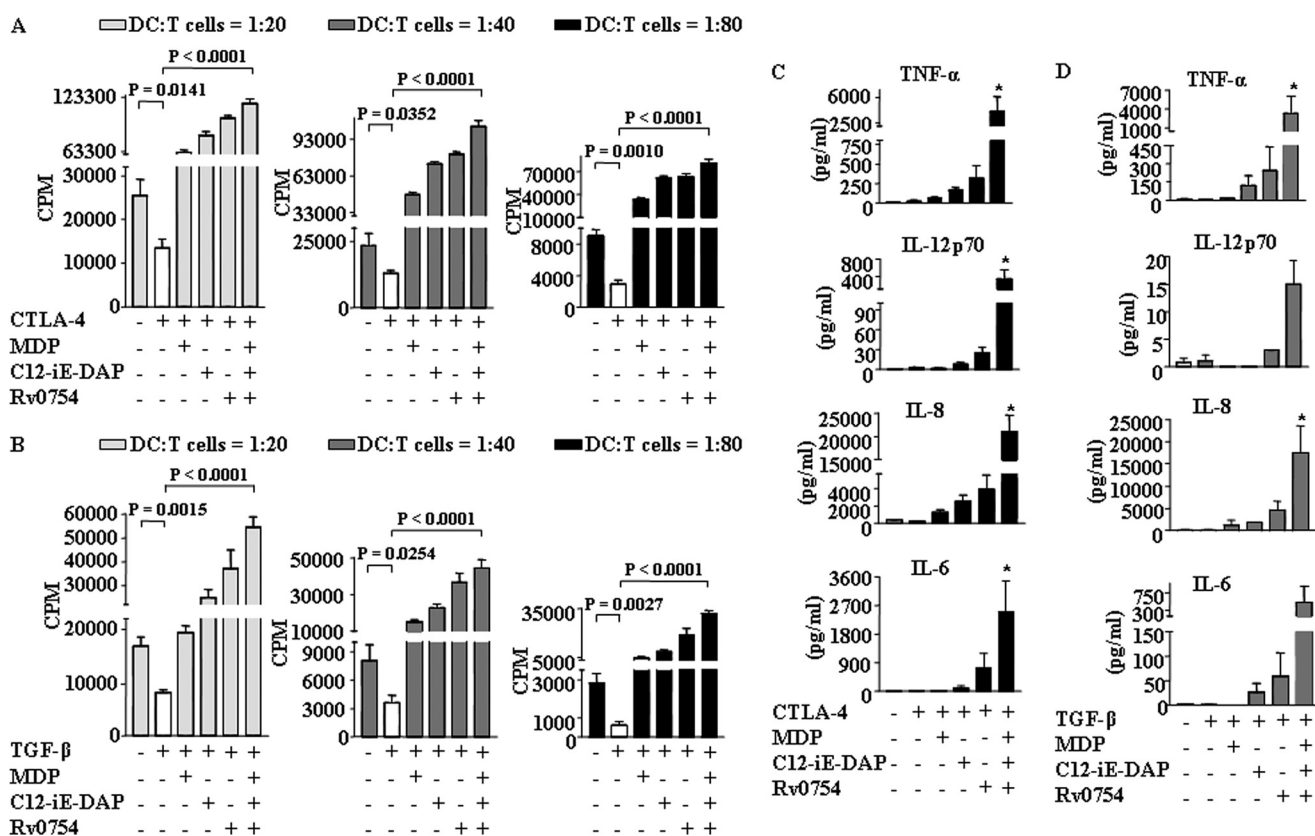


FIGURE 5. Synergistic activation of TLR2, NOD1, and NOD2 renders human DCs to trigger strong T cell response as well as to secrete pro-inflammatory cytokines under CTLA-4- and TGF- β -induced immunosuppressive conditions. A and B, DCs were treated as indicated in Fig. 3, A–F, and were co-cultured with allogenic CD4⁺ T cells at different DC to T cell ratios. After 4 days of co-culture, cells were pulsed overnight with 0.5 μ Ci of [³H]thymidine to quantify T cell proliferation. Radioactive incorporation was expressed as counts/min (mean \pm S.E. of quadruplet values). Data are presented as mean \pm S.E. from four independent donors. C and D, DCs were cultured with GM-CSF and IL-4 followed by treatment with CTLA-4 (1 μ g/ml) (C) or TGF- β (10 ng/ml) (D) for 6 h. DCs were further treated for 42 h with Rv0754 or C12-iE-DAP or MDP alone as well as with a combination of Rv0754, C12-iE-DAP, and MDP and secretion of IL-6, IL-8, IL-12p70, and TNF- α in cell-free culture supernatants was analyzed. *, $p < 0.05$ versus CTLA-4 or TGF- β .

μ M), PKC ϵ inhibitor (V2 peptide) (50 μ M), PKC ζ inhibitor (PKC ζ pseudosubstrate inhibitor, myristoylated) (20 μ M), U0126 (10 μ M), SB203580 (1 μ M), SP600125 (10 μ M), Bay11-7082 (20 μ M); DMSO at 0.1% concentration was used as the vehicle control. Immature DCs were treated for 1 h prior to DC challenge with TLR2 and NOD ligands. Specificity of given pharmacological inhibitor was addressed by treating human DCs with the respective inhibitor and looking for abrogation of its effector molecules.

Immunoblotting Analysis—Cells were lysed in 1 \times RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μ g/ml each aprotinin, leupeptin, pepstatin, 1 mM Na₃VO₄, 1 mM NaF) after washing briefly with ice-cold PBS. Equal amounts of proteins were resolved on SDS-PAGE followed by transfer of proteins to polyvinylidene difluoride membranes (Millipore). After blocking with 5% nonfat dried milk in TBST buffer (0.02 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.1% Tween 20), membranes were probed with primary antibodies overnight at 4 $^{\circ}$ C. After washing with TBST, membranes were incubated with secondary antibody linked to HRP (Jackson ImmunoResearch). The blots were then developed with an enhanced chemiluminescence detection system (PerkinElmer Life Sciences) as per the manufacturer's instructions.

Nuclear and Cytosolic Subcellular Fractionation—DCs were cultured in 35-mm dishes and treated as indicated. After treatment, cells were washed twice with ice-cold PBS followed by resuspension in ice-cold Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF). After incubation on ice for 15 min, cell membranes were disrupted with 10% Nonidet P-40, and the nuclear pellets were recovered by centrifugation at 13,000 rpm for 15 min at 4 $^{\circ}$ C. The supernatants from this step were used as cytosolic extracts. Nuclear pellets were lysed with ice-cold Buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF), and nuclear extracts were collected after centrifugation at 13,000 rpm for 20 min at 4 $^{\circ}$ C.

Transfection Studies—Human DCs were transfected with NOTCH1, RIP2K, or control siRNA at a final concentration of 100 nM using Lipofectamine (Invitrogen) as the transfection agent as per manufacturer's instructions. Transfection efficiency was determined by counting the number of siGLO Lamin A/C (Dharmacon)-positive cells in a microscopic field using a fluorescent microscope. Transfection efficiency was more than 50% through all the experiments. After 72 h, DCs were treated with either CTLA-4 or TGF- β for 6 h followed by stimulation with TLR and NLR agonists and processed for expression analysis.

TLR2/NOD Regulate DCs Resistance to TGF- β /CTLA-4

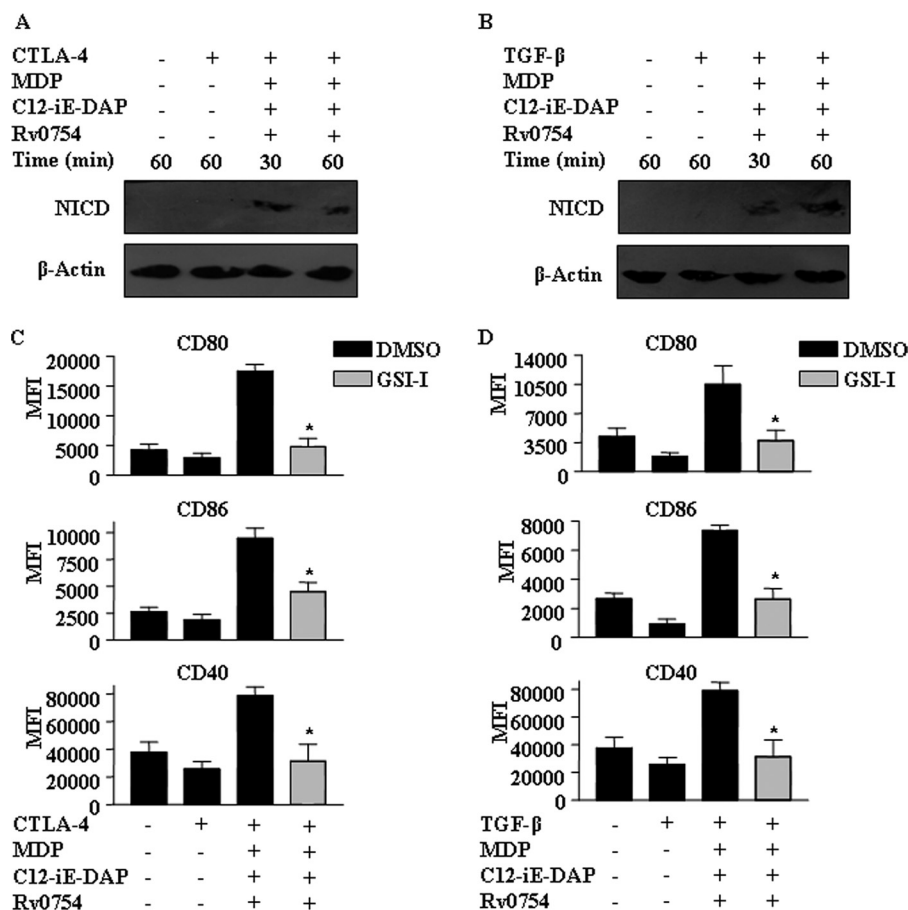


FIGURE 6. Critical role for NOTCH1 signaling in TLR2-, NOD1-, and NOD2-mediated maturation of human DCs under CTLA-4- and TGF- β -induced immunosuppressive conditions. *A* and *B*, immature DCs were pretreated with CTLA-4 (1 μ g/ml) (*A*) or TGF- β (10 ng/ml) (*B*) for 6 h followed by treatment with combination of Rv0754, MDP, and C12-iE-DAP for 30 or 60 min, and activation of NOTCH1 (NICD) was analyzed by immunoblotting. *C* and *D*, pretreatment with GSI-I abrogates Rv0754, MDP, and C12-iE-DAP-triggered maturation of DCs under CTLA-4-induced (*C*) or TGF- β -induced (*D*) immunosuppressive conditions as assessed by analysis of surface expression of maturation markers CD80, CD86, and CD40. The immunoblots are representative of three independent experiments, and *bar diagrams* represent data as mean \pm S.E. from three independent donors. *, $p < 0.05$ versus CTLA-4 and MDP-C12-iE-DAP-Rv0754 or TGF- β and MDP-C12-iE-DAP-Rv0754.

