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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*[S]

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Dendr fectious importa respons stimulat tors, inc immu- tion don (PRRs) iting fac ceptors robust i ors like ing" the odulate demonst rocess, regulate on, and synergis esenta- enhance –7). In impairm osed of tion dat -driven assume t influ- diated su mmune uration hibits a naling a thogen- virtue of surface, study p inflam- TLR2- a OD1 or tions 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-

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.

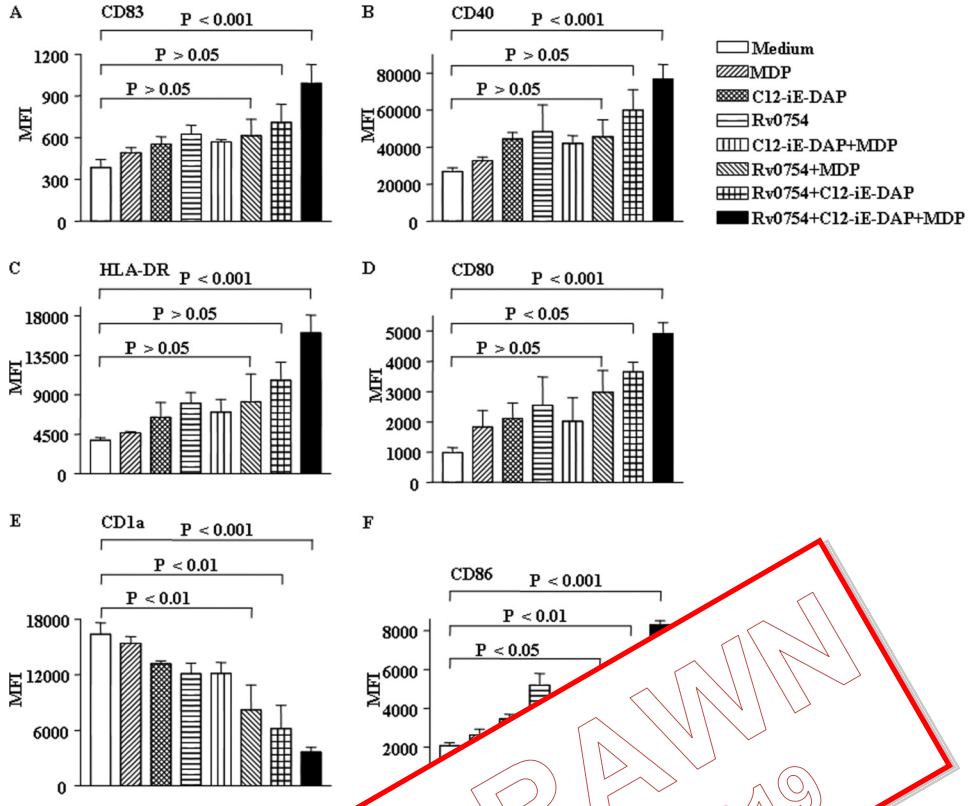

FIGURE 1. **TLR2, NOD1, and NOD2 cooperatively regulate** cells/ml) were cultured with GM-CSF and IL-4 and left untreated (*Medium*) or treated with TLR μg/ml) or NOD2 ligand MDP (1 μg/ml) as well as with combinations of TLR2, NOD1, and NOD2 on of maturation markers CD83 (*A*), CD40 (*B*), HLA-DR (*C*), CD80 (*D*), CD1a (*E*), and CD86 (*F*) by f ensities (*MFI*) ± S.E. from six independent donors.

NOD1 and NOD2 operate a our data may represent mechanisms by which matching pathways upon re processes integrate multiple signals from PRRs muramyl dipeptide (M equired for functional maturation of human DCs as well as to doglycan (15–20). Althou impart refractoriness to DCs against various immunosuppressive stimuli.
signaling events culminate i slocation of NF-κB, transcriptome to TLR2 or NOD receptors could markedly gh TLR2 signaling utilizes MyD88 and TRIF ad executing signaling cascades, NOD receptor oligomer ation 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-κB (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-PKCδ-dependent activation of ERK1/2, p38 MAPK, and NF-κB during TLR2 and NOD receptor-driven maturation of human

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 μg/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 μg/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,

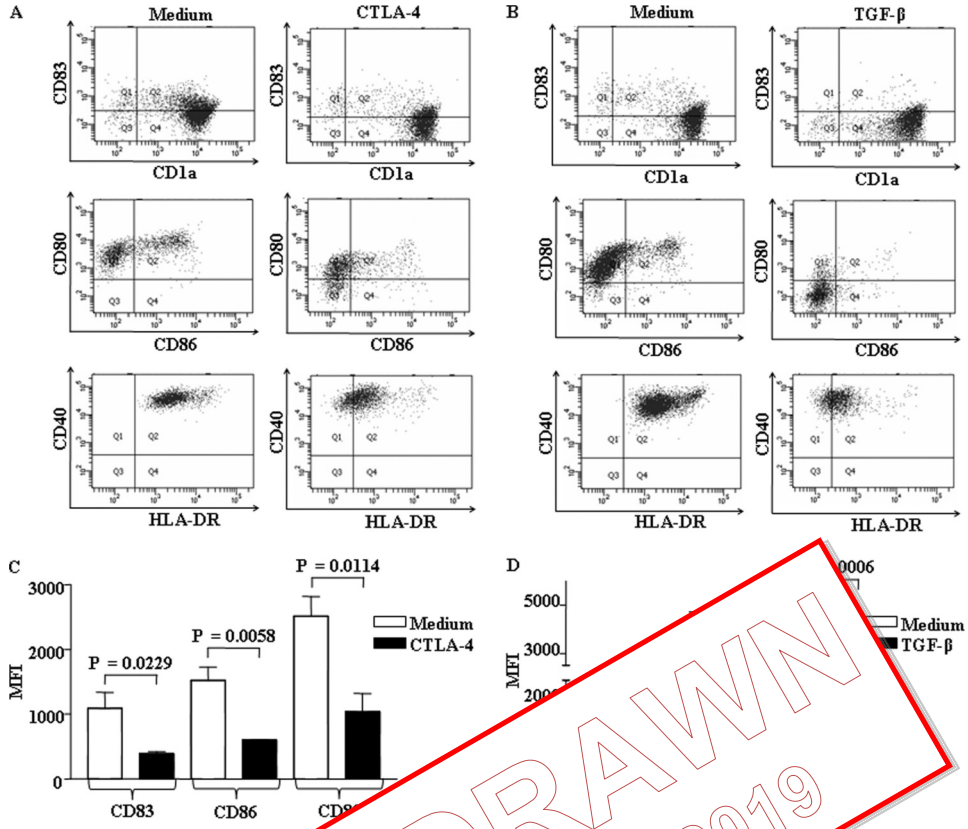

FIGURE 2. **CTLA-4 and TGF-β markedly inhibit human D** GM-CSF and IL-4 and were treated with CTLA-4 (1 μg/ml) (*A*), TGF-β (10 ng/ml) (*B*), or left unt flow cytometry for expression of CD83, CD1a, CD80, CD86, CD40, and HLA-DR. Representative nd *D*, representative mean fluorescence intensities (*MFI*) of CD83, CD86, and CD80 expression g/ml) (*C*) or TGF-β (10 ng/ml) (*D*) are shown. Data in bar diagrams are represented as mean ± S

