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Negative regulation of TLR9-mediated IFN-α induction by a small-molecule, synthetic TLR7 ligand

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Abstract: Toll-like receptors (TLRs) are a family of molecules that function as sensors for the detection of foreign pathogens through the recognition of nonvariable microbial motifs. Although numerous studies have focused on singular TLRs, less attention has been focused on how simultaneous signaling of multiple TLRs may result in counter-regulation of the effects of each. Here, we examine the counter-regulation that occurs during simultaneous stimulation of TLR7 and TLR9 on human plasmacytoid dendritic cells (PDCs) and B cells. Interestingly, we observed that the capacity for potent IFN-α-induction by TLR9 ligands like CpG-C and CpG-A is markedly reduced by concurrent small molecule TLR7 stimulation. However, this inhibition is specific to particular CpG motif, containing immunostimulatory sequence (ISS) functions such as IFN-α induction and BDCA-2 down-regulation. Other ISS activities such as PDC expression of CD80/CD86, secretion of IL-6, and B cell proliferation are not altered by the presence of TLR7 ligands (TLR7Ls). In concordance with the ability of TLR7Ls to decrease IFN-α secretion induced by ISS, we also find that the expression of interferon regulatory factor-7 (IRF-7), a transcriptional factor critical for IFN-α expression, is reduced. Furthermore, down-regulation of TLR9 mRNA expression is accelerated after TLR7 stimulation. These data indicate that TLR7 and TLR9 costimulation do not combine synergistically for IFN-α induction and demonstrate that, instead, a negative feedback mechanism has evolved, possibly to prevent levels of IFN-α secretion potentially detrimental to the host. *J. Leukoc. Biol.* 82: 000–000; 2007.

Key Words: *TLR9 · TLR7 · PDCs · type I interferon*

INTRODUCTION

During an encounter with a microbial pathogen, recognition by the host is achieved primarily through early sentinels called pattern recognition receptors (PRRs), endogenous molecules that bind with high affinity to pathogen-associated molecular patterns (PAMPs), such as LPS, lipopeptides, flagellin, RNA, and DNA. The primary family of PRRs is the Toll-like receptor (TLR) family, transmembrane signaling molecules that play a key role in the initiation of innate immune responses and that also act as a link to the T cell-mediated adaptive response (reviewed in [1]). Although most TLRs are cell surface expressed, a subset of TLRs is localized to the endosomal compartment, where they are uniquely situated to recognize foreign nucleic acid ligands that have entered the cell through the endocytosis and subsequent breakdown of the foreign pathogen. Included within this subset are TLR3 (recognizes viral dsRNA), TLR7 and TLR8 (viral ssRNA), and TLR9 (CpG-rich viral and bacterial DNA). A common functional property of this subset is the induction of elevated IFN-α production from conventional DCs (TLR3) and plasmacytoid dendritic cells (PDCs) (TLR7, TLR9) [2].

Type I interferons are tightly regulated cytokines, the production of which is induced by a limited panel of microbial stimuli that interact with receptors on specific cell types: usually, subsets of dendritic cells. The important nature of this careful regulation becomes clear by observations that overexpression of IFN-α can be very detrimental to the host. Highly elevated levels of IFN-α have been implicated as etiologic for the onset of systemic autoimmune diseases, especially systemic lupus erythematosus (SLE) [3]. Increased serum concentrations of IFN-α have been shown to correlate directly with disease severity in human SLE [3]. Such high expression is thought to be induced by immune complexes composed of antibodies bound to host DNA with demethylated CpG motifs or to ribonucleoprotein (RNP), TLR9- and TLR7-mediated, respectively [4], [5].

Although signaling through either TLR7 or TLR9 is known to activate a variety of cell types and immune responses, these TLRs are perhaps best known for being strong promoters of type I interferon secretion from PDCs. Recombinant type I IFN is already in use as effective therapy for HCV infection [6] and for condylomata acuminata (genital warts) [7], and IFN-α-mediated effects are thought to be desirable for the treatment of cancer [8, 9] and asthma [10]. For this reason, TLR7Ls and TLR9Ls engineered for optimal IFN-α-inducing capacity are being developed as drugs and vaccine adjuvants for several indications, including allergy, HBV, HCV, and Non-Hodgkin's lymphoma [11, 12]. TLR9Ls include CpG-A, a form of ISS that is characterized by poly-guanosine sequences that enable G-tetrad binding with other CpG-A ODNs, allowing the formation

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of large molecular weight globular CpG-A structures that induce very high levels of IFN-α from PDCs but are inert on B cells. On the other hand, CpG-C ODNs are another class of TLR9Ls that contain 5′-TCG motifs and palindromic, self-annealing sequences that harbor multiple CpG motifs. This class of ODN can form a duplex that appears to be required for high IFN-α induction. A potent TLR7L is R848, or resiquimod, a small guanosine analog molecule, known to signal through either TLR7 or TLR8, which mimics natural ligands, fragments of viral ssRNA, in a manner yet unclear. Like ISS, R848 is known to induce IFN-α from PDCs, but it also induces high levels of inflammatory cytokines such as IL-6, TNF-α, IL-12, and IL-10 from monocytes and myeloid dendritic cells (MDCs) [2, 13, 14, 15]. To better understand the ability of these compounds to promote high expression of type I interferon from PDCs, we examined the ability of TLR7Ls and TLR9Ls to each counter-regulate the immune activities of the other.

MATERIALS AND METHODS

Preparation of peripheral blood mononuclear cells, PDCs, and B cells

Peripheral blood was collected from healthy volunteers by venipuncture using heparinized syringes. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation through a Ficoll (Pharmacia, Uppsala, Sweden) density gradient at 2,500 rpm for 25 min at room temperature. Enrichment of PDCs was performed using the Diamond Plasmacytoid Dendritic Cell Isolation kit (Miltenyi Biotec, Auburn, CA, USA). Specifically, PBMCs were initially labeled with a biotinylated antibody cocktail specific for non-PDCs (i.e., T cells, B cells, NK cells, myeloid dendritic cells, monocytes, granulocytes, and erythroid cells). Cells were washed, resuspended in MACS buffer, and magnetically labeled with anti-biotin Microbeads (Miltenyi) for 15 min at 4°C. Cells were again washed and resuspended in adequate volume and passed over LD depletion columns placed within a MidiMACS or QuadroMACS magnet. Cells that did not bind to the column were then incubated with anti-BDCA-4/CD304 Microbeads and positively selected over two sequential MS columns. Cell purity of >99% was consistently obtained for the BDCA-2$^+$, CDw123$^+$ PDC phenotype. Isolation of B cells was performed by double-positive selection using CD19 Microbeads (Miltenyi Biotec) and two sequential LS columns. B cell purity was consistently ≥98%.

Cell culture and reagents

Generally, PBMCs were cultured at 5×10^5 cells/well in 96-well flat-bottom plates. For isolated PDCs, cells were cultured at $3–5 \times 10^4$ cells/well in 96-well round-bottom plates. For isolated B cells, cells were cultured at 2×10^5 cells/well in 96-well round-bottom plates. PBMCs, PDCs, and B cells were cultured using RPMI 1640 (BioWhittaker Cambrex, Walkersville, MD, USA) supplemented with 10% heat-inactivated human AB serum (Gemini, Woodland, CA, USA) plus 50 U/ml penicillin, 50 μg/ml streptomycin, 300 μg/ml glutamine, 1 μM sodium pyruvate, and 1X nonessential amino acids (all BioWhittaker). R848 (TLR7/8L), imiquimod (TLR7L), and poly(I:C) (TLR3L) (all from InvivoGen, San Diego, CA, USA) were used at 1 μM, 10 μM, and 5 μg/ml, respectively, previously determined to be optimal concentrations for the induction of IFN-α from human PBMCs. CpG ODNs (ISS) were synthesized as described [16] and used at 1–20 μg/ml. All ODNs were phosphorothioate and had <5 endotoxin units/mg of ODN, determined by *Limulus* amebocyte lysate assay (BioWhittaker). Sequences: 1018 (CpG-B): 5′-TGACTGTGAACGTTCGAGATGA; C274 (CpG-C): 5′-TCGTCGAACGTTCGAGATGAT; D19 (CpG-A): 5′-GGtgcatcgatgcagGGGGG; C695 (CpG-C):5′-TCGAACGTTCGAACGTTCGAACGTT; C792 (CpG-C): 5′- TCGAACGTTCGAACGTTCGAACGTTCGAAT; control C ODN: 5′-TGCTTGCAAGCTTGCAAGCA. Upper case letters represent PS linkages and lowercase letters represent PO linkages. Polymyxin B (PMXB, Sigma, St. Louis, MO, USA) was used at 100 μg/ml. Herpes Simplex Virus-1 (kindly provided by Dr. Rick Pyles, University of Texas Medical Branch, Texas) was UV-inactivated and used at a multiplicity of infection (MOI) of 10. Influenza virus (H1N1, strain A/PR/8/34) from American Type Culture Collection (Manassas, VA, USA) was inactivated for 30 min at 56°C and used at a MOI of 10.