Statistical Analysis—Levels of significance for comparison between samples were determined by the Student's *t* test distribution. The data in the graphs is expressed as the mean \pm S.E. GraphPad Prism 3.0 software (GraphPad software) was used for all statistical analyses.

RESULTS

TLR2 and NOD Receptors Cooperatively Regulate Maturation of Human DCs—Immature DCs (0.5×10^6 /ml) were cultured with agonists for TLR2, Rv0754 (200 ng/ml), NOD1, and C12-iE-DAP (1 μ g/ml), or NOD2, MDP (1 μ g/ml) for 48 h, and expression of various surface markers on cells was analyzed by flow cytometry. We have previously demonstrated that Rv0754, a prototype member of the PE_PGRS family of *M. tuberculosis* recognizes TLR2 and induces maturation and activation of human DCs (15). Furthermore, NOD1 and NOD2 agonists in concert with TLRs have been shown to direct Th1 lineage commitment of ensuing immune responses (6). In this perspective, as a first step, we studied the maturation process of human DCs initiated by engagements of TLR2, NOD1, and NOD2 receptors. We have utilized the above-mentioned concentrations of receptor agonists after carrying out titration analysis. Although TLR2 agonists could trigger expression of maturation markers,

concomitant engagement of TLR2 and NOD receptors induced robust maturation of human DCs as evaluated by significantly increased expression of co-stimulatory molecules CD80, CD86, and CD40, antigen presenting molecule HLA-DR, and DCs terminal maturation marker CD83 along with simultaneous decrease in the expression of DCs differentiation marker CD1a (Fig. 1, *A–F*, and [supplemental Fig. 1, A–F](#)). Furthermore, the combination of TLR2, NLR1, and NLR2 agonists compared with individual agonists or TLR2 and NLR1 or TLR2 and NLR2 agonists significantly enhanced the maturation of human DCs. In these experiments, we substantiated that the stimulatory effects of Rv0754 protein on DCs were not due to endotoxin or LPS contamination in the protein preparations. For all the experiments, we have used agonist preparations that were passed through a polymyxin B-agarose column. Accordingly, we could not detect endotoxins in agonist preparations as analyzed by E-Toxate kit (Sigma). Furthermore, as demonstrated previously, unrelated mycobacterial lipase protein produced and processed by the same procedure did not demonstrate the ability to induce expression of maturation markers on DCs (15). Significantly, treatment of Rv0754 with proteinase K abolished the ability of Rv0754 to trigger maturation of DCs suggesting

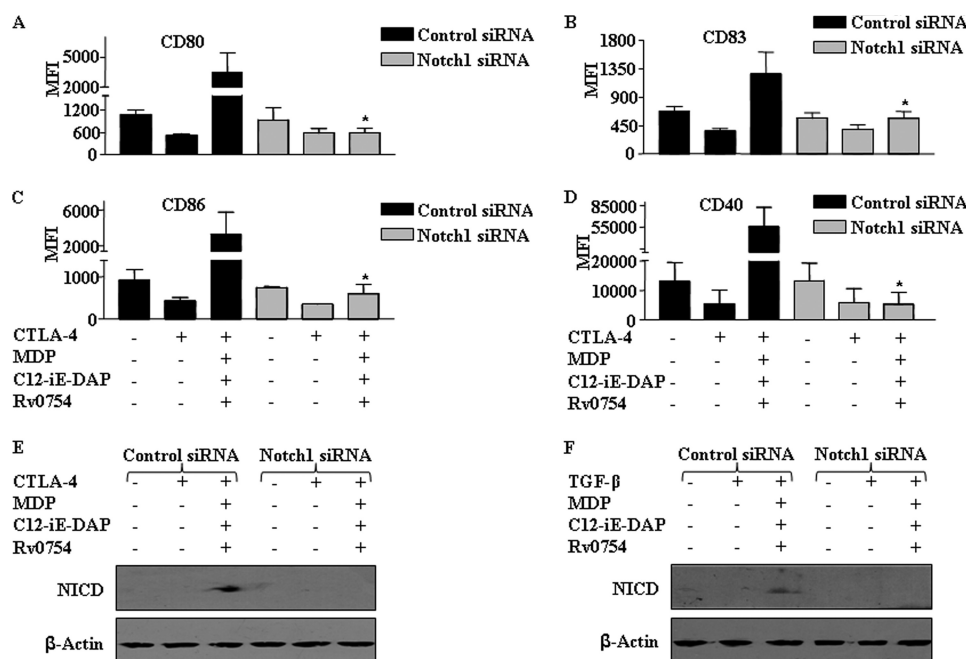


FIGURE 7. NOTCH1 signaling axis controls TLR2-, NOD1-, and NOD2-triggered maturation rescue of human DCs under CTLA-4- and TGF- β -triggered immunosuppressive conditions. A–D, expression of NOTCH1 in human DCs was knocked down by transfecting human DCs with NOTCH1 siRNA at a final concentration of 100 nM. After 72 h, DCs were treated with CTLA-4 for 6 h, and human DC maturation was assayed by flow cytometry for the expression of CD80 (A), CD83 (B), CD86 (C), and CD40 (D). Data are presented as means \pm S.E. from three independent donors. *, $p < 0.05$ versus CTLA-4 and MDP-C12-iE-DAP-Rv0754. E and F, inactivation of NICD formation in NOTCH1 siRNA-transfected human DCs is shown under either CTLA-4-mediated (E) or TGF- β -mediated (F) suppressive conditions using Western blotting. Blots are representative of three independent experiments.

the requirement of intact protein in its native form for inducing the maturation of DCs (15).

Cooperative Stimulation by TLR2 and NOD Receptors Renders Enhanced Refractoriness to TGF- β - or CTLA-4-mediated Impairment of Human DC Maturation—DC activation and subsequent maturation are often tightly modulated by several factors, including TGF- β , CTLA-4, and tumor-derived or microbially derived factors (12–14). Importantly, several studies have clearly established an inhibitory role for TGF- β in DC maturation as DCs derived in the presence of TGF- β exhibited significant reductions in IL-12/IL-23 secretion with concomitant induction of Foxp3⁺ regulatory T cells (Tregs) as well as T cell anergy (13, 14, 25, 26). Similarly, CTLA-4 expressed by Tregs, in addition to inhibiting direct T cell activation, strongly inhibits T cell-mediated immunity by interaction with B7 molecules (CD80 and CD86) expressed by DCs (12, 27–29). Thus, a dichotomous engagement and bidirectional effect of CTLA-4 on T cells and B7 molecules on DCs effectively inhibit the initiation as well as ongoing immune responses. In view of these observations, we attempted to explore whether concomitant engagement of NOD1, NOD2, and TLR2 renders enhanced refractoriness to CTLA-4- or TGF- β -mediated impairment of human DC maturation. As shown in Fig. 2, A–D, CTLA-4 and TGF- β markedly inhibited DC maturation as evaluated by the expression of various maturation markers, including CD80, CD86, CD40, HLA-DR, and CD83. Importantly, synergistic activation of TLR2, NOD1, and NOD2 reversed the inhibitory effects of CTLA-4 and TGF- β on maturation of DCs (Fig. 3, A–C and D–F). Although TLR2 triggering by Rv0754 demonstrated significant rescue, cooperative NLR engagement with respective agonists, C12-iE-DAP (NOD1) and MDP (NOD2),

significantly surmounted CTLA-4- and TGF- β -mediated suppression of DC maturation (Fig. 3, A–C and D–F). The activation of NOD receptors leads to recruitment and association with RIP2 kinase (RIP2K) through CARD-CARD domain interaction, and Glu-69, Asp-70, and Glu-71 amino acid residues of CARD domain of NOD2 are critical for mediating NOD2 interaction with RIP2K (30–33). Thus, RIP2K forms a crucial link in signal transduction downstream of NLR2 (34, 35). From this perspective, we have addressed critical involvement of NLR2 signaling to surmount CTLA-4- or TGF- β -mediated DC maturation in combination with TLR2 signaling. Importantly, NLR2 signaling is critical as shown in Fig. 4, where signaling perturbation of NLR2 by RIP2K siRNA markedly inhibited the ability of TLR2 agonists to subvert CTLA-4- and TGF- β -induced suppression of human DC maturation (Fig. 4, A–C, and supplemental Fig. 4).

