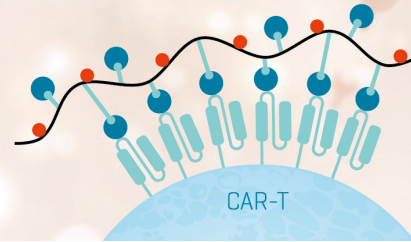


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# Synthetic TLR Agonists Reveal Functional Differences between Human TLR7 and TLR8

Keith B. Gorden, Kevin S. Gorski, Sheila J. Gibson, Ross M. Kedl,<sup>1</sup> William C. Kieper, Xiaohong Qiu, Mark A. Tomai, Sefik S. Alkan, and John P. Vasilakos<sup>2</sup>

Although TLR7 and TLR8 are phylogenetically and structurally related, their relative functions are largely unknown. The role of TLR7 has been established using TLR7-deficient mice and small molecule TLR7 agonists. The absence of TLR8-selective agonists has hampered our understanding of the role of TLR8. In this study TLR agonists selective for TLR7 or TLR8 were used to determine the repertoire of human innate immune cells that are activated through these TLRs. We found that TLR7 agonists directly activated purified plasmacytoid dendritic cells and, to a lesser extent, monocytes. Conversely, TLR8 agonists directly activated purified myeloid dendritic cells, monocytes, and monocyte-derived dendritic cells (GM-CSF/IL-4/TGF- $\beta$ ). Accordingly, TLR7-selective agonists were more effective than TLR8-selective agonists at inducing IFN- $\alpha$ - and IFN-regulated chemokines such as IFN-inducible protein and IFN-inducible T cell  $\alpha$  chemoattractant from human PBMC. In contrast, TLR8 agonists were more effective than TLR7 agonists at inducing proinflammatory cytokines and chemokines, such as TNF- $\alpha$ , IL-12, and MIP-1 $\alpha$ . Thus, this study demonstrated that TLR7 and TLR8 agonists differ in their target cell selectivity and cytokine induction profile. *The Journal of Immunology*, 2005, 174: 1259–1268.

One of the ways that the innate immune system recognizes pathogens is through TLRs. Specifically, TLRs recognize structurally conserved pathogen-associated molecular patterns, such as LPS and CpG containing bacterial DNA. There are at least 10 known human TLRs. All TLRs are type I integral membrane proteins with extracellular leucine-rich regions and intracellular regions that are homologous to the IL-1R signaling Toll IL-1R domain (1, 2). To date, all TLRs are linked to an adaptor protein, such as MyD88, Toll IL-1R adaptor protein, Toll IL-1R domain-containing adaptor protein inducing IFN- $\beta$ , and Toll IL-1R domain-containing adaptor protein inducing IFN- $\beta$ -related adaptor molecule (3–7). Activation of TLRs results in downstream activation of NF- $\kappa$ B and other transcription factors, culminating in transcription of numerous genes, including cytokines, chemokines, and costimulatory markers (8).

Although innate immune cells express the highest levels of TLRs, their mRNA expression profile differs from one cell type to another. For instance, human blood-derived myeloid dendritic cells (mDC)<sup>3</sup> express TLR2, -3, -4, and -5, whereas plasmacytoid DC (pDC) express TLR9 (9–12). Consistent with these mRNA expression patterns, myeloid blood DC produce cytokines in re-

sponse to their respective ligands, such as peptidoglycan and lipopeptides (TLR2), poly I:C (TLR3), LPS (TLR4), and flagellin (TLR5), but do not respond to CpG DNA (TLR9). Conversely, pDC produce cytokines in response to CpG DNA, but do not respond to PGN, poly I:C, LPS, or flagellin (13–18). Therefore, one can evaluate the specificity of TLR agonists using specific populations of cells with defined TLR expression patterns.