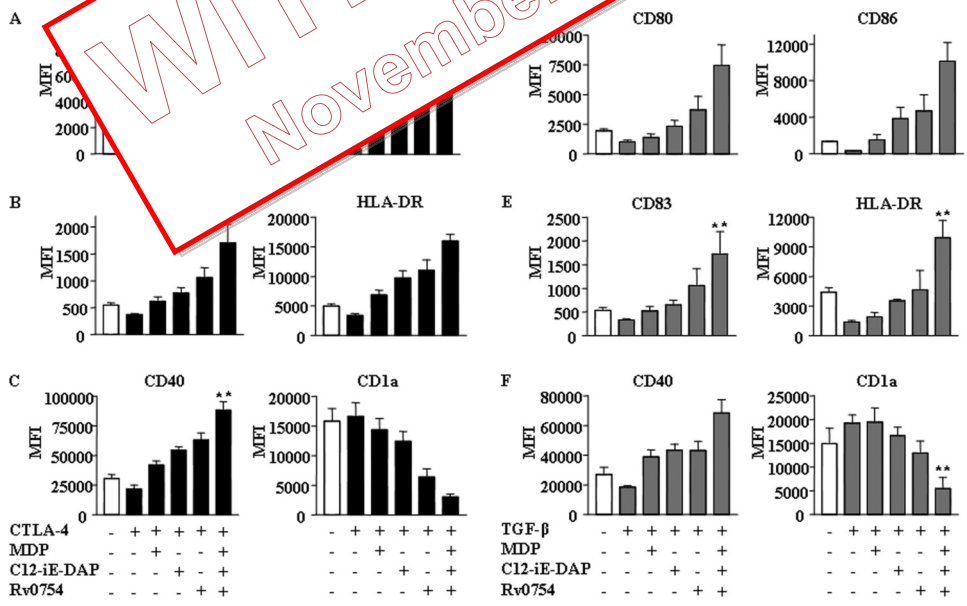

FIGURE 3. **TLR2, NOD1, and NOD2 synergistically surmount CTLA-4- and TGF-β-mediated suppressed maturation of human DCs.** *A–C,* DCs were pretreated with CTLA-4 (1 μg/ml) for 6 h followed by treatment with Rv0754 or C12-iE-DAP or MDP as well as with a combination of Rv0754, C12-iE-DAP, and MDP for an 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

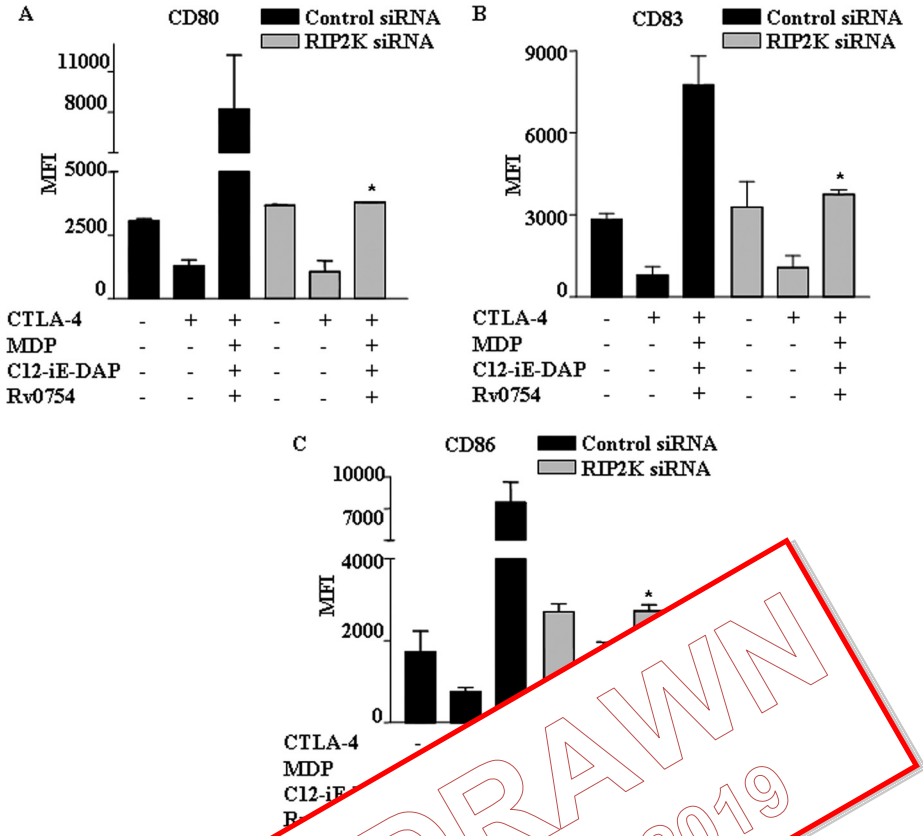

FIGURE 4. **NOD signaling collaborates with TLR2 to s… …an DC maturation.** *A–C*, human DCs were transfected with RIP2K siRNA or control siRNA at a … …with CTLA-4 for 6 h, and human DC maturation was assayed by flow cytometry by monitorin… …(*B*), and CD86 (*C*). Data represent mean ± S.E. from three independent donors. *, $p < 0.05$ *ve*…

kinase (pp70 S6K), and … …ed Lymphocyte Reaction*—CD4$^+$ T cells used in allogenic from Cell Signaling Te… …mixed lymphocyte reactions were isolated from peripheral (AC-15) was procured fr… …blood mononuclear cells of healthy donors by immunomagnetic separation using CD4-conjugated microbeads (Miltenyi siGLO Lamin A/C control s… …Dharmacon as siGENOME^{TM} SM… …ch contains a pool of four different double-… …oligonucleotides (siRNA). Oligofectamine transf… …eagent 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′-CGGGATCCATGTCATTTGTGATCGTGGCG-3′ (forward) and 5′-CCCAAGCTTTCATGGGATCAGGCTGGGCAG-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).