As described, compared with individual agonists, the combination of TLR and NLR agonists augmented robust rescue of DC maturation from inhibitory effects mediated by CTLA-4 and TGF- β (Figs. 1 and 3). These data clearly advocate a decisive role for NLR2 signaling to cooperate with TLR2 signaling to impart enhanced refractoriness to human DCs.

As reported, CTLA-4 or TGF- β modulate and prime the generation of tolerogenic DCs, which possess the ability to suppress a wide range of effector T cell responses and enhance Treg generation (36). In this regard, we assessed a key characteristic of DCs, the ability to prime T cells in terms of activation and proliferation of CD4⁺ T cells at a very low stimulator to responder ratio. As shown in Fig. 5, A and B, CTLA-4 and TGF- β treatment severely repressed CD4⁺ T cell proliferation in an allogeneic mixed lymphocyte reactions as analyzed by

TLR2/NOD Regulate DCs Resistance to TGF- β /CTLA-4

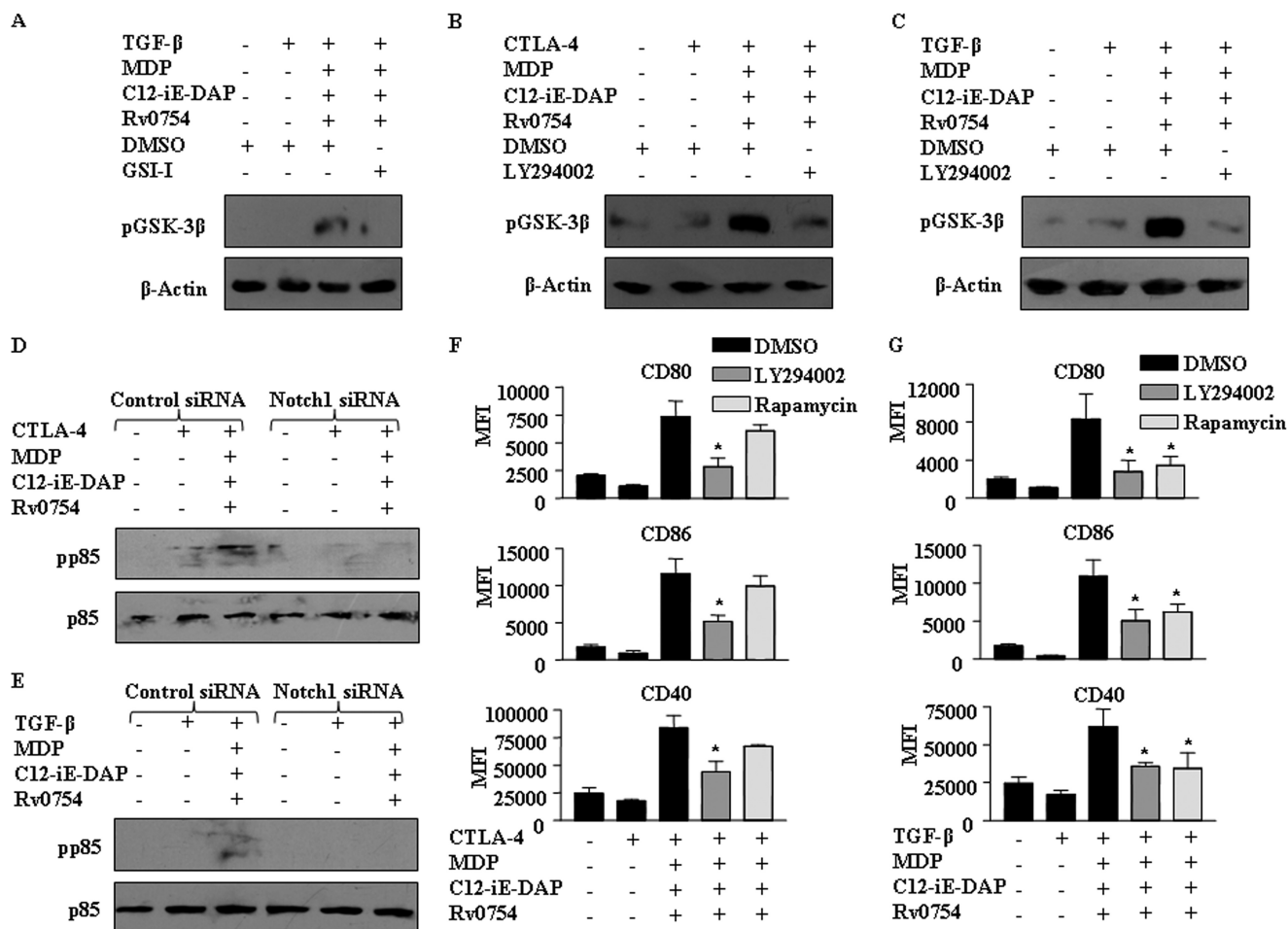


FIGURE 8. NOTCH1-PI3K signaling axis regulates TLR2-, NOD1-, and NOD2-triggered maturation rescue of human DCs. A–C, pretreatment of DC with GSI-I (γ -secretase inhibitor) (A) or LY294002 (B and C) abolishes Rv0754-, MDP-, and C12-iE-DAP-induced phosphorylation of GSK-3 β (pGSK-3 β) in DCs under CTLA-4-induced (B) or TGF- β -induced (A and C) immunosuppressive conditions. D and E, abrogation of NOTCH1 signaling by transfecting human DCs with NOTCH1 siRNA at a final concentration of 100 nM inhibit PI3K pathway activation under either CTLA-4-mediated (D) or TGF- β -mediated (E) suppressive conditions. PI3K pathway activation was assayed by phosphorylation of p85 using Western blotting. Blots are representative of three separate experiments. F and G, LY294002 or rapamycin inhibits Rv0754-, MDP-, and C12-iE-DAP-induced maturation of human DCs under CTLA-4-induced (F) or TGF- β -induced (G) immunosuppression. The immunoblots represent three independent experiments, and data in bar diagrams are represented as means \pm S.E. from three independent donors. *, $p < 0.05$ versus CTLA-4 and MDP-C12-iE-DAP-Rv0754 or TGF- β and MDP-C12-iE-DAP-Rv0754.

[3 H]thymidine incorporation. In accordance with previous results on maturation markers on DCs (Figs. 1 and 3), TLR2 and NOD receptor engagements restored the CD4 $^+$ T cell proliferations from CTLA-4- or TGF- β -mediated suppression (Fig. 5, A and B). In concordance with these data, CTLA-4 or TGF- β treatment compromised the ability of DC to secrete TNF- α , IL-6, IL-8, and IL-12, and TLR2 and NOD2 agonists reinstated the capacity of DC to secrete these cytokines in presence of CTLA-4 or TGF- β (Fig. 5, C and D).

NOTCH1-PI3K Signaling Dynamics Integrated into Signaling Cohorts That Influence TLR2 and NOD Receptor-triggered Maturation of Human DCs—The maturation of DCs often involves the spectrum of cellular signaling events, including TLR2-dependent activation of NOTCH signaling, which is suggested to play an important role in critical cell fate decisions during DC maturation and subsequent priming of effector T cell responses (37, 38). In this regard, we and others have previously shown that TLR2 stimulation leads to up-regulation of NOTCH1 and activation of the NOTCH1 signaling pathway by inducing the formation of a cleavage product of NOTCH1

(NICD) as well as robust activation of Jagged1 expression, a NOTCH1 receptor ligand (39–44). From this perspective, we addressed whether the ability of TLR2 NOD receptors to surmount the CTLA-4- and TGF- β -mediated suppression of DC maturation requires the involvement of activated NOTCH1 signaling. Significantly, TLR2 and NOD receptor agonists triggered the activation of NOTCH1 signaling under CTLA-4- or TGF- β -induced immunosuppressive conditions as evaluated by the formation of NICD (Fig. 6, A and B). Importantly, signaling perturbations with NOTCH1 activation inhibitor GSI-I or by NOTCH1-specific siRNA markedly inhibited DC maturation as evaluated by the surface expression of a multitude of DC maturation markers, including CD80, CD86, CD83, CD40, and HLA-DR during TLR2, and NOD receptors mediated the reversal of the inhibitory of effects of CTLA-4 and TGF- β (Figs. 6, C and D, and 7, A–D, and supplemental Fig. 2).

In addition, NOTCH1-specific siRNA markedly inhibited TLR2- and NLR-triggered activation of NOTCH1 signaling as evaluated by generation of NICD during rescue from immune suppression mediated by CTLA-4 and TGF- β (Fig. 7, E and F).