ELISA

Following a 20–24 h incubation at 37°C, PBMC, B cell, and PDC-derived supernatants were harvested and processed by ELISA for cytokines IFN-α, TNF-α, and IL-6. Human TNF-α and IL-6 were assayed with CytoSet antibody pairs (BioSource, Worcester, MA, USA). Limits of maximal/minimal detection were 4000/2 pg/ml for both assays. IFN-α was assayed with an ELISA using anti-human IFN-α antibodies (PBL Biomedical Laboratories, New Brunswick, NJ, USA), and the limit of maximal/minimal detection was 12,100/16 pg/ml.

Flow cytometry

To evaluate PDC maturation, PDCs were cultured as described above with TLR ligands for 20–24 h at 37°C and then harvested and stained for surface marker expression using CD80-FITC, CD86-APC, CCR7-PE.Cy7 (BD PharMingen, San Diego, CA, USA), and BDCA-2-FITC (Miltenyi Biotec). For the evaluation of intracellular IFN-α within PDCs, cells were cultured for 4 h (with R848) or 10 h (with C274), followed by an overnight incubation with 5 μg/ml brefeldin A (Epicentre, Madison, WI, USA). Culture times were previously determined to be optimal for those stimulations. Following BFA exposure, PDCs were washed, stained for BDCA-2, and fixed with 2% formaldehyde. Following fixation, PDCs were permeabilized with 0.5% saponin and then stained for intracellular IFN-α using anti-human IFN-α-PE (Chromaprobe, Maryland Heights, MO, USA). Acquisition was performed using a FACScaliber and FACScan (Becton Dickinson, San Diego, CA, USA), and data analysis was conducted through FlowJo (Ashland, OR, USA).

Gene expression assay and analysis

Human PBMCs and purified PDCs were stimulated with TLR7Ls and/or TLR9Ls and cultured for 10 h. Total RNA was extracted via the Qiagen RNeasy mini protocol (Qiagen, Valencia, CA, USA) and was converted to cDNA using oligo-dT (Promega, Madison, WI, USA), random hexamers (Promega), and SuperScript RT II (Invitrogen, Carlsbad, CA, USA). cDNA was diluted 1:10 for PBMC-derived samples and 1:3 for PDC-derived samples. Polymerase chain reaction (PCR) was conducted using QuantiTect SYBR green PCR master mix (Qiagen) and naked primers synthesized by Operon (Alameda, CA, USA) for human ubiquitin (F: 5′-CACTTGGTCCTGCGCTTGA, R: 5′-CAATTGGGAATGCAACAACTTTAT), IFN-α (F: 5′-CCCAGGAGGAGTTTGGCAA, R: 5′-TGCTGGATCATCTCATGGAGG), IRF-7 (F: 5′-GAGCCCTTACCTCCCCTGTTAT, R: 5′-CCACTGCAGCCCCTCATAG), TLR7, TLR9 (primer sequences proprietary). Human TNF-α was assayed using QuantiTect probe PCR master mix (Qiagen) and predeveloped TaqMan assay reagents (PDAR) primers with labeled probe (Applied BioSystems, Foster City, CA, USA). Reactions were conducted using the MyIQ Single Color RT-PCR Detection System (Bio-Rad, Hercules, CA, USA). Threshold cycle (C_T) values for each gene were normalized to ubiquitin using the Eq. $1.8^{(UBQ-GENE)}$ (100,000), where UBQ is the mean C_T of triplicate ubiquitin runs, GENE is the mean C_T of duplicate runs of the gene of interest, and 100,000 is arbitrarily chosen as a factor to bring all values above 0. The negative control for each experiment, stimulation with medium alone, is assigned a value of 1, and all data are expressed as fold induction over the negative control.

B cell proliferation

Purified B cells were cultured as described above for 4 days followed by an overnight incubation with ^{3}H-thymidine (1 μCi/well, GE Healthcare, Arlington Heights, IL, USA). Wells were then harvested through unifilter 96-well plates using a Packard Filtermate Harvester (Packard Bioscience, Meriden, CT, USA). Following the harvest, the wells of the filter plates were loaded each with 40 μl of scintillation cocktail and loaded on a TopCount NXT Microplate Scintillation Counter (Packard) for tritium detection.

Phospho-IκBα detection

Purified human PDCs were cultured with C274 (5 μg/ml) ± R848 (5 μM) for various time points (10–90 min). Following culture, cells were harvested and

assessed for phosphorylated IκBα S32 using the IκBα CASE kit, according to manufacturer's instructions (SuperArray Bioscience, Frederick, MD, USA).

Immunofluorescent microscopy

Purified human PDCs were cultured with Alexa-488-conjugated C274 and D19 for 3 h, fixed, permeabilized, and counterstained with CD107a-PE or CD71-PE (Becton Dickinson, San Jose, CA, USA). The labeled PDCs were mounted on microslides with a cytospin centrifuge and acquired on a Zeiss LSM 510 META confocal microscope and a 63X/1.4 N.A. objective. Analysis was conducted on Zeiss LSM software.

MTT cytotoxicity assay

Human PBMCs or PDCs were cultured overnight in 96-well plates at previously experimentally determined cell densities with titrated concentrations of R848, imiquimod (IMQ), and/or ISS. MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (R&D Systems, Minneapolis, MN, USA) was added to each well, and the cultures were incubated up to 18 h longer. When purple formazan precipitates were clearly visible, detergent reagent was added to each well. The plates were then left covered in the dark at room temperature overnight. When no purple crystals remained, the plate cover was removed, and absorbance was read at 570 nm with a reference wavelength of 650 nm.

Statistics

Statistical significance was calculated using either the paired t test or the one-way ANOVA with standard (parametric) methods (GraphPad InStat, San Diego, CA, USA); which test is specified in the figure legend. Symbols representing significance are ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant. In some cases, data were log-transformed before analysis. Error bars are standard error of means (SEM).

RESULTS

CpG-C induces a higher maximal IFN-α response from PBMCs than do small molecule TLR7Ls

Both CpG-C and small molecule TLR7Ls, such as R848 and IMQ, have been demonstrated as robust IFN-α-inducers for mouse and human PDCs [2, 17, 18]. When CpG-C C274 was used to stimulate human PBMCs at 20 μg/ml (3 μM), a standard concentration for observing in vitro ISS activity [16], IFN-α induction was comparable to that of R848 and imiquimod (IMQ) at their previously determined optimal concentrations (1 μM and 10 μM, respectively) (**Fig. 1**). However, when titrated to lower concentrations, C274 displays much more potent IFN-α-inductive ability than does R848 or IMQ, with optimal induction at <1 μg/ml (0.15 μM). This "bell-shaped curve" type of IFN-α response has been described previously for CpG-C [16, 17]. CpG-A D19 proved most potent of all stimuli for maximal IFN-α induction, while CpG-B 1018 induced marginal levels. These results indicate that the A and C classes of ISS induce higher maximal levels of IFN-α than synthetic TLR7Ls.