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 μM), LY294002 (50 μM), rapamycin (100 nM), PKCα inhibitor (Safingol) (50 μM), PKCβ inhibitor (50 μM), PKCδ inhibitor (Rottlerin) (10

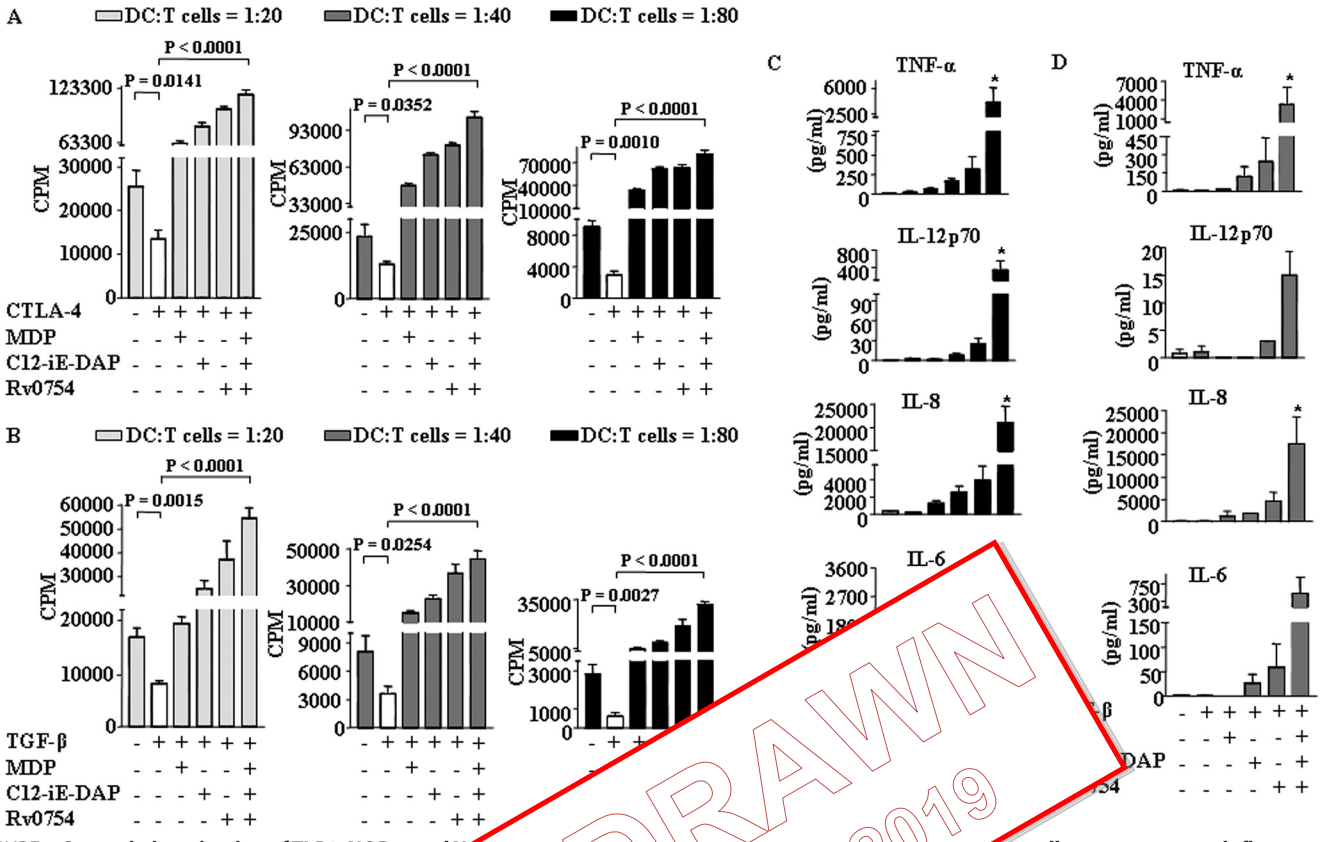

FIGURE 5. **Synergistic activation of TLR2, NOD1, and N… as well as to secrete pro-inflammatory cytokines under CTLA-4- and TGF-β-induced im…** …s indicated in Fig. 3, *A–F*, and were co-cultured with allogenic $CD4^+$ T cells at different DC to T… …ight with 0.5 μCi of [^{3}H]thymidine to quantify T cell proliferation. Radioactive incorporation … values). Data are presented as mean ± S.E. from four independent donors. *C* and *D*, DCs w… …th CTLA-4 (1 μg/ml) (*C*) or TGF-β (10 ng/ml) (*D*) for 6 h. DCs were further treated for 42 h with … …ation of Rv0754, C12-iE-DAP, and MDP and secretion of IL-6, IL-8, IL-12p70, and TNF-α in cell-f… …LA-4 or TGF-β.

μM), PKCϵ inhibitor (V2… …f (PKCζ pseudosubstrate in… …μM), U0126 (10 μM), SB203580 (1… …μM), Bay11-7082 (20 μM); DMSO at 0.1% … …was used as the vehicle control. Immature DCs w… …ed 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× 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 μg/ml each aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4, 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 °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 °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 °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.