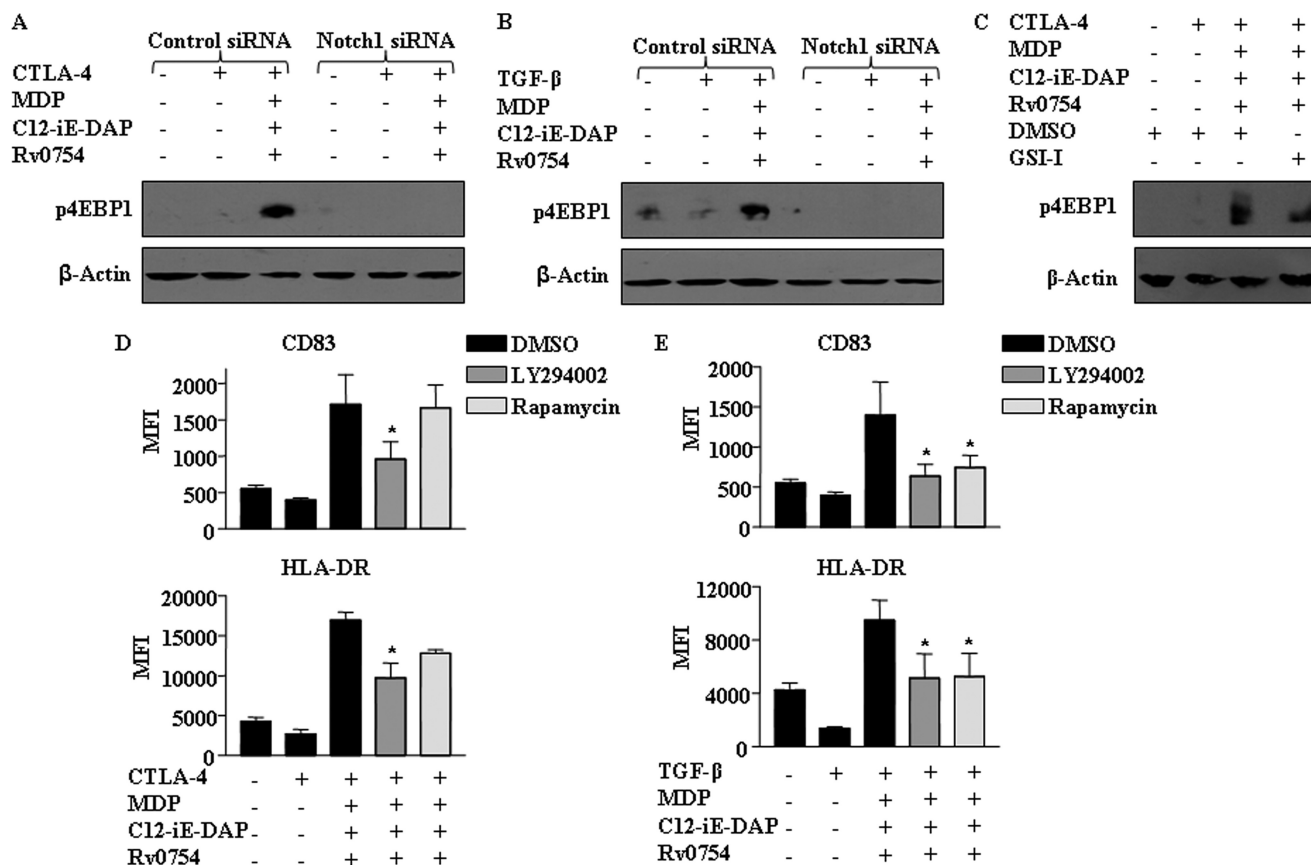


FIGURE 9. **Cross-talk of NOTCH1-PI3K signaling pathways during TLR2-, NOD1-, and NOD2-triggered maturation of human DCs.** A and B, human DCs were transfected with NOTCH1 siRNA at a final concentration of 100 nM. After 72 h, DCs were treated with CTLA-4 (A) or TGF- β (B) for 6 h and subsequently with a combination of TLR and NLR agonists. Activation of PI3K pathway by means of 4EBP1 phosphorylation was addressed by Western blotting. Blots are representative of three independent experiments. C, pretreatment with GSI-I abrogates Rv0754-, C12-iE-DAP-, and MDP-triggered phosphorylation of 4EBP1 under CTLA-4-induced immunosuppression of human DCs. D and E, inhibition of PI3K signaling axis by LY294002 or rapamycin curtails Rv0754-, C12-iE-DAP-, and MDP-triggered maturation of human DCs under CTLA-4-induced (D) or TGF- β -induced (E) immunosuppression. Immunoblot represents three independent experiments, and bar diagrams are representing mean \pm S.E. from three independent donors. *, $p < 0.05$ versus CTLA-4 and MDP-C12-iE-DAP-Rv0754 or TGF- β and MDP-C12-iE-DAP-Rv0754.

To address the involvement of different NOTCH ligands in our studies, we carried out expression level analysis of NOTCH ligands during TLR2 and NLR agonist stimulation of human DCs in the presence or absence of CTLA-4 and TGF- β treatment. As shown in supplemental Fig. 2, TLR2 and NLR stimulation significantly augmented expression levels of the DLL4 ligand of NOTCH receptor as well as expression of DLL1, DLL3, JAG1, and JAG2.

In addition to NOTCH signaling, a diverse set of signaling events, including the PI3K/AKT and MAPK pathways, as well as the active heterodimer p50/p65 form of nuclear factor- κ B (NF- κ B), have been suggested to play a central role in maturation of DCs by inducing expression of a variety of genes involved in maturation processes (45–48). In this regard, engagement of TLR2 and NOD receptors triggered the activation of the PI3K pathway under CTLA-4- or TGF- β -triggered immunosuppressive conditions as evaluated by phosphorylation status of p85, GSK-3 β , and 4EBP1 (Figs. 8, A–E, and 9, A–C). Significantly, inhibition of NOTCH1 signaling activation by NOTCH1-specific siRNA interference or by pharmacological inhibitor GSI-I, members of the PI3K pathway, PI3K by LY294002 and mTOR by rapamycin, abolished TLR2 and NOD receptor-triggered activation of p85 and GSK-3 β (Figs. 8, A and

E, and 9, A–C). Furthermore, inhibition of PI3K or mTOR abolished the ability of the TLR2 and NOD receptors to suppress the inhibitory effects of CTLA-4 and TGF- β on DC maturation (Figs. 8, F and G, and 9, D and E). Reports have suggested the activation of AKT by mTOR via a feedback activation loop. On the contrary, studies have also suggested the direct regulation of mTOR activity by NOTCH signaling and thus uncoupling NOTCH signaling from the AKT pathway (49). We have shown earlier that NOTCH1 can directly regulate PLD1, whose product phosphatidic acid can directly regulate the NOTCH1-responsive gene *SOCS3* (42). Importantly, this study clearly depicts a role for PI3K/AKT in TLR2-NLR-mediated DC maturation (Figs. 8 and 9 and supplemental Fig. 6). In the current stage, we are unable to distinguish the relative contributions of AKT activation either by PI3K or mTOR.

Integration of PKC-MAPK-NF- κ B Signaling Pathways during TLR2 and NOD Receptors Induced Maturation of Human DCs—Innate immune responses of DCs involving innate receptors frequently involve regulatory kinases that play a crucial role either downstream or upstream of MAPKs (48), and in this regard; characterization of signaling partners of NOTCH1-PI3K axis during DC maturation assumes critical importance. Significantly, PKCs are important kinases that often effectuate