The presence of TLR7Ls suppresses TLR9L-induced IFN-α

Because the IFN-α inductive capacities of CpG-C and TLR7Ls are similar in that both types of ligands signal through TLR family members and both act exclusively on PDCs, we determined whether any counter-regulation existed between them in their induction of IFN-α from PBMCs. **Figure 2A** shows that

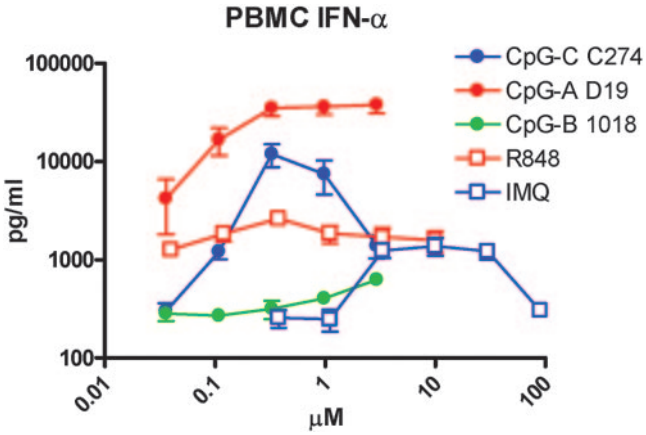

Fig. 1. Immunostimulatory sequence (ISS) is a more potent inducer of maximal IFN-α production from peripheral blood mononuclear cells (PBMCs) than Toll-like receptor 7 ligands (TLR7Ls). Human PBMCs were cultured at 5×10^5 cells/well for 20–24 h with titrated concentrations of CpG-A D19, CpG-B 1018, CpG-C C274, R848, and imiquimod (IMQ). Supernatants (SNs) were assayed for IFN-α via ELISA. Data are expressed as means ± SEM from 4 donors and is 1 of 3 representative experiments.

the TLR7Ls R848 and IMQ used at their optimal IFN-α-inducing dose markedly reduced the IFN-α-inducing capacity of C274, bringing total secreted IFN-α levels close to those observed with TLR7L alone, even at the most optimal concentration of C274. Subsequent dose titration experiments showed this inhibitory effect to be dose-dependent, with a declining concentration of TLR7L correlating with less inhibitory effect (data not shown). To determine whether this was a general phenomenon that applied to multiple types of TLR9L, we also stimulated PBMCs with CpG-A D19 in the presence and absence of TLR7Ls. Despite D19 exhibiting an even more potent IFN-α-inductive capacity than C274, we still observed that the presence of TLR7Ls inhibited D19's effect on IFN-α while retaining their own ability to induce modest levels of IFN-α (Fig. 2B). To extend these observations, we determined whether R848 could also inhibit IFN-α induction by a natural TLR9L, inactivated HSV-1, previously shown to promote IFN-α expression in a TLR9-dependent manner [19]. For comparison, we also included the inactivated influenza strain PR8, a known TLR7L and IFN-α-inducer. Interestingly, we observed that the presence of R848 inhibited the high IFN-α induction of HSV-1 but not PR8 (Fig. 2C). Even when we optimized the culture system for maximum TLR9L IFN-α-inducing capacity, by using the most potent examples of CpG-C (C695 and C792) complexed with PMXB, a polycationic carrier known to greatly enhance ISS-mediated IFN-α levels [20], and stimulated PBMCs enriched with purified PDCs added back to 3%, we still observed dramatic IFN-α reduction (Fig. 2D). We also stimulated cells with the TLR3L and known IFN-α-inducer poly(I:C) but found that R848 was markedly more potent for IFN-α induction and consequently could not measure any potential inhibitory effect on poly(I:C) in this assay (data not shown). These results indicate that synthetic TLR7Ls can interfere with the ability of potent TLR9Ls to induce IFN-α but do not interfere with potent natural TLR7Ls.

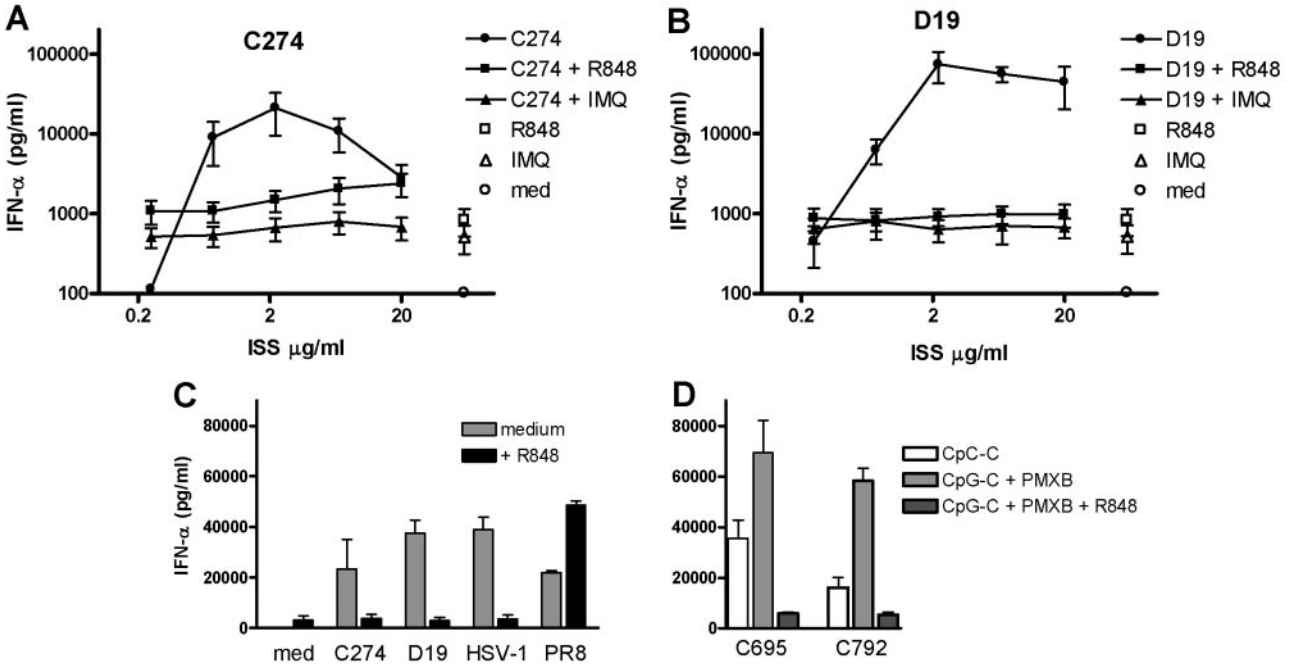

Fig. 2. The presence of R848 suppresses TLR9L-induced IFN-α. Human PBMCs were cultured at 5×10^5 cells/well for 20–24 h with (A) titrated concentrations of C274, (B) titrated D19, (C–D) high IFN-α-inducers HSV-1 (10 multiplicity of infection (MOI)), influenza PR8 (10 MOI), CpG-C C695/Polymyxin B (PMXB) (5/100 μg/ml), and CpG-C C792/PMXB (5/100 μg/ml), all in the presence or absence of 1 μM R848 or 100 μM IMQ. Supernatants were assayed for IFN-α using ELISA. Data are expressed as mean ± SEM from 4 donors and is 1 of 3 representative experiments (A–B) and mean ± SEM from 3 donors (C–D). Statistical significance was computed vs. C274 values using the paired *t* test.

R848 suppression is conducted through the PDC and is specific to IFN-α secretion and BDCA-2 expression

R848 has shown ligand specificity for both the TLR7 and TLR8 receptors [21]. Because non-PDC cell types like monocytes and MDCs are known to express TLR8 in humans [22] (TLR7 expression on human myeloid cells is controversial), it is possible that the negative influence of R848 on ISS-induced IFN-α might be carried out indirectly through another cell type in PBMCs other than the PDC. To address this question, we purified PDCs to >98% purity through BDCA-4 MACS microbead-positive selection and stimulated them with combinations of ISS and R848. As expected, we found that CpG-C C274, CpG-A D19, and R848 were all able to induce IFN-α production from PDCs, although the ISS ODNs were more potent (**Fig. 3A**). However, when R848 was used in conjunction with ISS, the induction of IFN-α by both C274 and D19 were inhibited, although the effect on D19 was more dramatic. In support, we also observed that IFN-α induced by combination R848 + C274 stimulation was detectable in PDCs via ICS at the optimal time point for stimulation by R848 alone, 4 h, and was not detectable at the optimal time point for C274 alone, 10 h (Fig. 3D). This indicates that the R848 molecule is acting directly on the PDC to inhibit the IFN-α-inducing activity of ISS and does not require the presence of secondary cell types to carry out this effect. In addition, since human PDCs are known to be $TLR7^+$ but $TLR8^-$ [23], this inhibitory activity appears to be mediated through TLR7. When TNF-α and IL-6 induction by ISS and R848 were also monitored, we did not observe any inhibitory regulation by R848 (Fig. 3, B and C).