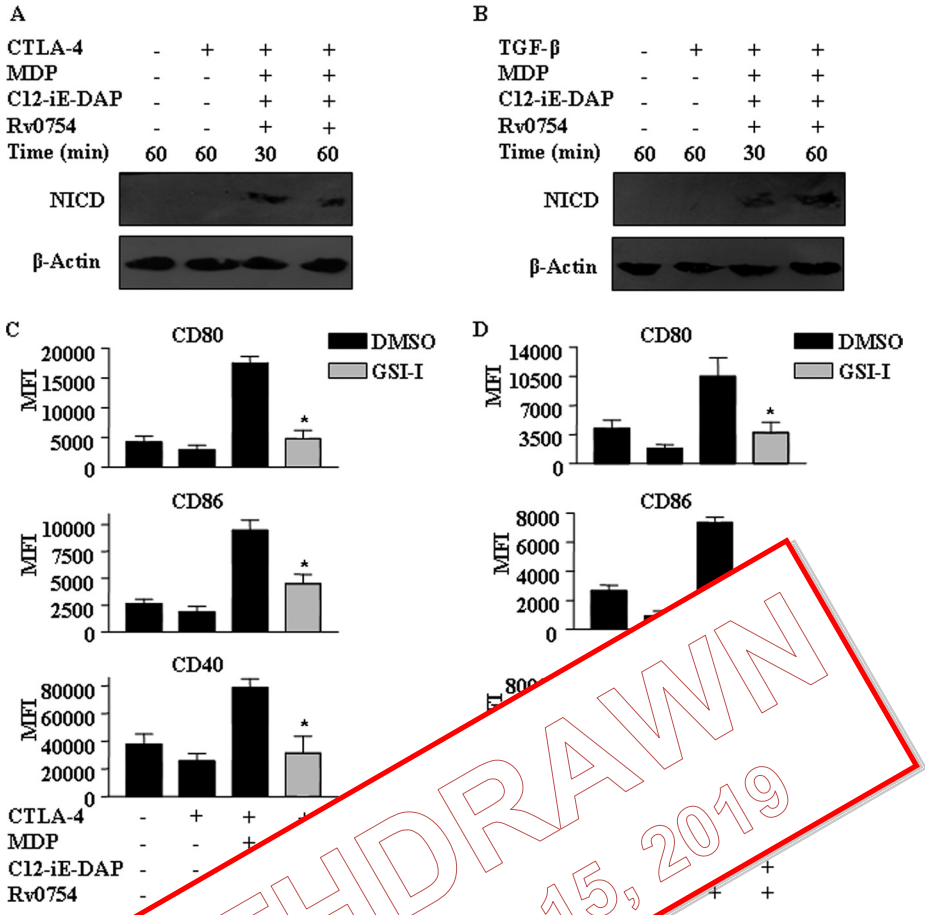

FIGURE 6. **Critical role for NOTCH1 sig… …on of human DCs under CTLA-4- and TGF-β-induced immunosuppressive conditions.** A… …l) (*A*) or TGF-β (10 ng/ml) (*B*) for 6 h followed by treatment with combination of Rv0754, MDP, a… …CD) was analyzed by immunoblotting. *C* and *D*, pretreatment with GSI-I abrogates Rv0754, MDP… …-4-induced (*C*) or TGF-β-induced (*D*) immunosuppressive conditions as assessed by analysis of surfac… …d CD40. The immunoblots are representative of three independent experiments, and *bar diagrams* repres… …nt 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… …comparison between samples were determin… …dent's *t* test distribution. The data in the graphs is … …sed as the mean ± 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 μg/ml), or NOD2, MDP (1 μg/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

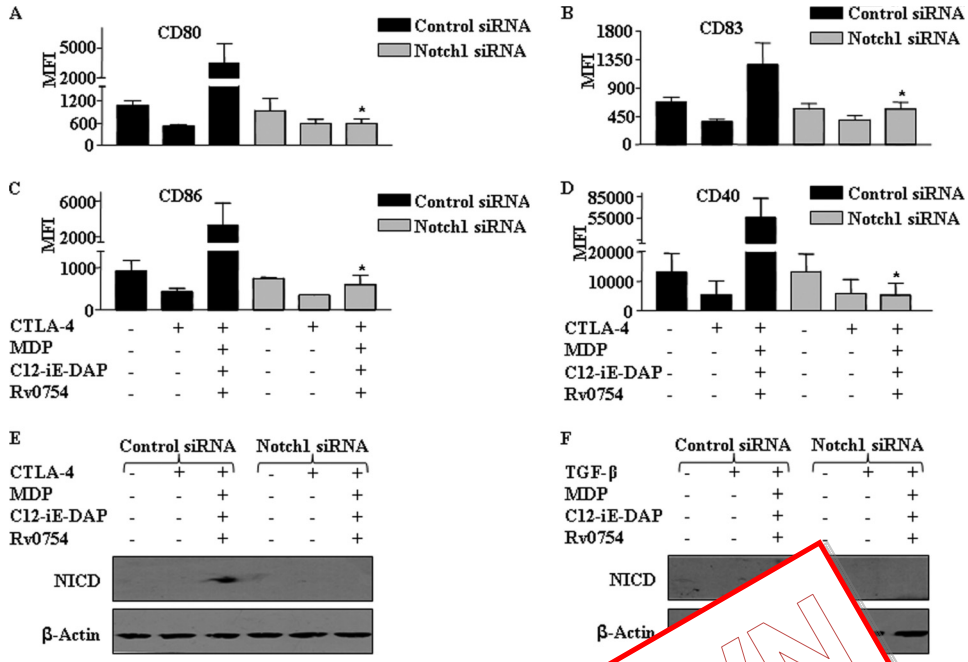

FIGURE 7. **NOTCH1 signaling axis controls TLR2-, NOD1-, and NOD2-triggered matur… CTLA-4- and TGF-β-triggered immunosuppressive conditions.** *A–D,* expression of NOTCH1 in human DCs was k… with NOTCH1 siRNA at a final concentration of 100 nM. After 72 h, DCs were treated with CTLA-4 for 6 h, and hu… for the expression of CD80 (*A*), CD83 (*B*), CD86 (*C*), and CD40 (*D*). Data are presented as means ± S.E. f… A-4 and MDP-C12-iE-DAP-Rv0754. *E* and *F,* inactivation of NICD formation in NOTCH1 siRNA-trans… (*E*) or TGF-β-mediated (*F*) suppressive conditions using Western blotting. *Blots* are represent…

the requirement of intact protein in its nati… CTLA-4- and TGF-β-mediated suppression of the maturation of DCs (15).

*Cooperative Stimulation by TL… ration (Fig. 3, A–C and D–F). The activa-ders Enhanced Refractorine… ceptors leads to recruitment and association Impairment of Humar… kinase (RIP2K) through CARD-CARD domain interaction subsequent maturation …, and Glu-69, Asp-70, and Glu-71 amino acid residues of factors, including TGF-β… ARD domain of NOD2 are critical for mediating NOD2 interaction with RIP2K (30–33). Thus, RIP2K forms a crucial link in microbially derived factors … studies have clearly established an… F-β in DC signal transduction downstream of NLR2 (34, 35). From this maturation as DCs derived in t… TGF-β exhibited perspective, we have addressed critical involvement of NLR2 significant reductions in IL-12/I… etion 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), 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