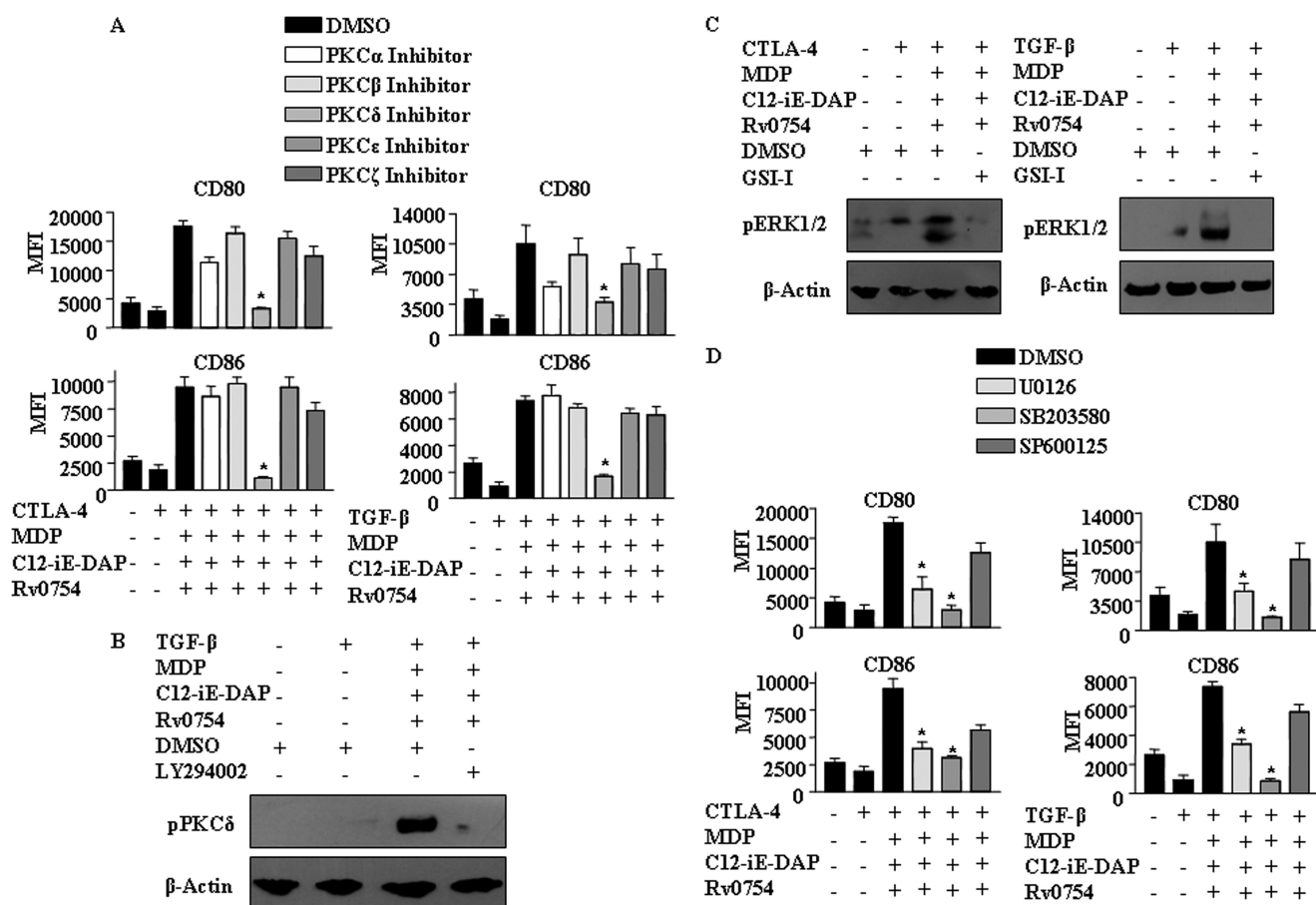


FIGURE 10. Signaling integration through cross-talk of NOTCH1, PI3K, PKC, and MAPK during TLR2-, NOD1-, and NOD2-triggered maturation of human DCs. *A*, inhibition of PKC δ abolishes Rv0754-, MDP-, and C12-iE-DAP-induced maturation of human DCs under CTLA-4 or TGF- β -induced immunosuppressive conditions. DMSO was used as a solvent control. *B*, pretreatment with LY294002 inhibits Rv0754-, MDP-, and C12-iE-DAP-triggered activation of PKC δ under TGF- β -induced immunosuppression of DCs. *C*, NOTCH1 signaling axis regulates Rv0754-, MDP-, and C12-iE-DAP-induced phosphorylation of ERK1/2 MAPK during CTLA-4- (*left panel*) or TGF- β -mediated (*right panel*) immunosuppressive conditions. *D*, pretreatment with U0126 (ERK1/2 inhibitor) or SB203580 (p38 MAPK inhibitor) abrogates Rv0754-, MDP-, and C12-iE-DAP-induced maturation of human DCs under CTLA-4- (*left panel*) or TGF- β -triggered (*right panel*) immunosuppression. The immunoblots are representative of three independent experiments, and *bar diagrams* represent data as mean \pm S.E. from three independent donors. *, $p < 0.05$ versus CTLA-4 and MDP-C12-iE-DAP-Rv0754 or TGF- β and MDP-C12-iE-DAP-Rv0754.

the effects of the PI3K pathway across diverse cell types (43, 50, 51). Thus, we examined the role for PKC in the contribution to the ability of TLR2 and NOD receptors in suppressing the inhibitory effects of CTLA-4 and TGF- β on DC maturation. In this regard, to identify a role, if any, for specific PKC isoform, we utilized well defined inhibitors for PKC α , PKC ζ , PKC β , PKC δ , and PKC ϵ . As shown, inhibition of PKC δ markedly abolished TLR2 and NOD receptor-mediated reversal of the inhibitory effects of CTLA-4 and TGF- β as evaluated by expression of DC maturation markers (Fig. 10A and supplemental Fig. 3). Importantly, inhibition of PI3K (LY294002) abolished the TLR2- and NOD-driven PKC δ phosphorylation implicating a role for the PI3K pathway in subsequent activation of PKC δ during DC maturation (Fig. 10B).

As described, MAPKs frequently act as important executioners of the DC maturation, and in this regard, MAPKs, including extracellular signal-regulated kinase (ERK) 1/2, p38, and JNK in concert with NF- κ B, have been recommended to assume a critical role in immunological processes by regulated expression of a variety of genes involved in inflammatory responses (48). In this regard, pharmacological inhibition data suggest the involvement of ERK1/2 and p38 MAPK in surmounting the inhibitory

effects of CTLA-4 and TGF- β by engagement of TLR2 and NOD receptors (Fig. 10D). Importantly, activation of ERK1/2 or p38 MAPK by TLR2 and NOD receptors could be repressed by inhibition of NOTCH1 signaling (GSI-I or NOTCH1 siRNA), PI3K (LY294002), or PKC δ (PKC δ Inhibitor) (Figs. 10C and 11, A-H; data not shown). These results strongly implicate a role for NOTCH1-PI3K-PKC δ signaling integration during TLR2 and NOD receptor-mediated reversal of the inhibitory effects of CTLA-4 and TGF- β on DC maturation.

The pharmacological inhibition of an intended signaling molecule was addressed by treating human DCs with the respective inhibitor and looking for inhibition of activation of its effector molecules. For example, inhibition of ERK1/2 by U0126 abrogated ERK1/2 activation, although p38 phosphorylation remained unaffected (supplemental Fig. 6E). Similarly, NOTCH signaling activation inhibitor GSI-I inhibited NICD generation (supplemental Fig. 6A) and PI3K inhibitor, LY294002, and PKC δ inhibitor, Rottlerin, abrogated specifically activation of AKT, 4EBP1, and GSK-3 β and PKC δ , respectively (supplemental Figs. 6, B and D).

The transcription factor NF- κ B tightly regulates distinct sets of genes involved in innate immune responses thus positioning

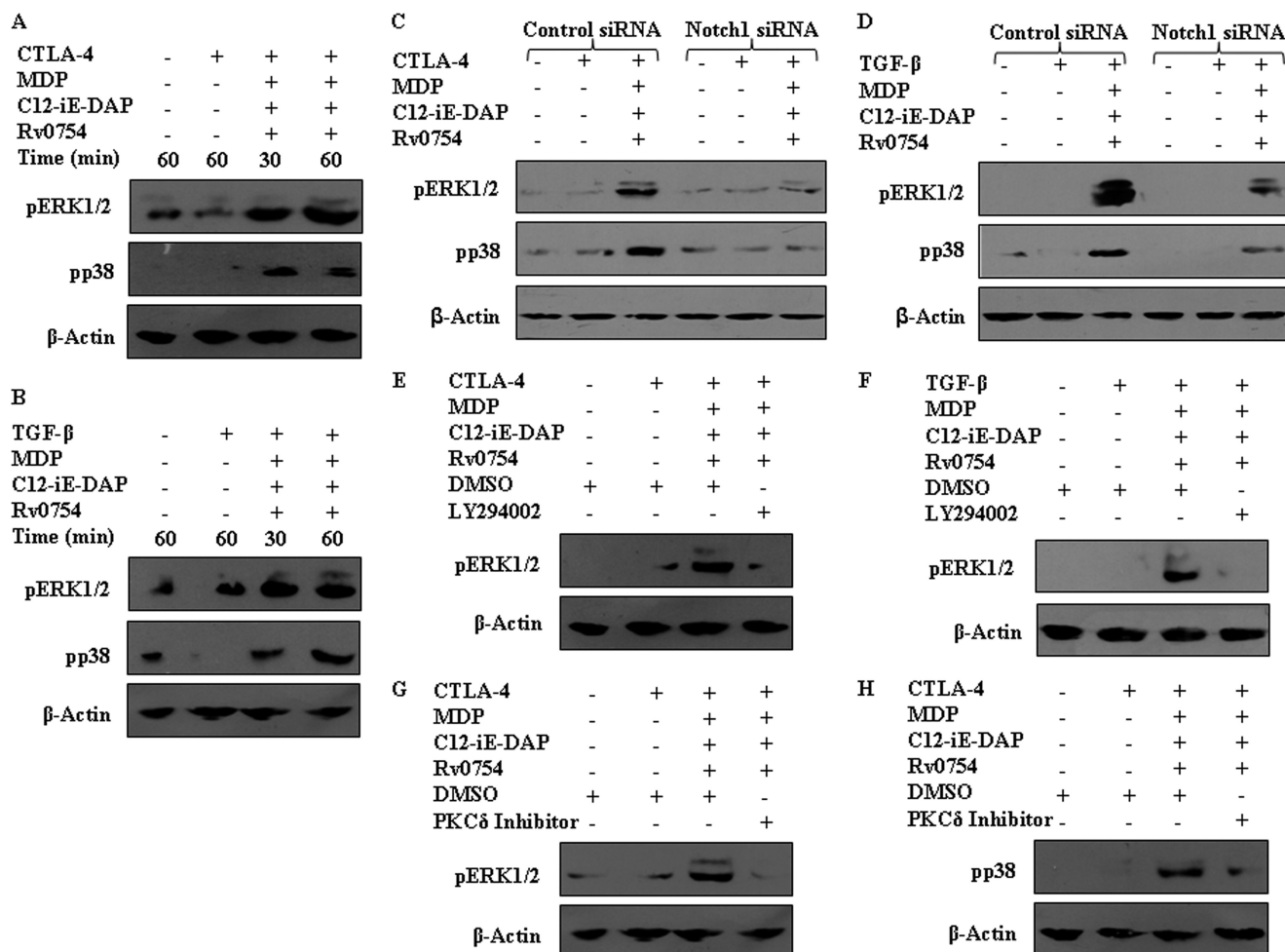


FIGURE 11. PI3K and PKC δ signaling axis integrate with MAPK pathway during TLR2-, NOD1-, and NOD2-triggered maturation of human DCs. *A* and *B*, Rv0754, C12-iE-DAP, and MDP induce activation of ERK1/2 and p38 MAPK during CTLA-4-triggered (*A*) or TGF- β -triggered (*B*) immunosuppression of DCs. *C* and *D*, inhibition of MAPK activation upon NOTCH1 signaling inactivation using NOTCH1 siRNA as assayed by Western blot under CTLA-4-mediated (*C*) and TGF- β -mediated (*D*) suppression of human DCs. *E–H*, inhibition of PI3K by LY294002 (*E* and *F*) or PKC δ by PKC δ inhibitor Rottlerin abrogates Rv0754-, C12-iE-DAP-, and MDP-triggered activation of ERK1/2 or p38 MAPK during CTLA-4-triggered (*E*, *G*, and *H*) or TGF- β -triggered (*F*) immunosuppression of DCs. The immunoblots are representative of three independent experiments.

itself as a novel executioner of DC maturation (45, 48). Significantly, promoters of various DC maturation marker genes such as CD83 and CD86 demonstrate the presence of canonical NF- κ B-binding sites thus implicating effects of NF- κ B on the functionality of DCs (52, 53). As rigorously established, I κ B, implied as a strong negative feedback, tightly regulates activation of NF- κ B, thus effectuating a speedy turn off of the NF- κ B responses (54). In this perspective, treatment with Bay11-7082, an I κ B inhibitor, effectively blocked TLR2 and NOD receptor-mediated reversal of inhibitory effects of CTLA-4 and TGF- β on DC maturation (Fig. 12, *A* and *B*, and supplemental Fig. 5). Furthermore, inhibition of NOTCH1 signaling (GSI-I), PI3K (LY294002), PKC δ (Rottlerin), ERK1/2 (U0126), or p38 MAPK (SB203580) abrogated TLR2 and NOD receptor-triggered translocation of p65 NF- κ B from the cytosol to the nucleus (Fig. 12C), thus further corroborating a critical role for NF- κ B in DC maturation.