Natural agonists for the TLRs have been identified for some, but not all, TLRs. Natural agonists for TLR2, TLR3, TLR4, TLR5, TLR2/6, and TLR9 include peptidylglycan, dsRNA (poly I:C), LPS, flagellin, macrophage-activating lipopeptide 2, and bacterial DNA-containing CpG motifs, respectively. Natural agonists for TLR7 and TLR8 have recently been identified as guanosine- and uridine-rich ssRNA (19, 20). In addition, synthetic imidazoquinoline-like molecules, imiquimod (R-837), resiquimod (R-848), S-27609, and guanosine analogues such as loxoribine have been shown to activate NF- $\kappa$ B through TLR7, whereas resiquimod also activates NF- $\kappa$ B through TLR8. Both imiquimod and resiquimod have been identified as TLR7 agonists based on their inability to induce DC maturation or TNF- $\alpha$ , IL-12, or IFN- $\alpha$  production in TLR7-deficient mice. Additionally, imiquimod and resiquimod induce NF- $\kappa$ B activation in HEK293 cells transfected with human or mouse TLR7 (21, 22). Resiquimod has also been identified as a TLR8 agonist based on its ability to activate NF- $\kappa$ B in HEK293 cells transfected with human, but not mouse, TLR8 (23). Therefore, imiquimod and resiquimod can tentatively be defined as human TLR7 or TLR7/8 agonists, respectively.

In this study we first identified small molecules that were selective for human TLR7 or human TLR8. Then we evaluated their effects on several primary human innate immune cells. Despite the phylogenetic and structural similarities between TLR7 and TLR8, we found that activation of TLR7 and TLR8 by these new agonists has distinct consequences on the innate immune cells.

## Materials and Methods

### Reagents and media

Small molecule imidazoquinoline TLR7, TLR8, and TLR7/8 agonists (3M-001, *N*-[4-(4-amino-2-ethyl-1H-imidazo[4,5-c]quinolin-1-yl)butyl]methanesulfonamide; formula, C<sub>17</sub>H<sub>23</sub>N<sub>5</sub>O<sub>2</sub>S; m.w., 361; 3M-002,

3M Pharmaceuticals, St. Paul, MN 55144

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<sup>3</sup> Abbreviations used in this paper: mDC, myeloid dendritic cell; cRPMI, complete RPMI (RPMI 1640, 25 mM, sodium bicarbonate, HEPES, 1 mM sodium pyruvate, 1 mM L-glutamine, 1% penicillin/streptomycin, and 10% heat-inactivated FCS); DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate; IP-10, IFN- $\gamma$ -inducible protein-10; M-001, small molecule imidazoquinoline TLR7 agonist; 3M-002, small molecule imidazoquinoline TLR8 agonist; 3M-003, small molecule imidazoquinoline TLR7/8 agonist; 3M-006, inactive analog of 3M-001, 3M-002, and 3M-003; Mo-DC, monocyte-derived DC; pDC, plasmacytoid dendritic cell; I-TAC, IFN-inducible T cell  $\alpha$  chemoattractant.

2-propylthiazolo[4,5-c]quinolin-4-amine; formula, C<sub>13</sub>H<sub>13</sub>N<sub>3</sub>S; m.w., 243; 3M-003, 4-amino-2-(ethoxymethyl)- $\alpha$ , $\alpha$ -dimethyl-6,7,8,9-tetrahydro-1H-imidazo[4,5-c]quinoline-1-ethanol hydrate (formula, C<sub>17</sub>H<sub>26</sub>N<sub>4</sub>O<sub>2</sub>; m.w., 318) and an inactive small molecule TLR7/8 analog, 3M-006, were prepared by 3M Pharmaceuticals. All imidazoquinolines were prepared in DMSO (sterile cell culture grade; Sigma-Aldrich) at a concentration of 10 mM and stored in aliquots at 4°C. LPS (Ultra Pure; *Salmonella minnesota*) was obtained from InvivoGen. CpG oligonucleotide (CpG2216, GGGG GACGATCGTCGGGGGG) was obtained from Invitrogen Life Technologies. Phosphothioate-protected ssRNA (ssRNA40; 5'-GCCCCUCU GUUGUGUGACUC; HIV-1 U5 region 108–127) was synthesized (MWG Biotech) as previously described (20). The cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP liposomal transfection reagent; Roche) was used to complex with ssRNA40 before addition to cell cultures. Abs linked to immunomagnetic beads were used for positive selection and depletion of various cell types. These include BDCA-4 for pDC, BDCA-1 for mDC, and CD14 for monocytes (Miltenyi Biotec). Fluorochrome-labeled Abs for flow cytometry include non-DC lineage Ab mixture-FITC, HLA-DR-PerCP, CD11c-allophycocyanin, and human IL-12-PE (BD Pharmingen). Nonspecific binding to FcRs was prevented with FcR blocking reagent (Miltenyi Biotec). Recombinant human cytokines GM-CSF, IL-4, and TGF- $\beta$ 1 were obtained from R&D Systems. Recombinant human TLR-7 (accession no. AF240467) and human TLR8 (accession no. AF246971) were cloned into the pIRES expression vector (BD Clontech). The NF- $\kappa$ B-luciferase reporter construct was obtained from BD Clontech.