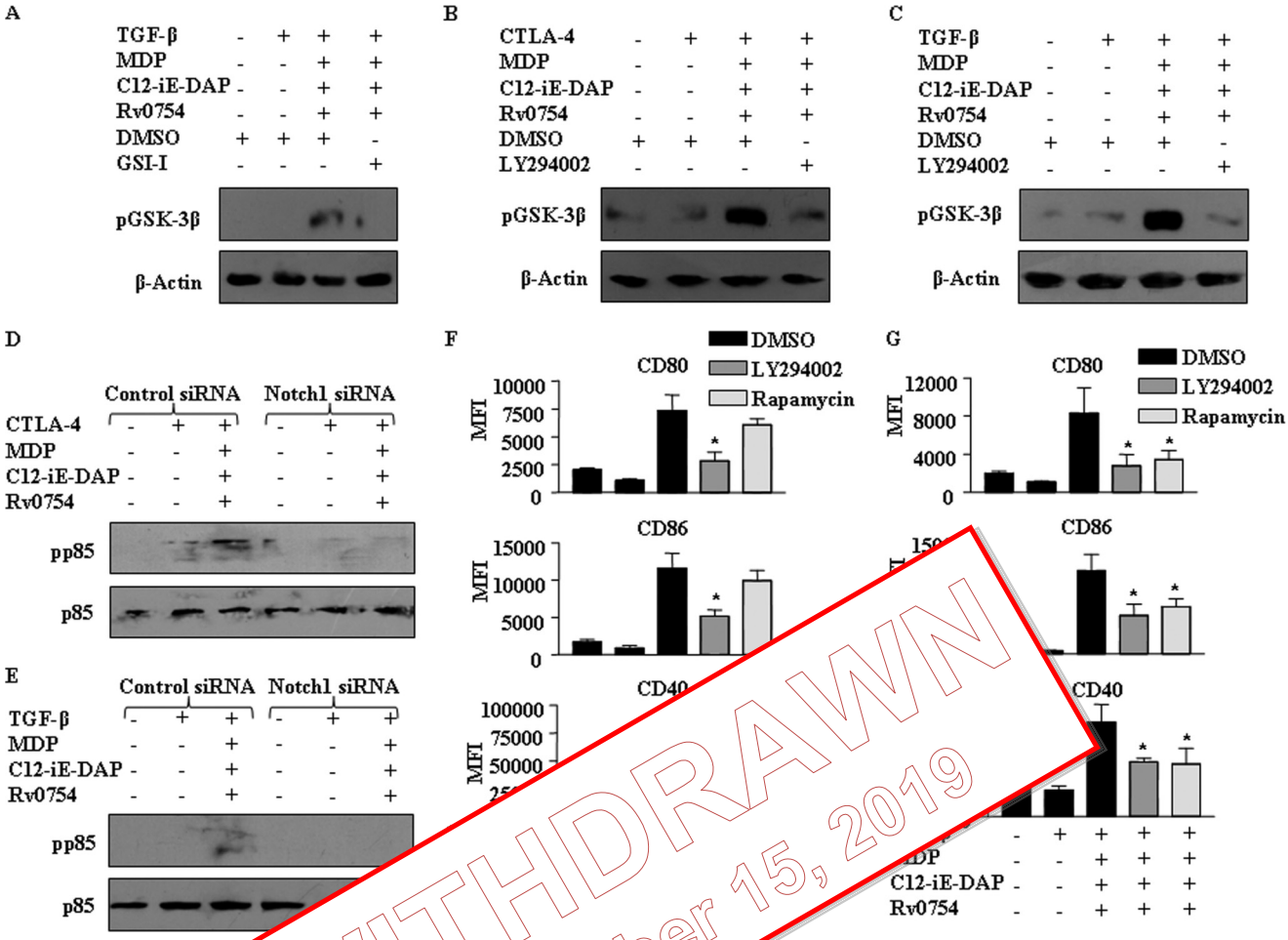

FIGURE 8. **NOTCH1-PI3K sig… …maturation rescue of human DCs.** *A–C*, pretreatment of DC with GSI-I (γ-secretase inhibitor) (… …C12-iE-DAP-induced phosphorylation of GSK-3β (pGSK-3β) in DCs under CTLA-4-induced (*B*) or TGF-β-… *D* and *E*, abrogation of NOTCH1 signaling by transfecting human DCs with NOTCH1 siRNA at a final conce… …ctivation under either CTLA-4-mediated (*D*) or TGF-β-mediated (*E*) suppressive conditions. PI3K pathway activati… …p85 using Western blotting. Blots are representative of three separate experiments. *F* and *G*, LY294002 or rapamycin inh… …E-DAP-induced maturation of human DCs under CTLA-4-induced (*F*) or TGF-β-induced (*G*) immunosuppression. The immunol… …pendent experiments, and data in *bar diagrams* are represented as means ± S.E. from three independent donors. *, $p < 0.05$ *versu*… …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*).