DISCUSSION

DCs are classified as critical regulators of host immune response to various cellular cues, including infection (3–5, 7). In

this perspective, PRRs, notably TLRs and NLRs, often execute innate molecular sensing functions with respect to intruding microbes, thus promoting signaling cohorts for effective initiation and execution for well organized immune responses (2, 10). Interestingly, roles played by TLRs or NLRs like NOD1 or NOD2 are often intriguing as TLRs are largely believed to be pro-inflammatory, whereas NODs have been implicated in the regulation of anti-inflammatory responses as well as polarization of T cells toward the skewed Th2 phenotype (8–11). Significantly, pro-inflammatory skewed diseases, including Crohn disease, Blau syndrome, and chronic inflammatory bowel disease, are linked to mutations in the *NOD2* gene; thus, polymorphism in *NOD2* predisposes subjects for an overabundance of inflammatory responses (55, 56). Despite these observations, information in regard to signaling cohorts or a battery of genes associated with TLR2, NOD1, and NOD2 receptor-mediated cellular functions remains imprecisely understood. This information will be of significance in TLR2 and NOD receptor-mediated DC responses during immunosuppressive conditions. For example, CTLA-4- or TGF- β -mediated down-regulation of

TLR2/NOD Regulate DCs Resistance to TGF- β /CTLA-4

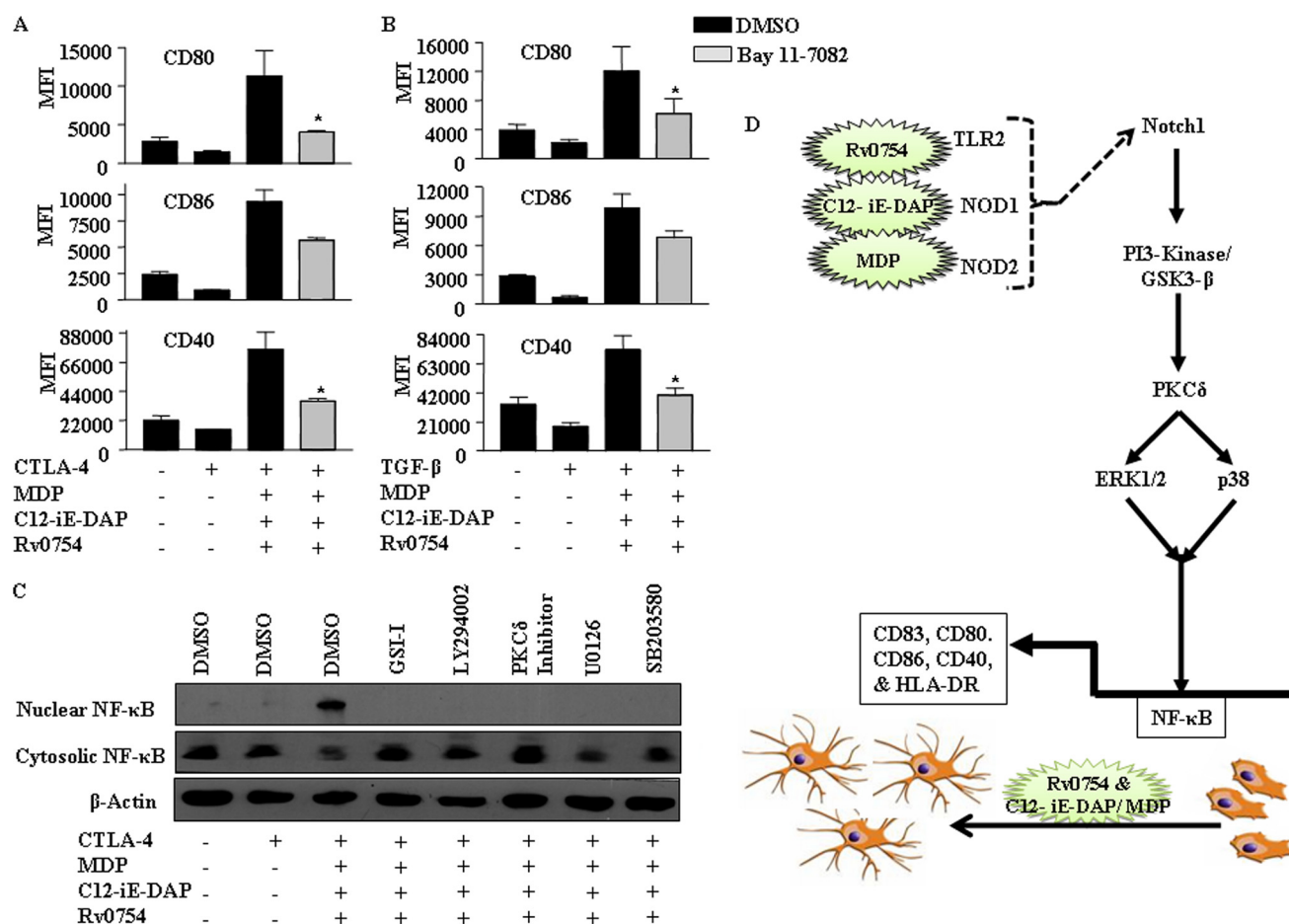


FIGURE 12. Requirement of NF- κ B activation during TLR2-, NOD1-, and NOD2-triggered maturation of DCs. A and B, inhibition of NF- κ B by Bay 11-7082 abrogated Rv0754-, MDP-, and C12-iE-DAP-triggered maturation of human DCs under CTLA-4-induced (A) or TGF- β -induced (B) immunosuppressive conditions. C, pretreatment of DCs with GSI-I or LY294002 or PKC δ inhibitor or U0126 or SB203580 abolishes Rv0754-, MDP-, and C12-iE-DAP-triggered nuclear translocation of p65-NF- κ B under CTLA-4-induced immunosuppression as analyzed by immunoblotting. Immunoblots represent three independent experiments, and data in bar diagrams are representing mean \pm S.E. from three independent donors. D, model depicting TLR2-, NOD1-, and NOD2-driven integration of NOTCH1-PI3K-PKC-MAPK-NF- κ B signaling during maturation of human DCs. *, $p < 0.05$ versus CTLA-4 and MDP-C12-iE-DAP-Rv0754 or TGF- β and MDP-C12-iE-DAP-Rv0754.

immune responses in various pathophysiological conditions such as infection with human immunodeficiency virus (HIV) predisposes infected individuals to a variety of chronic infectious diseases, including tuberculosis (12–14, 26, 28, 29, 36, 57–59). As described, CTLA-4 expressed by Tregs selectively down-regulates the expression of co-stimulatory molecules CD80/86 and pro-inflammatory cytokines by DCs, and it inhibits the potential of DCs to activate effector T cells, thus effectively contributing to tolerance or immune suppression (28, 29). Furthermore, immunosuppressive cytokine TGF- β is known to prevent maturation of DCs, in respect to MHC class II, CD80, CD86, and CD83 expression, as well as IL-12 and IL-10 production in response to TNF- α , LPS, IL-1 β , or haptens. Interestingly, the TGF- β -enriched immunoenvironment directs DCs toward a tolerogenic phenotype, which could be instrumental in the development of Tregs (13, 14, 26). Importantly, patients with HIV infection as well as *M. tuberculosis* exhibit DCs and CD4 T cell dysfunction associated with increased CTLA-4 and TGF- β expression indicating a critical role for CTLA-4- and TGF- β -mediated immunosuppression in the development of disease pathologies (60, 61). Paradoxically, recent reports suggest that *M. tuberculosis* contributes to HIV

pathogenesis by promoting a shift in the dynamic balance between antigen processing and presentation of intact virion particles favoring trans-infection of HIV to T cells. These findings clearly emphasize that HIV and *M. tuberculosis* act synergistically with each infection contributing specific immune aberrations (62). Because of the critical role of CTLA-4 and TGF- β in establishment and propagation of these infectious diseases, these observations stress the urgency of development of novel therapeutic intervention strategies for CTLA-4- and TGF- β -mediated impairment of the functional activity of DCs.

In this study, we demonstrate that cell surface and cytoplasmic immune surveillance PRRs, TLR2, NOD1, and NOD2, cooperatively regulate maturation of human DCs. Significantly, we observed that cooperative stimulation by TLR2 and NOD receptors renders enhanced refractoriness to CTLA-4- or TGF- β -mediated impairment of human DC maturation. Importantly, our data demonstrate the involvement of NOTCH1-PI3K signaling dynamics integrated into signaling cohorts that play a critical role in TLR2 and NOD receptor-mediated reversal of the inhibitory effects of CTLA-4 and TGF- β . As shown, signaling perturbations effectively blocked not only TLR2 and NOD receptor-mediated DC maturation, but also the ability of

TLR2 and NOD receptors to overcome inhibition of DC maturation by CTLA-4 and TGF- β . Critically, TLR2 and NOD receptor-mediated cellular functions involved unique participation of PKC δ among many PKC isoforms.