#### Transfection and reporter assay

Transient transfections were performed as previously described with some modifications (24). The day before transfection, HEK293 cells (CRL-1573; American Type Culture Collection) were seeded in six-well plates (BD Biosciences) at  $4 \times 10^5$  cell/well in DMEM supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin-streptomycin, and 10% heat-inactivated FCS and incubated at 37°C in 5% CO<sub>2</sub>. The cells were cotransfected with 1  $\mu$ g of human TLR7 or human TLR8 along with 0.1  $\mu$ g of NF- $\kappa$ B-luciferase reporter (BD Clontech) in a 10:1 ratio with 3  $\mu$ l of FuGene 6 transfection reagent (Roche) following the manufacturer's instructions. The plates were incubated 24 h after transfection and then stimulated with various concentrations of small molecule TLR agonists. The plates were then incubated an additional 15 h at 37°C in 5% CO<sub>2</sub>. NF- $\kappa$ B activation was determined by lysing the transfected HEK293 cells with reporter lysis buffer (Steadylyte HTS; PerkinElmer), and the lysate was assayed for luciferase activity using a LMax Luminometer (Molecular Devices). NF- $\kappa$ B activation is directly proportional to relative luciferase units.

#### Preparation of primary human cells

Whole blood anticoagulated with EDTA was obtained from healthy volunteers that had provided informed consent before donation (institutional review board no. 96-046). PBMC were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich) as recommended by the manufacturer. The mononuclear cells were washed twice with HBSS (Celox) and resuspended in cRPMI (RPMI 1640, 25 mM HEPES, sodium bicarbonate, 1 mM sodium pyruvate, 1 mM L-glutamine, 1% penicillin/streptomycin, and 10% heat-inactivated FCS (BioSource International)). PBMC were cultured at  $2 \times 10^6$ /ml in 0.25 ml in 96-well plates or in 0.5 ml 48 well, flat-bottom plates.

Plasmacytoid DC, mDC, and monocytes were isolated from PBMC by immunomagnetic bead positive selection using BDCA-4, BDCA-1, and CD14, respectively, according to manufacturer's recommendations. Briefly, PBMC were incubated with immunomagnetic microbeads, and the labeled cells were collected with Miltenyi LS or LD columns (25). The positively selected cells were resuspended in cRPMI and cultured at  $1 \times 10^6$  cells/ml in 0.25 ml in 96-well, flat-bottom plates. In some experiments pDC and monocytes were depleted using BDCA-4 and CD14 immunomagnetic beads, respectively. The remaining PBMC population was cultured at  $2 \times 10^6$  cells/ml in 0.2 ml in 96-well, flat-bottom plates. Cell purity from positively isolated cells and populations of cells was determined by flow cytometry.

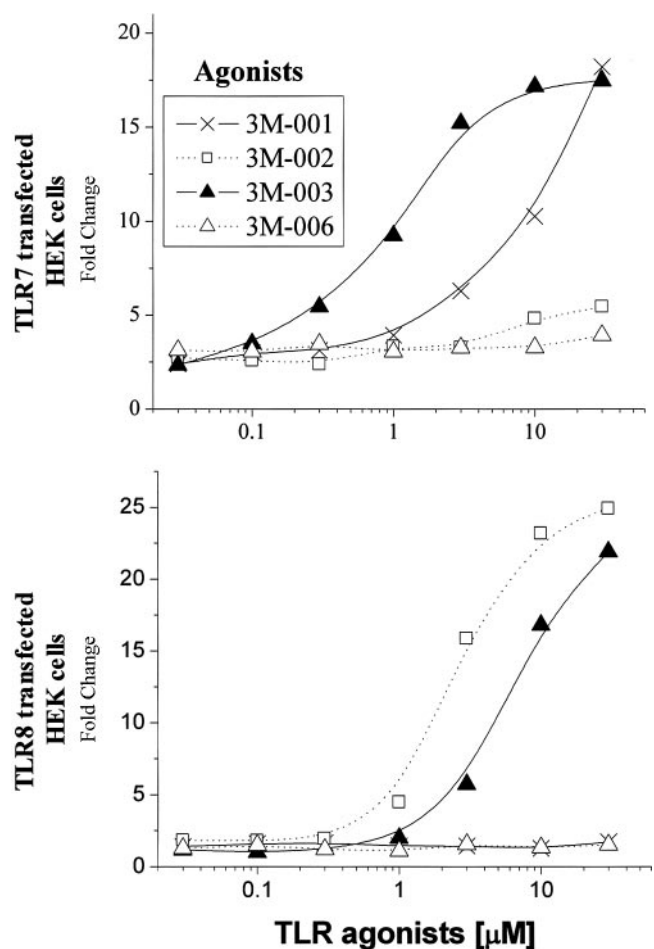
#### Determination of secreted cytokines and chemokines