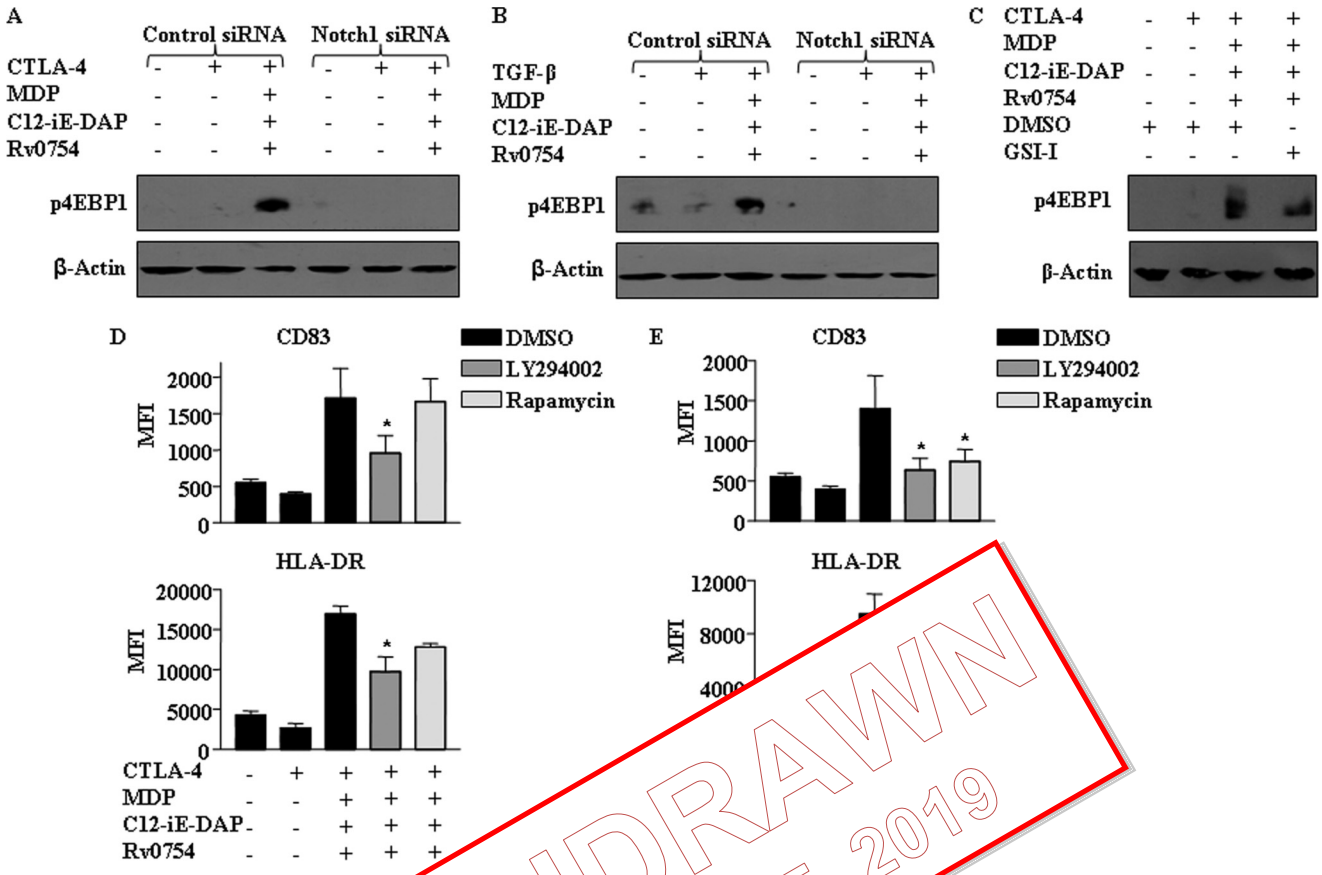

FIGURE 9. **Cross-talk of NOTCH1-PI3K signalin… …maturation of human DCs.** *A* and *B*, human DCs were transfected with NOTCH1 siRNA at a fi… …CTLA-4 (*A*) or TGF-β (*B*) for 6 h and subsequently with a combination of TLR and NLR agonist… …horylation was addressed by Western blotting. Blots are representative of three independe… …4-, C12-iE-DAP-, and MDP-triggered phosphorylation of 4EBP1 under CTLA-4-induced immun… …aling axis by LY294002 or rapamycin curtails Rv0754-, C12-iE-DAP-, and MDP-triggered maturat… …duced (*E*) immunosuppression. Immunoblot represents three indepen- dent experiments, and *bar dia*… …ependent donors. *, $p < 0.05$ *versus* CTLA-4 and MDP-C12-iE-DAP-Rv0754 or TGF-β and MDP-C12-iE-DAP-Rv…

To address the involvement o… …gands in our studies, we carried out expres… …lysis of NOTCH ligands during TLR2 and NLR a… …timulation of human DCs in the presence or absence o… …TLA-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-κB (NF-κB), 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-3β, 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-3β (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-κB 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