We also examined the maturation state of the PDCs after ISS and R848 stimulation. When PDCs encounter a maturative signal, they typically increase the expression of molecules involved with antigen presentation to and costimulation of T cells, such as CD80, CD86, CD54, CD40, and MHC class II, while down-regulating the PDC marker and C-type lectin receptor BDCA-2 and augmenting the chemokine receptor CCR7, which allows homing to secondary lymphoid tissue [2, 24]. We stimulated purified PDCs with CpG-C C274 and/or R848 for 24 h and found that both TLRLs enhanced the expression of CD80 and CD86 to equivalently maximal levels (data not shown). C274 substantially down-regulated BDCA-2 by 50%, while increasing CCR7 expression 2.5-fold (**Fig. 4**). R848 was less effective at decreasing BDCA-2 (21%), but more effective at augmenting CCR7 (4.7-fold). In combination, C274 + R848 stimulation was additive for CCR7 enhancement (6.5-fold), and the effect on BDCA-2 (19%) was similar to that of R848 alone. In contrast, the natural TLR7L PR8 had little effect on PDC maturation markers, and the effect of C274 + PR8 was essentially the same of C274 alone, indicating this effect may be limited to synthetic TLR7Ls. To determine whether the effects of C274 or R848 on BDCA-2 were dependent on IFN-α, we blockaded type I IFN signaling using a neutralizing Ab cocktail, and observed no change in the effects on BDCA-2 expression, indicating IFN-α-independence of this function. As PDCs mature, they lose IFN-α productive capacity as well as BDCA-2 expression, and a slow-down of BDCA-2 diminishment by R848 may represent a delay in differentiation to mature DC status. These data suggest that the triggering of TLR9 in the midst of TLR7 signaling by R848 may result in two functional changes in the ability of the TLR9L to stimulate

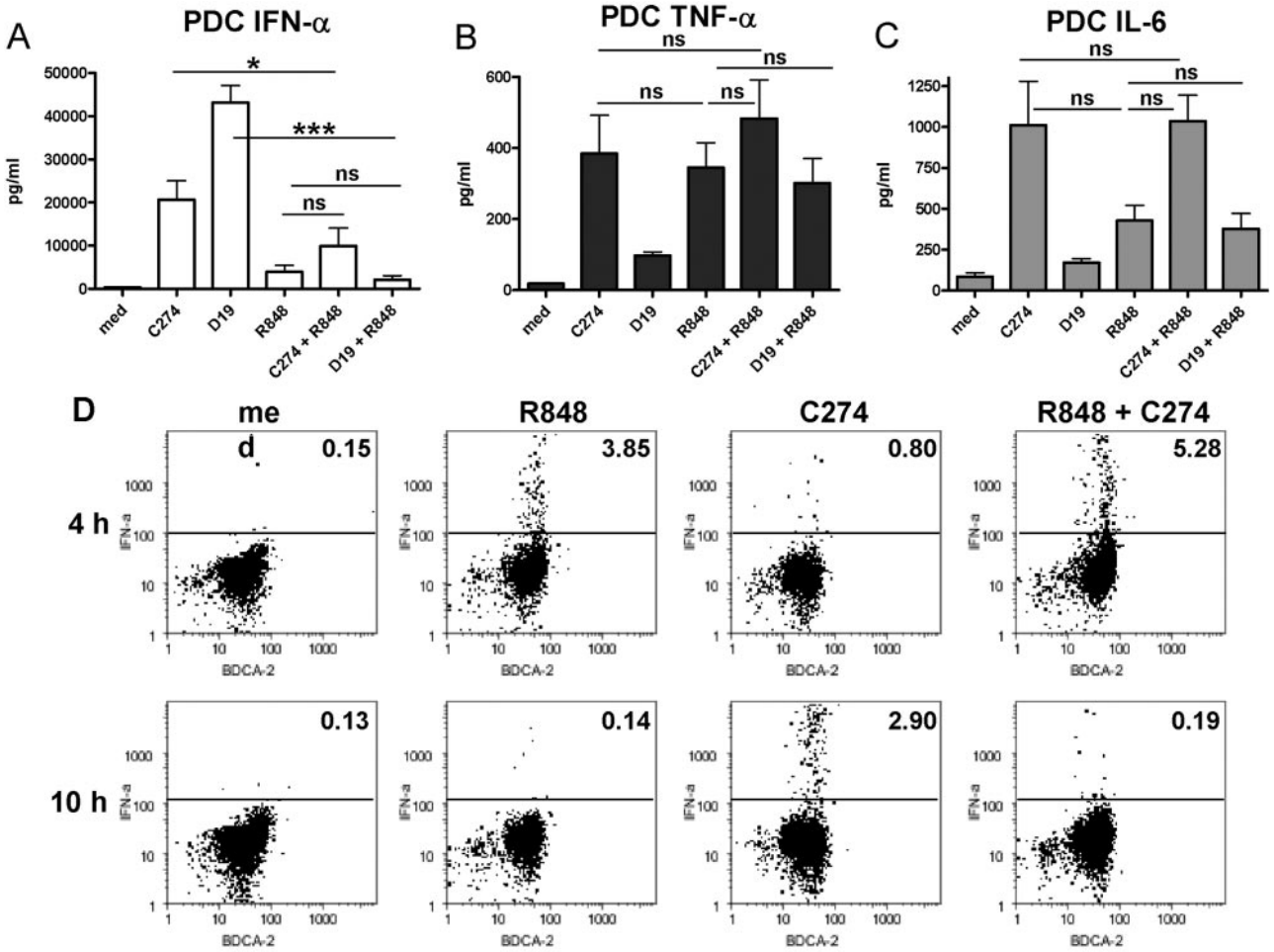

Fig. 3. R848 suppression is conducted through the PDC. (A–C) Human PDCs were cultured at 3–5 × 10^4 cells/well for 20–24 h with 5 μg/ml C274 ± 1 μM R848 and 5 μg/ml D19 ± 1 μM R848. SNs were assayed for IFN-α, tumor necrosis factor-α (TNF-α), and IL-6 via ELISA. Data are expressed as mean ± SEM from 4 donors and is 1 of 3 representative experiments. Statistical significance was calculated using one-way ANOVA. (D) Human purified PDCs (pooled from 2 donors) were cultured at 3-5 × 10^4 cells/well for 4 h and 10 h with C274 ± R848 followed by BFA addition and overnight culture as described in Methods. Intracellular IFN-α was assayed via flow cytometry. Results shown are representative of 3 experiments.

the PDC: *1*) a reduced ability to induce IFN-α production, and *2*) an altered ability to induce PDC maturation.

R848 has no effect on ISS-induced B cell functions

Additionally, we examined the activity of R848 and C274 on B cells, the other $TLR9^+$ subset contained within PBMCs. Figure 4 demonstrates that both C274 and R848 are able to induce proliferation of and IL-6 production by purified B cells. R848, however, is substantially less potent than C274. Moreover, the presence of R848 had no impact on the inductive abilities of C274 (**Fig. 5**). Therefore, the lack of inhibition by R848 on CpG-C-induced B cell functions or on PDC IL-6 secretion indicates that the inhibitory effect of R848 on C274 is a selective one, primarily for PDC IFN-α secretion.

R848 suppression is dominant over TLR9L-induced IFN-α

The inhibitory effect of R848 could be exerted through competition with ISS for binding to TLR9 or through the modulation of critical factors in the TLR9 signaling pathway. To determine whether R848 needs to be present simultaneously with ISS to mediate its inhibitory effects, we stimulated PBMCs with R848 or C274 for 4 h before washing the cells and restimulating them with TLR9Ls. PBMCs that had been previously exposed to R848 were no longer able to respond to the TLR9Ls CpG-C, CpG-A, or HSV-1 with IFN-α production (**Fig. 6A**). On the other hand, prestimulation for 4 h with C274 did not significantly impact subsequent stimulation with either R848 or other TLR9Ls in regards to IFN-α induction. Interestingly, pre-exposure to the natural TLR7L PR8, an inactivated influenza strain, did not result in subsequent resistance to ISS stimulation and in fact led to an additive effect on IFN-α induction (Fig. 6B). Pre-exposure to R848 also made the cells resistant to subsequent restimulation with R848 itself, with the levels of IFN-α maintained at a level 60% lower than control cultures. This may be an example of tolerance to TLR stimulation, as has been shown with LPS. In conclusion, pre-exposure to the synthetic TLR7L R848, but not the natural TLR7L PR8, rendered IFN-α-producing cells unable to respond to later stimulation with TLR9Ls.