Overall the cellular responses of immune cells, including DCs triggered with a wide variety of stimuli, are often suggested to involve extensive cross-talk between PI3K-AKT, PKC, and MAPK signaling cascades (45–48). In this perspective, TLR2 and NOD receptor-driven maturation of human DCs involved NOTCH1-PI3K-PKC δ -dependent activation of ERK1/2 and p38 MAPK. Intriguingly, transcription factor NF- κ B plays a central role in DC-mediated innate immune responses by modulating the induction of diverse sets of genes involved in inflammatory responses (45, 48). Furthermore, surface markers such as CD83 and CD86 that are associated with maturation of DCs are reported to have canonical NF- κ B-binding sites in their promoter suggesting the role of NF- κ B in functionality of DCs upon maturation (52, 53). In this regard, engagement of TLR2 and NOD receptors by their cognate ligand resulted in significant activation of NF- κ B during CTLA-4- or TGF- β -enriched immunosuppressive conditions. Furthermore, signaling perturbation data suggest that triggering of TLR2 and NOD receptors brings signaling integration through cross-talk of the NOTCH1-PI3K-PKC δ signaling axis to activate NF- κ B, which plays a crucial role in the regulation of a multitude of gene-associated maturation of human DCs (Fig. 12D). In regard to the source of the NOTCH ligand, NOTCH receptor ligands are reported to be expressed on human blood conventional DCs and plasmacytoid DCs. Even though unstimulated DCs are shown to express low levels of NOTCH receptors ligands, Delta and Jagged, different stimuli, including TLR engagements, have been shown to augment the expression levels of Notch ligands. However, in case of mouse immune cells, NOTCH ligands like Delta-like 1 (DLL1) and Jagged 1 (JAG1) are expressed by follicular DCs but not by B cells in the germinal centers. As shown in this study, TLR2 and NLR stimulation markedly induced the expression levels of DLL4 ligand as well as expression of DLL1, DLL3, JAG1, and JAG2. In conclusion, our study provides mechanistic and functional insights into TLR2 and NOD receptor-mediated development of refractoriness against various immunosuppressive stimuli in human DCs and establishes a conceptual framework for the development of novel therapeutic measures.

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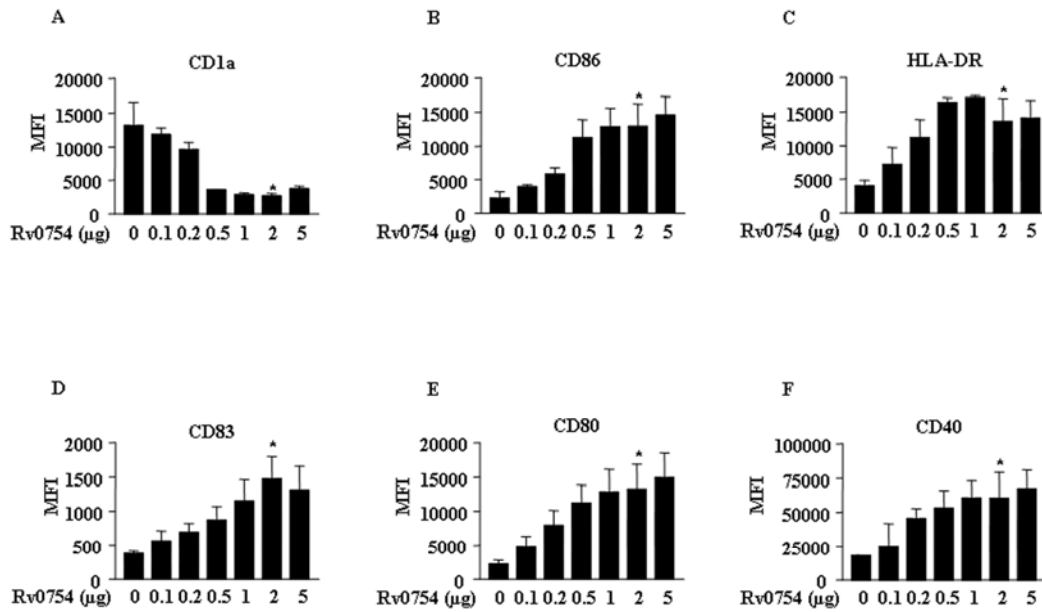
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TLR2/NOD Regulate DCs Resistance to TGF- β /CTLA-4

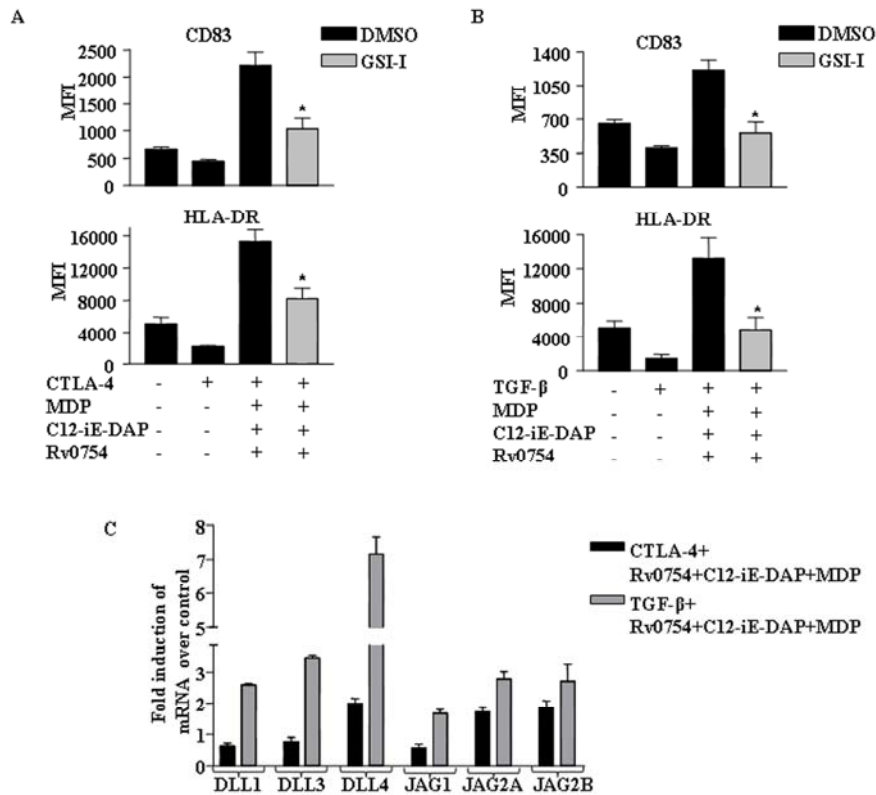
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Supplemental Fig. 1



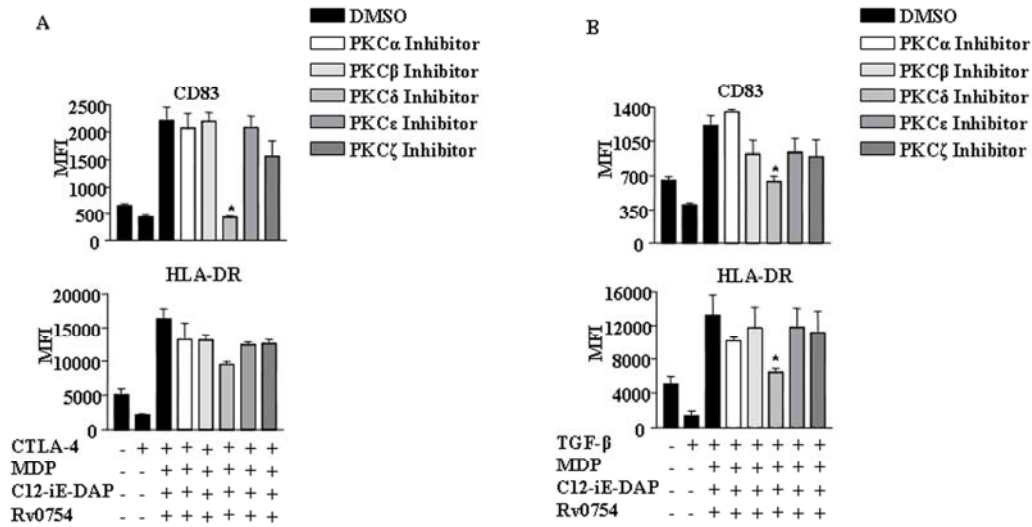
Supplemental Figure 1. TLR2 stimulation by Rv0754 induces maturation of human DCs. A-F. Dose dependent increase in maturation of human DCs upon treatment with TLR2 ligand Rv0754 as analyzed by surface expression of maturation markers (A) CD1a, (B) CD86, (C) HLA-DR, (D) CD83, (E) CD80 and (F) CD40 by flow cytometry. Data are presented as mean \pm SEM from six independent donors. *, $P < 0.05$ versus Medium.