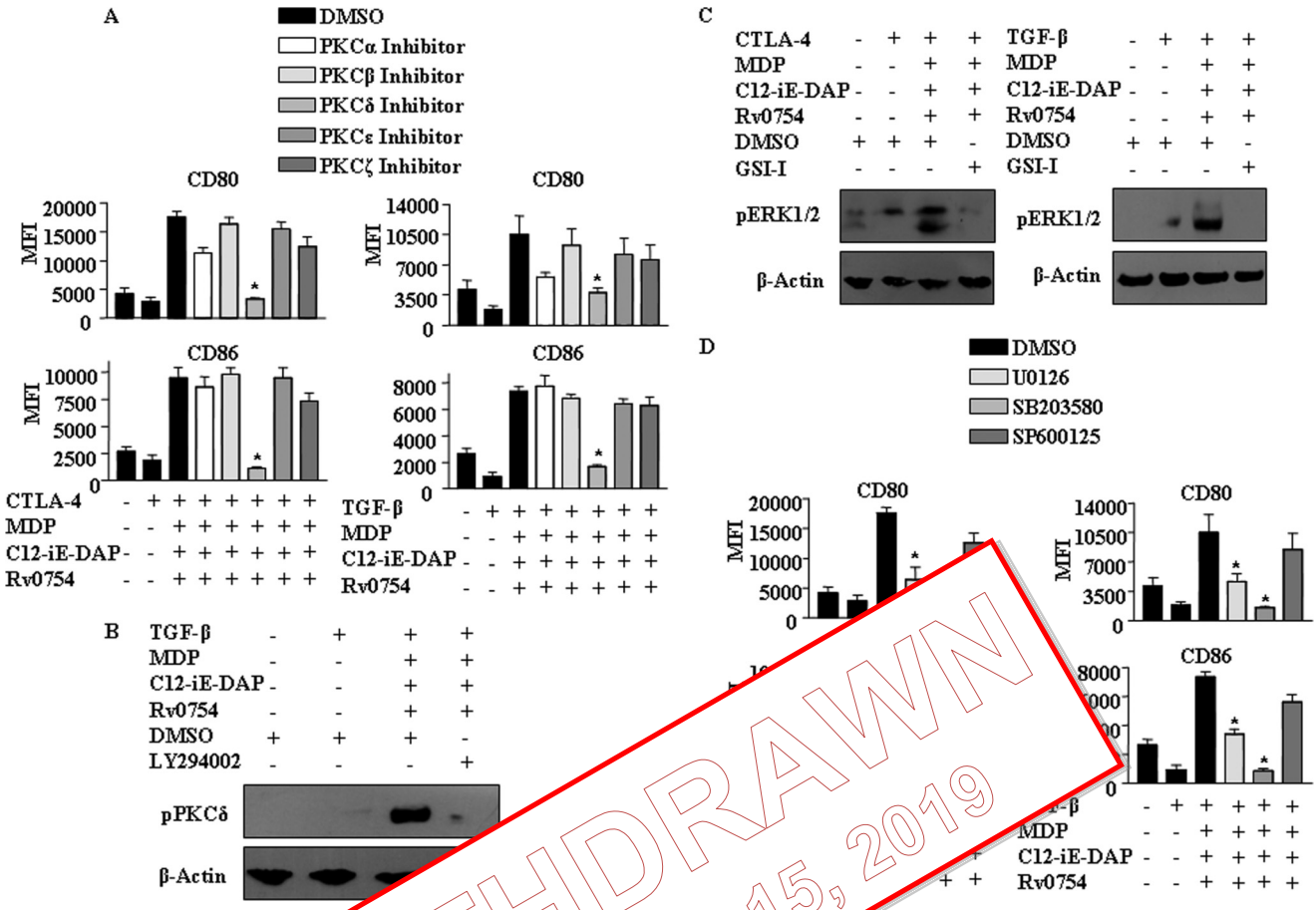

FIGURE 10. **Signaling integration thr… …g TLR2-, NOD1-, and NOD2-triggered maturation of human DCs.** *A,* inhibition of PKCδ … …ion of human DCs under CTLA-4- or TGF-β-induced immunosuppressive conditions. DMSO … …02 inhibits Rv0754-, MDP-, and C12-iE-DAP-triggered activation of PKCδ under TGF-β-induced … …egulates Rv0754-, MDP-, and C12-iE-DAP-induced phosphorylation of ERK1/2 MAPK during CTLA-4… …osuppressive conditions. *D,* pretreatment with U0126 (ERK1/2 inhibitor) or SB203580 (p38 MAPK inhibitor … …duced maturation of human DCs under CTLA-4-(*left panel*) or TGF-β-triggered (*right panel*) immunosuppressio… …of three independent experiments, and *bar diagrams* represent data as mean ± S.E. from three independent donors. * … C12-iE-DAP-Rv0754 or TGF-β and MDP-C12-iE-DAP-Rv0754.

the effects of the PI3K pathway … …cell types (43, 50, 51). Thus, we examined the role … …in the contribution to the ability of TLR2 and NOD re…ptors 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 PKCα, PKCζ, PKCβ, PKCδ, and PKCε. As shown, inhibition of PKCδ 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. 10*A* and supplemental Fig. 3). Importantly, inhibition of PI3K (LY294002) abolished the TLR2- and NOD-driven PKCδ phosphorylation implicating a role for the PI3K pathway in subsequent activation of PKCδ during DC maturation (Fig. 10*B*).

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-κB, 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. 10*D*). 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 PKCδ (PKCδ Inhibitor) (Figs. 10*C* and 11, *A–H*; data not shown). These results strongly implicate a role for NOTCH1-PI3K-PKCδ 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. 6*E*). Similarly, NOTCH signaling activation inhibitor GSI-I inhibited NICD generation (supplemental Fig. 6*A*) and PI3K inhibitor, LY294002, and PKCδ inhibitor, Rottlerin, abrogated specifically activation of AKT, 4EBP1, and GSK-3β and PKCδ, respectively (supplemental Figs. 6, *B* and *D*).

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

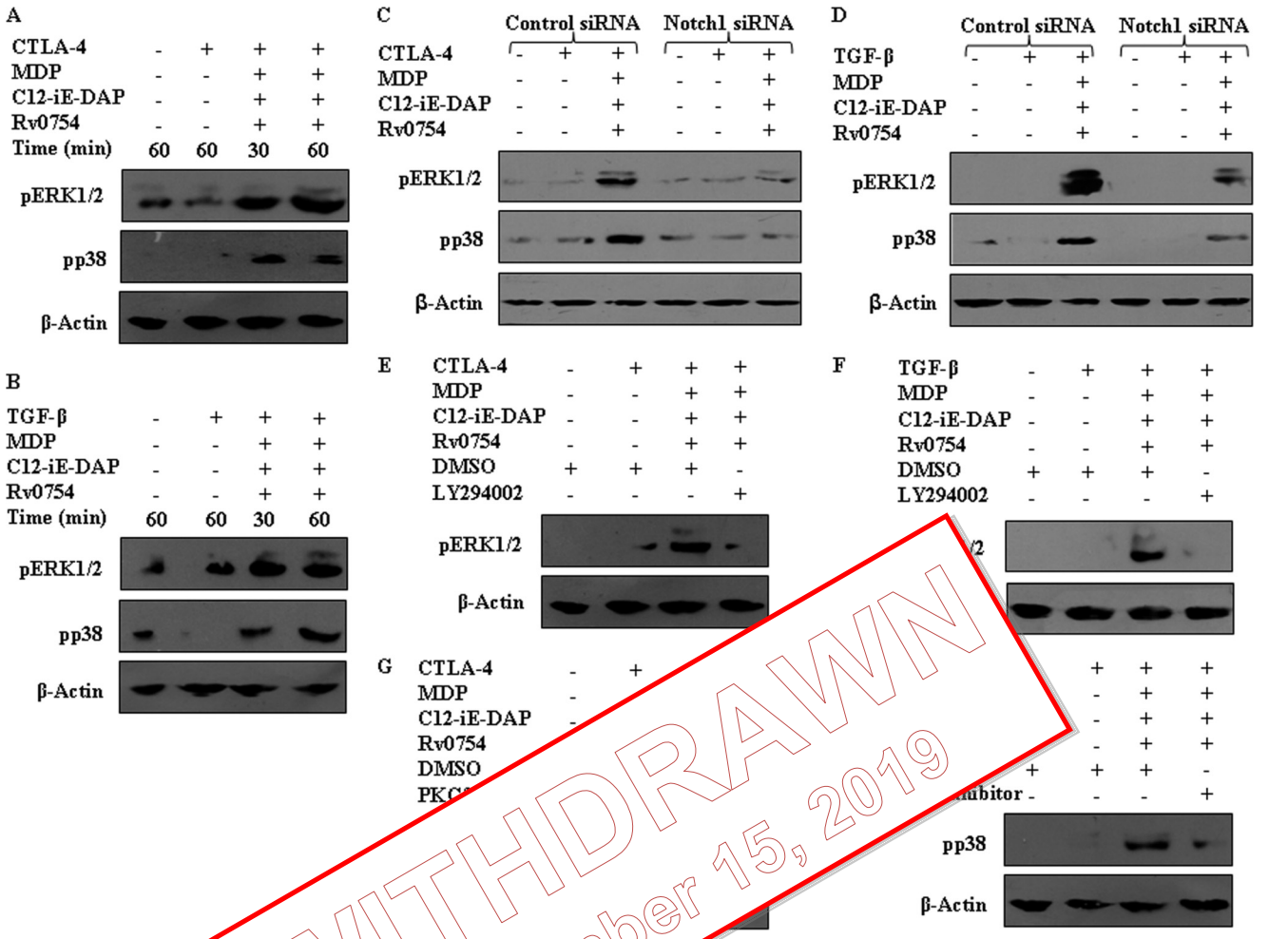

FIGURE 11. **PI3K and PKCδ s… …R2-, NOD1-, and NOD2-triggered maturation of human DCs.** *A* and *B*, Rv0754, C12-iE-DAP, and MDP… …CTLA-4-triggered (*A*) or TGF-β-triggered (*B*) immunosuppression of DCs. *C* and *D*, inhibition of MAPK activatio… …g NOTCH1 siRNA as assayed by Western blot under CTLA-4-mediated (*C*) and TGF-β-mediated (*D*) suppression… …y LY294002 (*E* and *F*) or PKCδ (*G* and *H*) by PKCδ inhibitor Rottlerin abrogates Rv0754-, C12-iE-DAP-, and MDP-triggered a… …during CTLA-4-triggered (*E, G,* and *H*) or TGF-β-triggered (*F*) immunosuppression of DCs. The immunoblots are representativ… …periments.