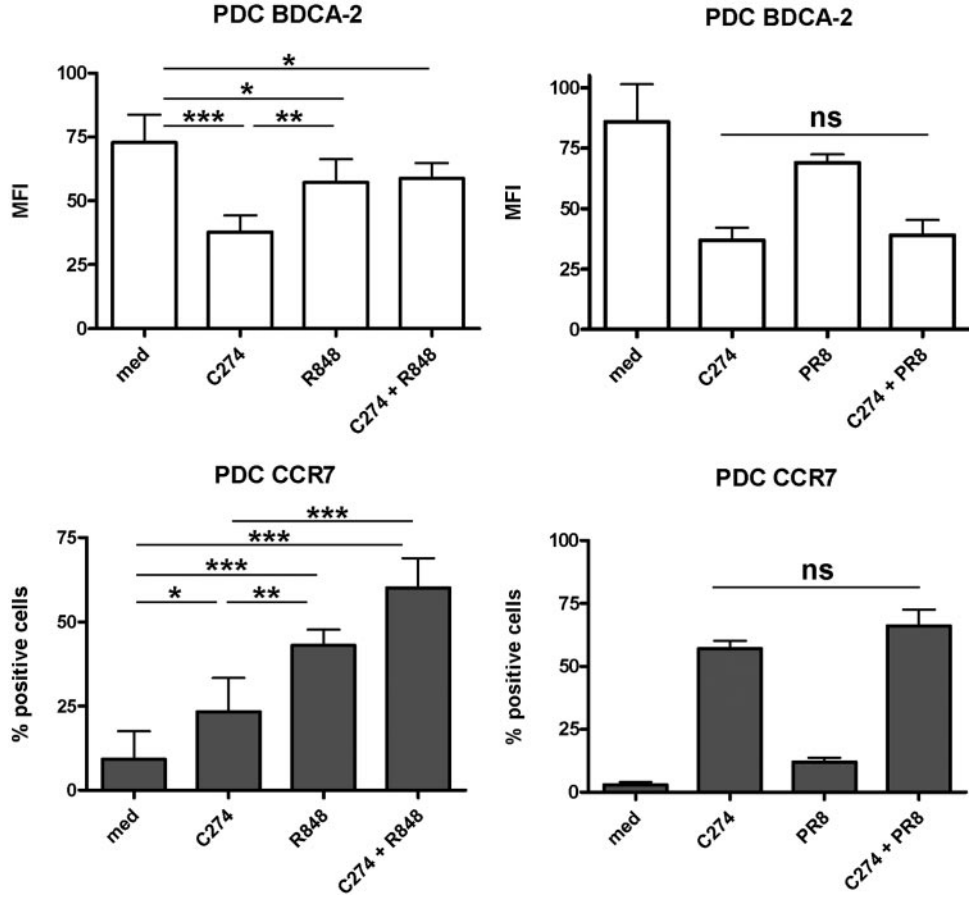

Fig. 4. R848 blocks ISS effect on BDCA-2 on PDCs. PDCs were cultured at 3-5 × 10^4 cells/well for 20–24 h with 5 μg/ml C274 ± 1 μM R848 or 10 MOI PR8. PDCs were harvested, stained for surface marker expression, and assessed by flow cytometry. Data are expressed as mean fluorescence intensity (MFI) ± SEM from 6 donors, and statistical significance was calculated using one-way ANOVA.

R848 induces a TLR7^{hi} TLR9^{lo} expression profile with faster kinetics than TLR9Ls

Since we had found the ability of ISS to induce IFN-α to be severely impaired in the presence of R848, we examined TLR9 expression in cells that had been stimulated with TLR7Ls. Potent TLR7Ls, such as R848 and PR8, were observed to quickly, if modestly, up-regulate TLR7 expression in PBMCs by 2 h while simultaneously down-regulating TLR9 expression, detectable by 1 h and very substantially by 4 h (**Fig. 7, A** and **B**). By 10 h, TLR7 levels induced by PR8 are still elevated, while R848-induced TLR7 is approaching baseline expression. However, TLR9 levels remain low by 10 h. IMQ was equivalently potent to the other TLR7Ls for TLR9 reduction, although not for TLR7 enhancement. Interestingly, C274 also induces a similar TLR7^{hi} TLR9^{lo} profile by 10 h, but its kinetics are considerably slower, and 10 h is the optimal time point for this effect compared with the 2–4 h observed with the TLR7Ls. Therefore, although both TLR7Ls and CpG-C eventually induce a similar TLR expression profile (TLR7^{hi} TLR9^{lo}) in PBMCs, TLR7Ls achieve this much more rapidly. These more rapid kinetics are also observed with IFN-α RNA expression (Fig. 7C). R848, IMQ, and PR8 all markedly enhanced IFN-α expression by 1 h, while C274 exhibited slower kinetics, reaching its maximal expression at 10 h.

Within bulk PBMCs, TLR9$^+$ cells include PDCs and B cells [25, 26]. TLR7 is synthesized by those same cell types and has sometimes been reported to be expressed by myeloid lineage cells like monocytes and MDCs [26]. To determine in which PBMC cell type compartment these modifications in TLR7/9 expression are occurring, we isolated purified PDCs and B

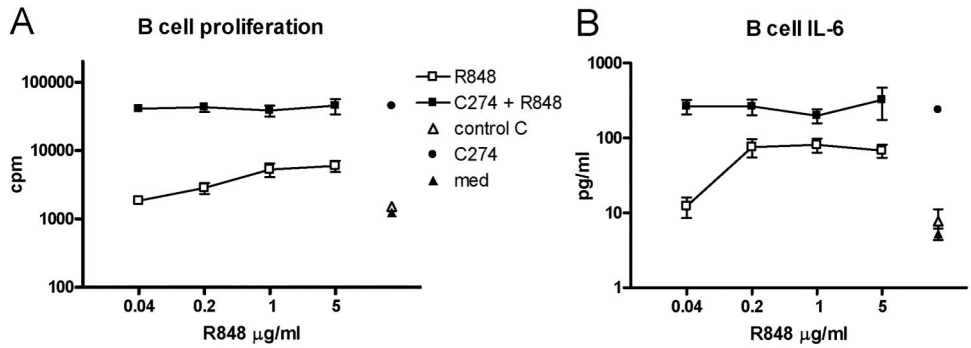

Fig. 5. R848 does not suppress C274 functions on B cells. Human purified B cells were cultured at 2 × 10^5 cells/well for 4 days with 5 μg/ml C274 ± titrated R848 and control type-C ODN. (A) B cell proliferation was assessed by ^{3}H-thymidine incorporation, and (B) B cell SNs were assayed for IL-6 via ELISA. Data are expressed as mean ± SEM from 4 donors and is 1 of 2 representative experiments. None of the C274 + R848 points were significantly different from C274 in both assays, using the paired *t* test.

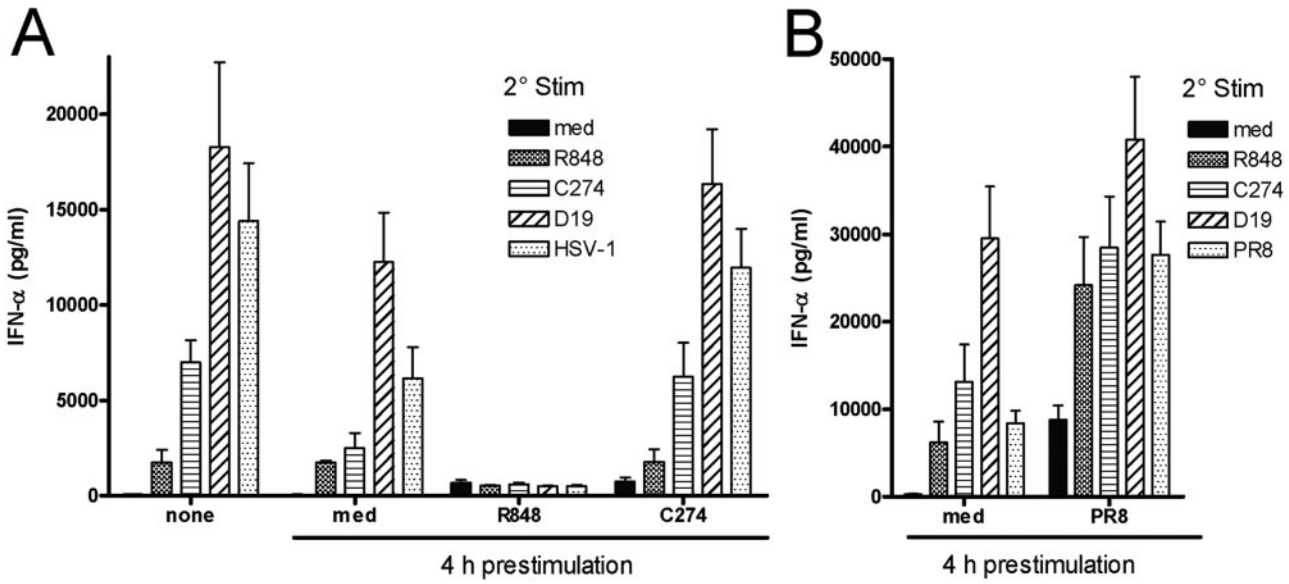

Fig. 6. R848 suppression is dominant over TLR9L-induced IFN-α. Human PBMCs were pretreated for 4 h with (A) C274, R848, or (B) PR8 and then secondarily stimulated overnight with various TLR7Ls or TLR9Ls. SNs were assayed for IFN-α content via ELISA. Data are expressed as mean ± SEM from 4 donors and is 1 of 2 representative experiments.