Supplemental Fig. 2



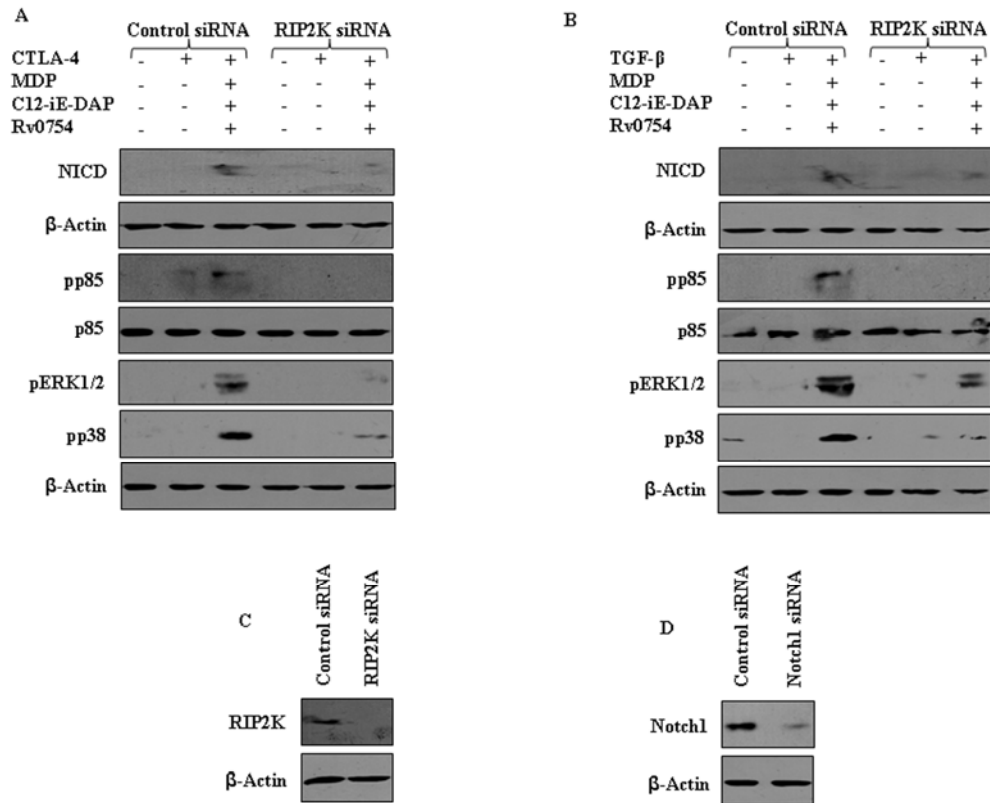
Supplemental Figure 2. Notch1 signaling is indispensable to mediate rescue of human DCs maturation. *A* & *B*. Pretreatment with GSI-I abrogates Rv0754, C12-iE-DAP and MDP-induced maturation of human DCs under (*A*) CTLA-4 or (*B*) TGF- β triggered immunosuppressive conditions. Data are presented as mean \pm SEM from three independent donors. *, $P < 0.05$ versus CTLA-4 and MDP-C12-iE-DAP-Rv0754 or TGF- β and MDP-C12-iE-DAP-Rv0754. *C*. Human DCs were treated with either CTLA-4 or TGF- β for 6 hours and then treated with combination of TLR2 (Rv0754), NOD1 (C12-iE-DAP), NOD2 (MDP) agonists for 48 hours. Expression of various ligands of Notch receptor as mentioned was quantified by real time PCR. The data represent three independent experiments and is expressed over and above the medium.

Supplemental Fig. 3



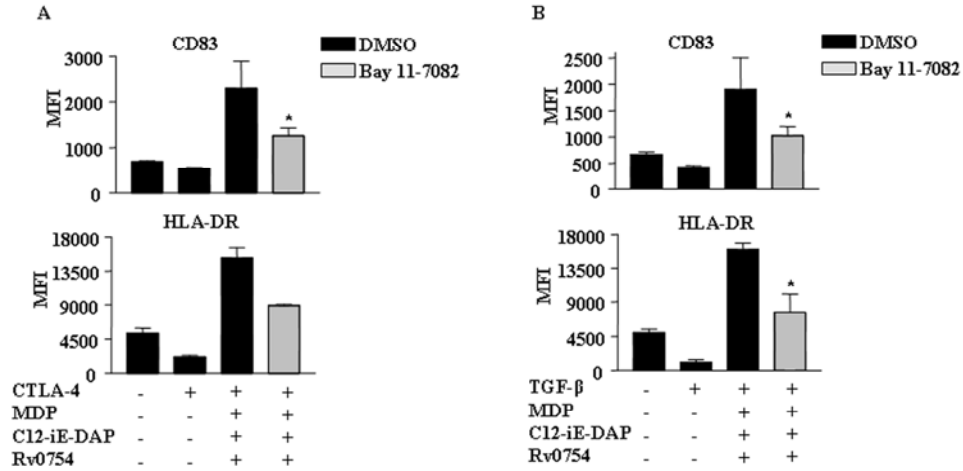
Supplemental Figure 3. Crucial role for PKC δ in TLR2, NOD1 and NOD2 triggered maturation of human DCs. *A & B.* Inhibition of PKC δ subverts Rv0754, C12-iE-DAP and MDP induced synergistic maturation of human DCs under (A) CTLA-4 or (B) TGF- β triggered immunosuppressive conditions. Data are presented as mean \pm SEM from three independent donors. *, $P < 0.05$ versus CTLA-4 and MDP-C12-iE-DAP-Rv0754 or TGF- β and MDP-C12-iE-DAP-Rv0754.

Supplemental Fig. 4



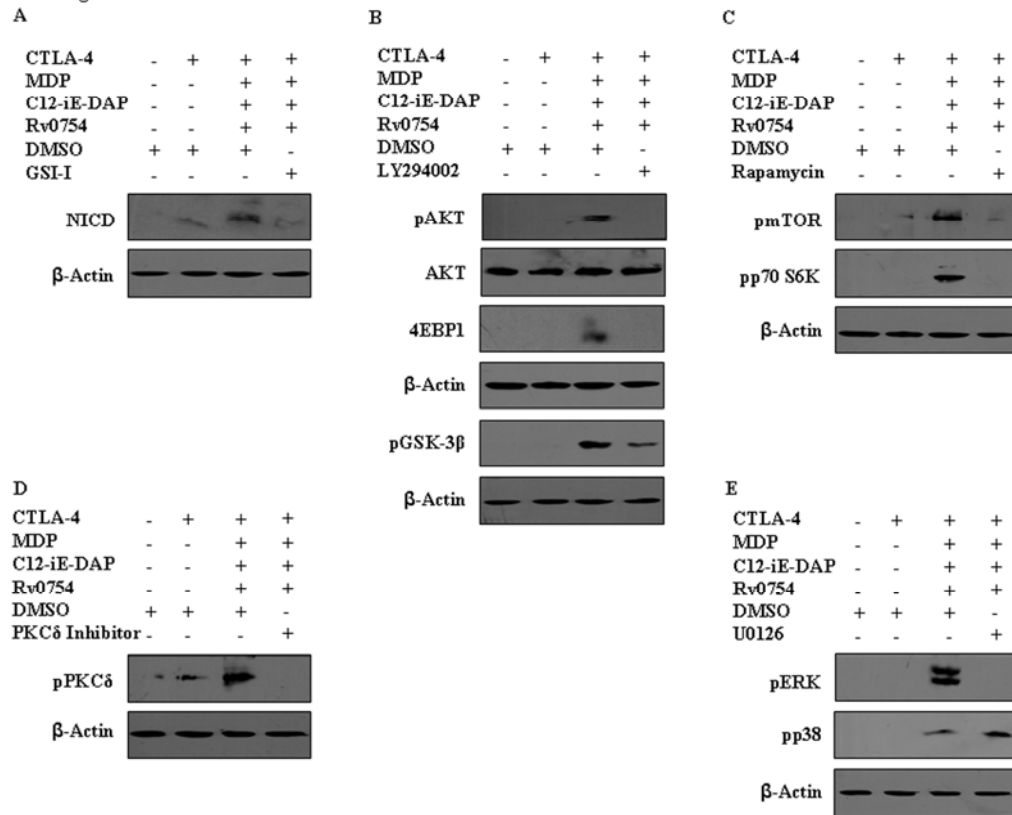
Supplemental Figure 4. NLR signaling critically regulates Notch1/PI3K-MAPK signaling cohort during TLR2, NOD1 and NOD2 mediated maturation of human DCs. *A & B*. Human DCs were transfected with RIP2K siRNA or control siRNA at a final concentration of 100nM. After 72 hours DCs were treated with (A) CTLA-4 and (B) TGF- β for 6 hours and effect of abolishing NLR signaling on Notch1 activation and subsequent activation of PI3K and MAPKs was assayed by western blotting. Data is representative of three independent experiments. *C & D*. RIP2K-specific siRNA and Notch1-specific siRNA mediated inhibition of (C) RIP2K and (D) Notch1 is shown. Data is a representative of three separate experiments.

Supplemental Fig. 5



Supplemental Figure 5. Critical role of NF- κ B during TLR2, NOD1 and NOD2 mediated maturation of human DCs. *A* & *B*. Pretreatment with Bay 11-7082 inhibits Rv0754, C12-iE-DAP and MDP triggered maturation of human DCs under (A) CTLA-4 or (B) TGF- β induced immunosuppressive conditions as analyzed by surface expression of maturation markers CD83 and HLA-DR. Data are presented as mean \pm SEM from three independent donors. *, $P < 0.05$ versus CTLA-4 and MDP-C12-iE-DAP-Rv0754 or TGF- β and MDP-C12-iE-DAP-Rv0754.

Supplemental Fig. 6



Supplemental Figure 6. Pharmacological inhibitors used in current study are specific. *A-E*. Specificity of given pharmacological inhibitor was addressed by treating human DCs with respective inhibitor and looking for abrogation of its effector molecules such as (*A*) GSI inhibits generation of NICD, (*B*) use of LY294002 abrogates activation of downstream AKT, 4EBP1 and GSK-3 β ; (*C*) Rapamycin suppresses mTOR and p70S6K phosphorylation, (*D*) PKC δ inhibitor prevents activation of PKC δ and (*E*) U0126, a MEK kinase inhibitor abrogates ERK1/2 activation while p38 phosphorylation remains unaffected.

Cooperative Regulation of NOTCH1 Protein-Phosphatidylinositol 3-Kinase (PI3K) Signaling by NOD1, NOD2, and TLR2 Receptors Renders Enhanced Refractoriness to Transforming Growth Factor- β (TGF- β)- or Cytotoxic T-lymphocyte Antigen 4 (CTLA-4)-mediated Impairment of Human Dendritic Cell Maturation

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