itself as a novel executioner of DC… …uration (45, 48). Significantly, promoters of various DC maturation marker genes such as CD83 and CD86 demonstrate the presence of canonical NF-κB-binding sites thus implicating effects of NF-κB on the functionality of DCs (52, 53). As rigorously established, IκB, implied as a strong negative feedback, tightly regulates activation of NF-κB, thus effectuating a speedy turn off of the NF-κB responses (54). In this perspective, treatment with Bay11-7082, an IκB 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), PKCδ (Rottlerin), ERK1/2 (U0126), or p38 MAPK (SB203580) abrogated TLR2 and NOD receptor-triggered translocation of p65 NF-κB from the cytosol to the nucleus (Fig. 12*C*), thus further corroborating a critical role for NF-κB 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

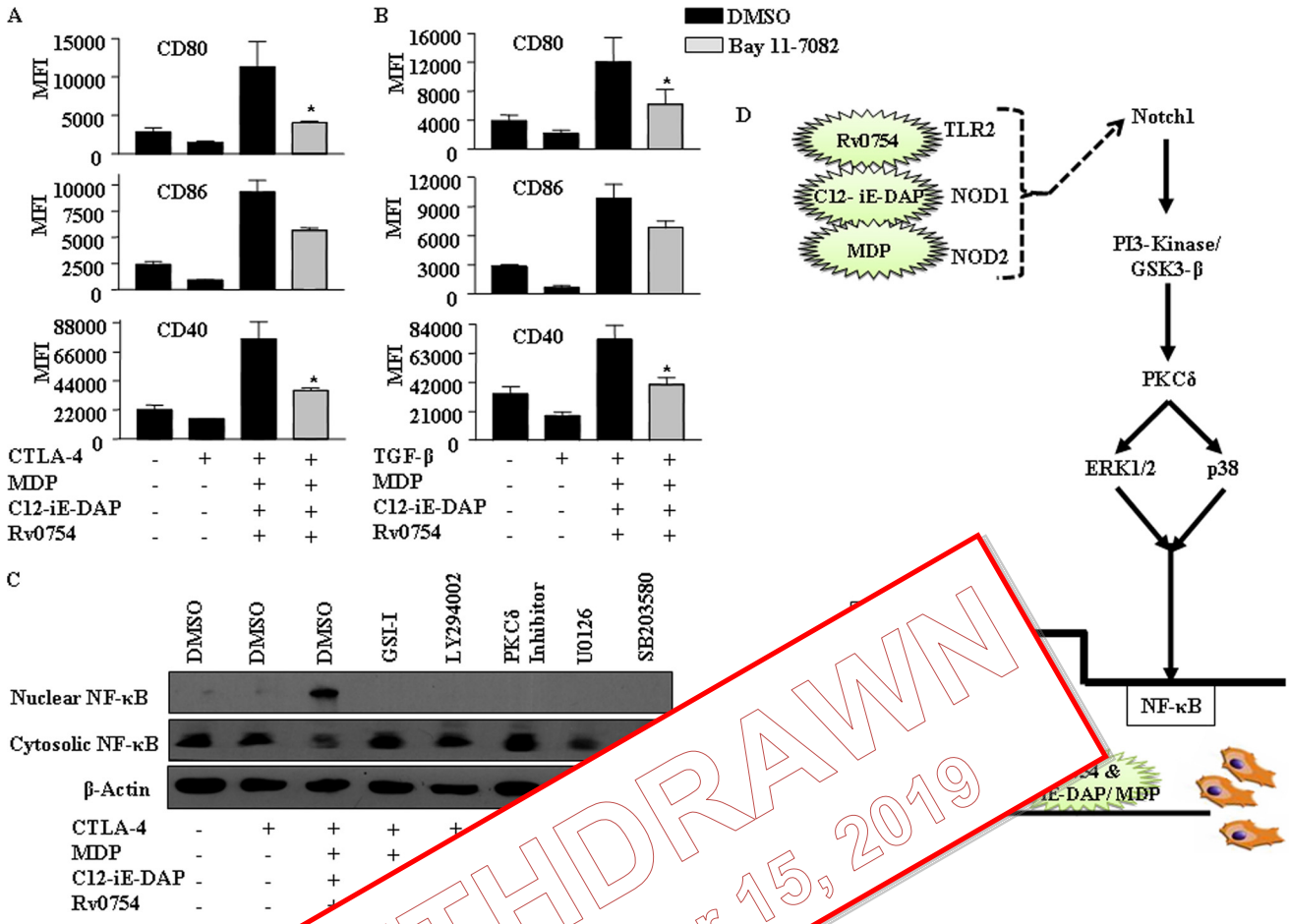

FIGURE 12. **Requirement of NF-κB … aturation of DCs.** *A* and *B*, inhibition of NF-κB by Bay 11-7082 abrogated Rv0754-, MDP-, and … A-4-induced (*A*) or TGF-β-induced (*B*) immunosuppressive conditions. *C*, pretreatment of DC… B203580 abolishes Rv0754-, MDP-, and C12-iE-DAP-triggered nuclear translocation of p65-NF-κB u… ed by immunoblotting. Immunoblots represent three independent experiments, and data in *bar diagram*… pendent donors. *D*, model depicting TLR2-, NOD1-, and NOD2-driven integration of NOTCH1-PI3K-PKC-MAPK-NF… man 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 … gical conditions such as infection with human i… eficiency virus (HIV) predisposes infected individuals t… 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-1β, 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 PKCδ 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-PKCδ-dependent activation of ERK1/2 and p38 MAPK. Intriguingly, transcription factor NF-κB 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-κB-binding sites in their promoter suggesting the role of NF-κB 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-κB 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-PKCδ signaling axis to activate NF-κB, which plays a crucial role in the regulation of a multitude of g associated maturation of human DCs (Fig. 12*D*). In the source of the NOTCH ligand, NOTCH recep reported to be expressed on human blood and plasmacytoid DCs. Even though shown to express low levels of NO and Jagged, different stimuli been shown to augment However, in case of mou Delta-like 1 (DLL1) and J licular DCs but not by B cell shown in this study, TLR2 and NLR s induced the expression levels of DLL4 ligan pression of DLL1, DLL3, JAG1, and JAG2. In co our study provides mechanistic and functional insight 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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