cells from PBMCs and examined TLR expression after stimulation with C274 or TLR7Ls. PDCs displayed the same shift toward TLR7 elevation and TLR9 reduction that we observed in PBMCs (Fig. 7, D–E). Notably, the reduction in TLR9 appeared earlier with TLR7Ls than with C274. Similarly, rapid kinetics were likewise found for TLR7Ls with regard to IFN-α expression in PDCs (Fig. 7F). Both R848 and PR8 were more potent than C274 for IFN-α RNA induction by 2 h, although C274 was much more effective than R848 by the 10 h time point, which accounts for the higher levels of secreted IFN-α protein induced by C274 compared with R848 (Fig. 1). Similarly to PDCs, B cells also demonstrated a shift to a $TLR7^{hi}$ $TLR9^{lo}$ profile after stimulation with either R848 or C274 (Fig. 7G), demonstrating that the reduction in TLR9 expression and elevation in TLR7 expression observed in PBMCs was contributed to by both PDCs and B cells, the only components of PBMCs that express both TLR7 and TLR9. It seems unlikely that this rapid down-regulation of TLR9 expression in PDCs by TLR7Ls is solely responsible for the mechanism of R848-mediated suppression of ISS-induced IFN-α; however, since other TLR9-mediated functions of PDCs (IL-6) and of B cells are not inhibited by R848. Nonetheless, it is clear that signaling through TLR7 leads to counter-regulation of TLR9 activity on multiple fronts, both directly on IFN-α induction during simultaneous ISS and TLR7L exposure, and on TLR9 expression itself, which would prevent further TLR9 signaling subsequent to the initial TLR7L exposure.

Maintenance of high levels of IRF-7 mRNA by ISS is disrupted by R848

In an attempt to define the mechanism of action of R848's inhibitory activity, we examined the intracellular compartmentalization of ISS within the PDC in the presence of R848. Previous studies have demonstrated that potent IFN-α induction by certain ISS ODNs is dependent upon localization to the early endosomal compartment, whereas the induction of other ISS activities such as PDC maturation occurs through TLR9 signaling from the late endosomal compartment [27]. The localization of fluoresceinated ISS to these compartments can be determined by costaining with anti-transferrin receptor (early endosome) and anti-LAMP-1 (late endosome) and visualization by confocal microscopy. We examined purified human PDCs that had been incubated with fluoresceinated C274 or D19 in the presence or absence of R848 and found that R848 did not alter the ability of D19 and C274 to localize to the early endosome (data not shown), arguing against modifying the compartmentalization of ISS as a mechanism for inhibition of IFN-α.

The determination of whether the inhibition of ISS-induced IFN-α secretion is mediated by a soluble factor induced from PDCs by TLR7Ls is difficult, because analysis of the activity of supernatants (SNs) from TLR7L-stimulated PDCs will be confounded by unknown quantities of residual TLR7L. However, we did seek to neutralize IL-10 in our cultures with blocking Ab to the cytokine and receptor, since IL-10 is a well-characterized inhibitory cytokine that has a known ability to counter the effects of IFN-α [28, 29]. We found that neutralization of IL-10 did not affect the inhibitory properties of SNs derived from R848- or IMQ-stimulated PBMCs (data not shown), ruling out IL-10 as the mediator of TLR7L effect.

Focusing on internal cellular events, we also examined the expression of molecules critical for signal transduction through TLR9. Triggering of TLR9 is known to activate two signaling pathways dominated by the transcription factors IRF-7 and nuclear factor κB (NFκB) [30, 31]. The IRF-7-mediated pathway of signal transduction is responsible for the transcription of IFN-α and IFN-β, which then promote the transcription of downstream genes, including cytokines and antiviral factors [32, 33]. On the other hand, activation of the NFκB pathway by TLR9 triggering leads to the up-regulation of activation mark-

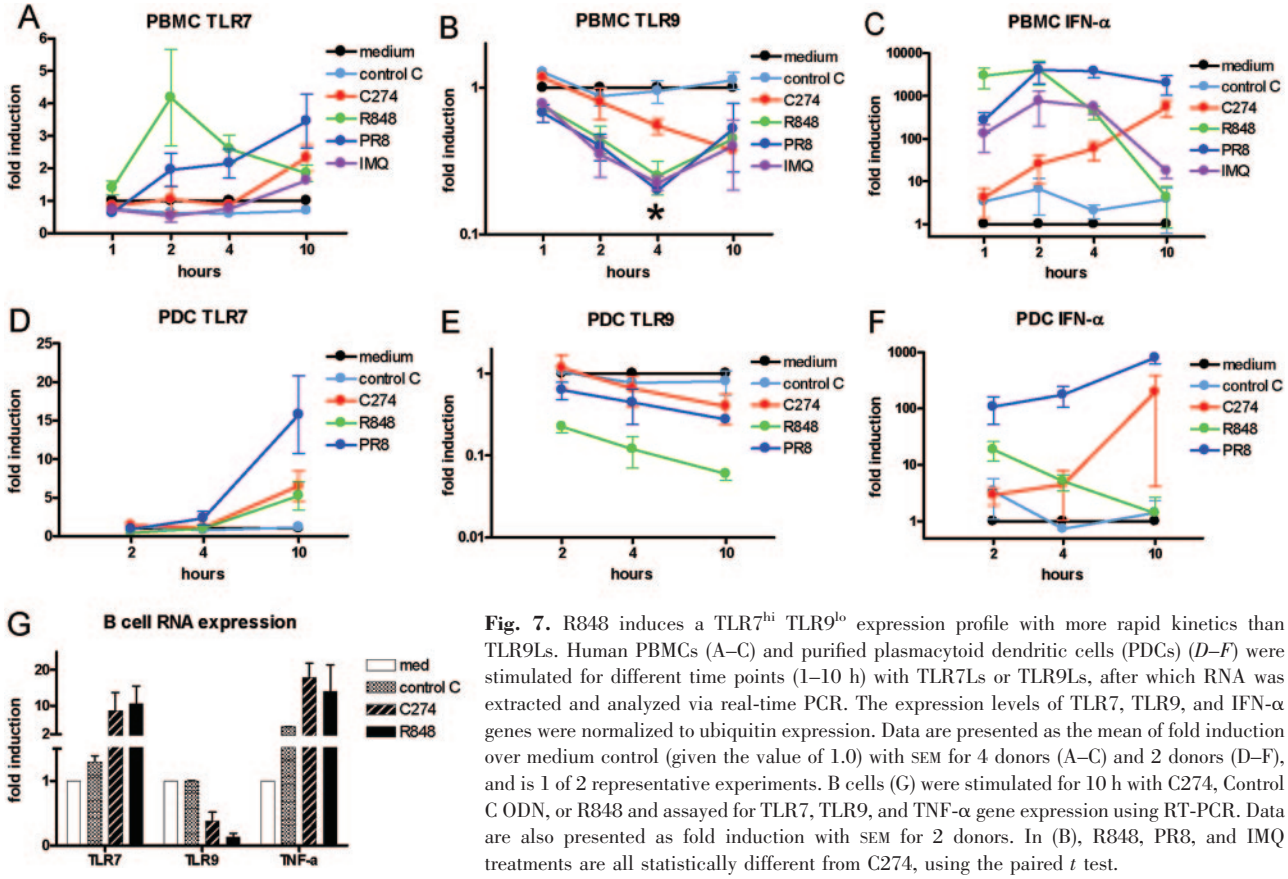

Fig. 7. R848 induces a TLR7^{hi} TLR9^{lo} expression profile with more rapid kinetics than TLR9Ls. Human PBMCs (A–C) and purified plasmacytoid dendritic cells (PDCs) (*D–F*) were stimulated for different time points (1–10 h) with TLR7Ls or TLR9Ls, after which RNA was extracted and analyzed via real-time PCR. The expression levels of TLR7, TLR9, and IFN-α genes were normalized to ubiquitin expression. Data are presented as the mean of fold induction over medium control (given the value of 1.0) with SEM for 4 donors (A–C) and 2 donors (D–F), and is 1 of 2 representative experiments. B cells (G) were stimulated for 10 h with C274, Control C ODN, or R848 and assayed for TLR7, TLR9, and TNF-α gene expression using RT-PCR. Data are also presented as fold induction with SEM for 2 donors. In (B), R848, PR8, and IMQ treatments are all statistically different from C274, using the paired *t* test.

ers like CD80 and CD86 and the secretion of cytokines like IL-6 and TNF-α from PDCs and B cells [31, 34]. We determined whether the activation of these two pathways by ISS was altered in the presence of R848. PDCs were purified from PBMCs, and IRF-7 mRNA expression was measured over a time course of stimulation with CpG-C C274 and/or R848. Stimulation with C274 kept IRF-7 peak expression at levels ~2-3-fold higher than those induced by R848 (**Fig. 8A**). However, IRF-7 mRNA levels in PDCs stimulated by the combination of C274 and R848 were not substantially different than those stimulated by R848 alone. Accordingly, the pattern of IFN-α mRNA expression over time confirms the dominance of the effect of R848 over C274 (Fig. 8B). This inhibitory effect was dose-dependent since decreasing doses of R848 resulted in diminished capacity to suppress IRF-7 or IFN-a mRNA induction by C274 (Fig. 8, C and D). These data suggest that R848 down-regulates the IFN-α-inducing activity of ISS by eroding the ability of ISS to up-regulate IRF-7 expression.

To measure activation of the NFκB pathway, we detected expression of the phosphorylated form of IκBα in purified human PDCs after ISS and R848 stimulation. IκBα is an inhibitor protein that binds to NFκB and prevents its translocation across the nucleus. Once phosphorylated, IκBα releases NFκB, which can translocate and activate genes with NFκB site-containing promoters. The levels of phosphorylated IκBα induced by C274 was not affected by the presence of R848 (data not shown), verifying that the NFκB signal transduction pathway is still activated to equivalent levels and thus does not account for diminished ISS activity. This result agrees with our findings that those ISS-induced activities that are mediated by IRF-7 (e.g., IFN-α induction), and not by NFκB (e.g., B cell functions), are reduced by TLR7Ls.

DISCUSSION

The majority of experimental work performed to date to examine the function of TLRs have focused on individual members of the TLR family by using specific ligands, both natural and synthetic. Solitary TLR activation rarely occurs in vivo; however, because microbes present a host of PAMPs that are recognized by the host virtually simultaneously. Thus, the normal context in which TLR signaling is experienced in vivo is simultaneous with the activation of other PRRs and specifically other TLRs. For this reason, the elucidation of TLR counter-regulation is important toward an understanding and prediction of the host immune response during pathogen attack. Some investigators have recently examined the issue of counter-regulation among the TLR repertoire. Synergistic coactivation has been observed between TLR3 and TLR7 [35–38], TLR4 and TLR7 [36–38], and TLR3 and TLR9 [39] and both synergism [40] and cross-tolerance [41] have been reported between TLR9 and TLR2/4. Simultaneous triggering of TLR7/8 and TLR9, however, is a less likely combination to be encountered in vivo, as a pathogen has yet to be described that displays both TLR7/8- and TLR9-specific PAMPs. The DNA genomes from viruses like HSV-1, HSV-2, and murine cytomegalovirus (MCMV), and the DNA from bacteria are rich in

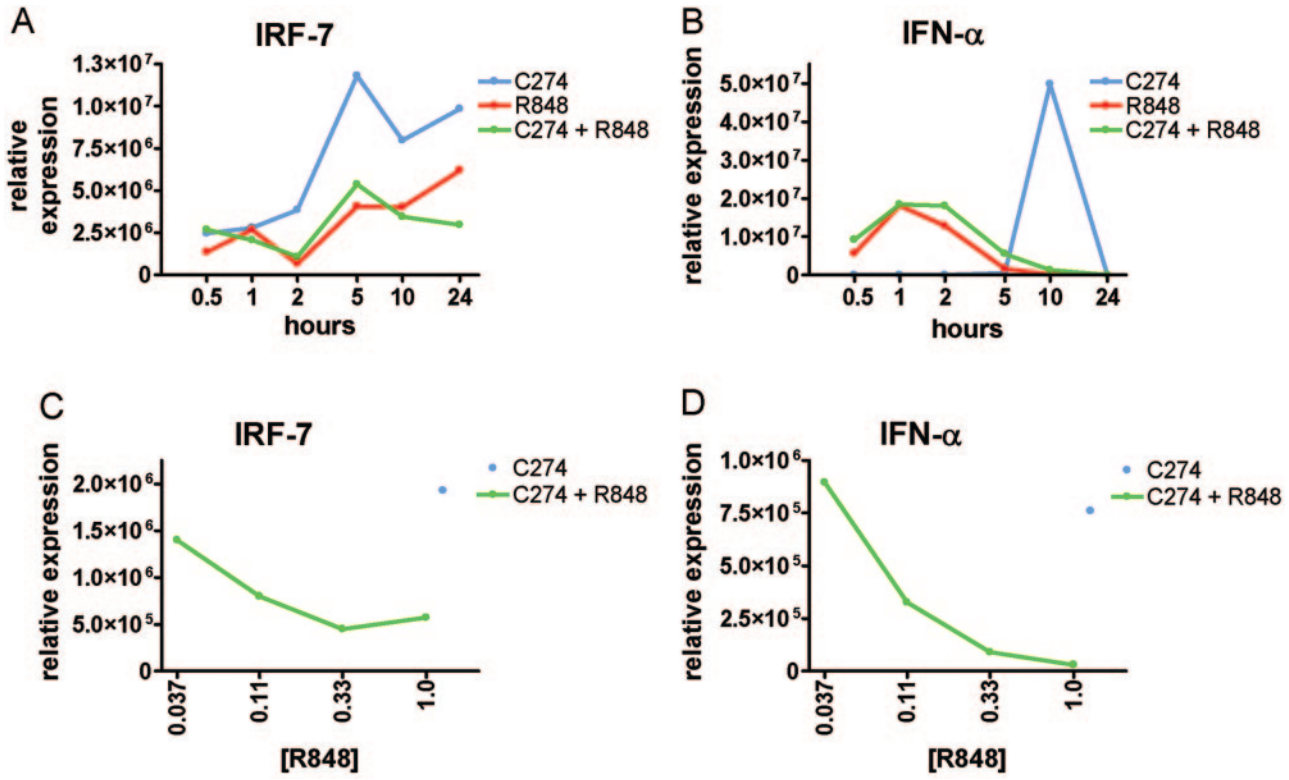

Fig. 8. R848 abrogates the ISS-induced increase in IRF-7 mRNA expression. (A–B) Human purified PDCs (pooled from 3 donors) were stimulated for different time points (1–24 h) with 5 μg/ml C274 ± 1 μM R848, after which RNA was extracted and analyzed via real-time PCR. (C–D) PDCs were stimulated with C274 ± a dose titration of R848 for 5 h, followed by RT-PCR analysis. The expression levels of IRF-7 and IFN-α genes were normalized to ubiquitin expression. Data are presented as relative expression and are from 1 of 3 representative experiments.

CpG motifs, which may be presented in single-strand (ss) or double strand (ds) DNA fragments to activate TLR9 [42, 43]. On the other hand, TLR7 and TLR8 recognize ssRNA from RNA viruses like influenza and VSV [42]. Therefore, activation of both TLR7 and TLR9 by a single pathogen is not currently predicted, and concurrent infection with a DNA virus and an RNA virus is a rare event. However, since these two TLRs (along with TLR3) rank among the most powerful inducers of type I IFN secretion, and given the clinical benefits of interferon therapy observed for indications like viral infection (HCV, HPV) and cancer (melanoma, renal cell carcinoma), it behooves us to investigate the possibility of the combined activation of TLR7 and TLR9 resulting in super-enhanced IFN-α induction.

Interestingly, our results indicate that activating the TLR7 pathway via small synthetic guanosine analogs simultaneously with activation of TLR9 can result in a down-modulation of the potential for TLR9-mediated IFN-α induction by PDCs, without affecting other TLR9-mediated PDC functions such as IL-6 secretion and CD80/CD86 expression. We show that when the TLR7/8L R848 is present, multiple types of TLR9Ls are inhibited from inducing IFN-α, including CpG-C C274, CpG-A D19, and the natural TLR9L HSV-1. This effect appears to be mediated directly through the PDC, and thus through TLR7 and not TLR8, since PDCs are $TLR8^{-}$. The state of PDC maturation induced by the triggering of both TLR7 and TLR9 is also dominated by TLR7, namely, the marked elevation in numbers of PDCs expressing CCR7 and the diminished loss of BDCA-2 expression. However, the levels of IL-6 induced by ISS from PDCs and the activation of B cells by ISS is not significantly altered by the presence of R848, indicating that the dominant effects of R848 on ISS-stimulated functions are selective. R848 does not appear to mediate its effect through blocking ISS from binding to TLR9, since pretreated cells retain their unresponsiveness to ISS even 4 h after exposure to R848, nor does R848 alter the endosomal localization of ISS within the PDC. R848 does prevent ISS from inducing higher levels of IRF-7 expression without affecting activation of the NFκB pathway, thus indicating interference with TLR9 signaling as a possible mechanism. Finally, in addition to counter-regulating IFN-α expression induced by ISS, TLR7Ls also stimulate for rapid up-regulation of TLR7 and down-regulation of TLR9 expression in PDCs and B cells within the first few hours of exposure, further contributing to a down-regulation of the TLR9 signaling pathway.

The mechanism by which synthetic TLR7Ls achieve their inhibitory effect is unclear. We have ruled out that R848 is inhibiting ISS-induced IFN-α simply through a cytopathic effect on the cells. MTT cell viability assays conducted on purified PDCs showed that R848 and IMQ did not exert detectable toxicity either alone or in the presence of ISS (data not shown). This is consistent with Schlaepfer et al., who found that R848 did not alter the metabolic activity of human tonsillar cells after 7 days of treatment in vitro [44]. We also determined that R848 did not interfere with the localization of ISS to the

early endosome, critical for IFN-α signaling [27], and that R848 was not suppressing IFN-α induction via IL-10.

The signaling capabilities of TLR7Ls and TLR9Ls appear to be very similar, in that they both up-regulate at least two main signaling pathways, the critical factors of each being IRF-7 and NFκB. IRF-7 is expressed constitutively in PDCs, and after stimulation by TLR7Ls or TLR9Ls becomes phosphorylated by TANK binding kinase 1 (TBK1) and IκB kinase ε (IKKε), then associates with MyD88 in the endosome, leading to sustained signaling. As an activated homodimer, IRF-7 translocates to the nucleus and binds to positive regulatory domain (PRD) sites in the promoter regions of type I interferon genes (reviewed in [45]), leading to the elevated expression of many IFN-α subtypes, IFN-β, and IFN-ω. High levels of type I interferon are, in turn, responsible for the up-regulation of numerous antiviral genes such as 2,5-oligoadenylate synthetase (OAS), interferon-stimulated gene-54K (ISG-54K), guanylate binding protein-1 (GBP-1), Mx-A (ortholog of mouse myxoma resistance gene-A), and GTPases [32, 33]. On the other hand, triggering of TLR7/TLR9 also results in the activation of the Ser/Thr kinase IKKα, which phosphorylates the inhibitor protein IκBα, releasing it from its association with NFκB. NFκB subunits are free to translocate to the nucleus, where they associate with NFκB binding sites and activate a wide range of genes, including cytokines like TNF-α, IL-6, and IP-10, and costimulatory receptors such as CD80, CD86, and CD40. Therefore, it appears that the activation by TLR7Ls and TLR9Ls of these two signaling pathways (IRF-7 vs. NFκB) in PDCs corresponds with the two major arms of immune functions induced by TLR7/9 triggering in that cell type (type I IFN production and IFN-α-dependent downstream effects vs. proinflammatory cytokines, costimulatory molecules, and antigen presentation).

It is unclear whether these two signaling pathways may positively or negatively counter-regulate each other. It has been suggested that potent IFN-α-inducers such as CpG-A may be inhibited by efficient activation of the NFκB pathway, especially since CpG-A itself is an inefficient activator of NFκB [46], which may be indicative of an antagonistic relationship. If so, R848 may be exerting its effects on ISS-induced IFN-α by amplifying the NFκB pathway, which, in turn, may cause a suppression of the IRF-7 pathway and type I interferon expression. As TLR7 and TLR9 appear to trigger very similar signaling pathways with similar functional outcomes, this suggests that they also employ similar programs for termination of signal.

Since we have observed that the kinetics of the response triggered by TLR7 (e.g., type I IFN mRNA transcription, intracellular and secreted IFN-α protein, alterations in TLR mRNA expression) are much more rapid when compared with the kinetics of the TLR9-mediated response, it is probable that the down-regulatory mechanism induced through TLR7, whatever it is, also has more rapid kinetics, and that this is responsible for inhibiting the ability of subsequent TLR9 triggering to induce IFN-α. However, this suppressive mechanism appears to be specific to the IRF-7 signaling pathway and the induction of type I interferon, since we have shown that NFκB-mediated functions induced by ISS (e.g., B cell activity, PDC CD80/86 expression) are unaffected by the presence of TLR7L. Viral infection of PDCs is known to induce the phosphorylation of constitutively expressed IRF-3, which, in turn, promotes the expression of IFN-β. IFN-β autocrinely activates the PDC through IFN-α/βR and causes the formation of interferon-stimulated gene factor (ISGF3) (composed of STAT1, STAT2, and IRF-9), which then binds to ISRE promoter sites in the IRF-7 gene, enhancing IRF-7 expression. Enhanced IRF-7 expression participates in a positive feedback loop for augmented IFN-α production (reviewed in [45, 47]). Thus, the TLR7L-mediated suppression of ISS-induced IFN-α that we observe may take the form of reduced expression of IRF-7 mRNA, which we observe is elevated to high levels by ISS but is lowered more than twofold in the presence of R848 (Fig. 8). Furthermore, reduction in the level of IRF-7 phosphorylation, required for activation of the IRF-7 pathway, may be another method by which TLR7Ls can reduce ISS-induced IFN-α expression.

We observed that TLR7 signaling accelerated the down-regulation of TLR9 expression in PDCs (Fig. 7). It seems unlikely that this accounts for the mechanism of R848's action on ISS-induced IFN-α induction, because *1*) if it were, it would be expected that all TLR9-dependent ISS activities, not just IFN-α induction, should be inhibited by R848, *2*) the reduction in TLR9 protein expression would have to occur immediately after the TLR9 mRNA decline to have an effect, requiring extremely rapid turnover kinetics, and *3*) the natural TLR7L PR8 exhibits similarly rapid kinetics yet does not exert dominant effects on ISS signaling in the way that R848 and IMQ do. However, it is still probable that the down-regulation of TLR9 does serve to limit subsequent ISS functionality after TLR7 signaling. Indeed, TLR7 and TLR9 show further similarity in their regulatory processes in that TLR9 signaling itself, like TLR7, also leads to a down-regulation of TLR9 and further ISS unresponsiveness (Fig. 7; [48]). This may be indicative of tight control over IFN-α secretion, whether instigated by TLR9 or TLR7, to prevent the triggering of autoimmunity such as SLE [3].

Counter-regulation between TLR7/8Ls and oligonucleotides has also been recently reported by Jurk et al. [49], who observed that R848-induced NFκB activation in HEK293 TLR7-transfectants, as well as loxoribine-induced IFN-α induction from human PBMCs and PDCs could be reduced and inhibited by the presence of PS ODNs. The authors show this to be a CpG-independent phenomenon in the case of the HEK293 cells, although it was observed that an oligo-dT(17) sequence was more potent than randomized sequences. The CpG dependency of the effect on IFN-α induction is unclear, as no CpG oligos were used in those studies, presumably because since they themselves induce IFN-α, a simultaneous reduction in TLR7L-induced IFN-α would be difficult to discern. Nevertheless, Jurk et al. observe that TLR7 signaling can be dominated by the presence of a ssDNA oligo (CpG-containing or not), which appears to contradict our observations that TLR7L signaling is dominant over TLR9Ls (CpG ODNs). However, those authors did not use R848 in their PDC IFN-α studies, while we did not use loxoribine in our studies, so it may be that different TLR7Ls can differentially regulate or be regulated by TLR9Ls, as supported by our observations of differential activity between synthetic TLR7L R848 and natural TLR&L

PR8. Furthermore, Jurk et al. speculate that they are observing the interference of TLR7L binding to TLR7 by the presence of poly-T ODNs. However, steric interference seems an unlikely possibility to explain our findings, since we do not observe that R848 inhibits other ISS functions mediated by TLR9, such as B cell activation, suggesting a selective inhibition and not a complete block, as prevention of ISS binding to TLR9 would imply.

Our study demonstrates that small-molecule TLR7Ls can negatively regulate the effects of TLR9 stimulation when both types of ligands are present to stimulate their main target cell, the PDC. The negative regulatory effects of TLR7Ls like R848 are specific, however, for the IRF-7-mediated pathway of ISS-induced functions, primarily characterized by massive up-regulation of type I interferon expression. However, other activities of ISS independent of the IRF-7 pathway are not affected by TLR7 coactivation. Because the kinetics of some TLR7L-induced responses are much more rapid than those induced by CpG ODNs, we speculate that the natural down-regulatory mechanism that limits TLR7 induction of IFN-α also serves to essentially halt TLR9-mediated activation of IFN-α before it gets started. Since activation of multiple strong inducers of IFN-α might lead to debilitating effects such as autoimmunity, this compensatory mechanism may have evolved as a preemptive defense system. These observations should serve to shed light on the unknown effects of combination therapy with TLR7Ls and TLR9Ls.

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