TNF- $\alpha$  and IL-12p40/70 protein levels were measured from tissue culture supernatants by Origen (IGEN) assays (BioVeris), a sandwich-type immune assay based on electrochemiluminescence. Cytokine-specific Ab was mixed with tissue culture supernatants in 96-well plates. A biotin-labeled, cytokine-specific Ab captured the cytokine, and an ORI-TAG (BV-TAG)-labeled secondary Ab was used for detection. Streptavidin-coated beads

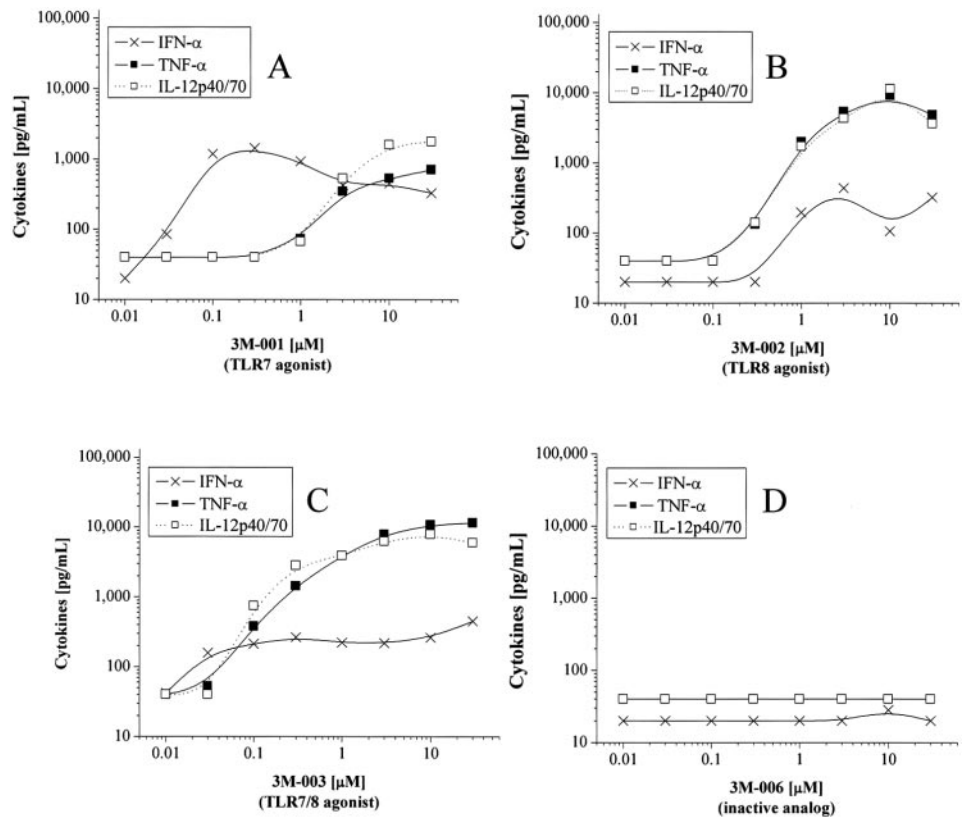
captured the immune complexes. The plate was then put into an IGEN M8 workstation (BioVeris), where the cytokine levels in the culture supernatant were determined by measuring the light emitted from ORI-TAG-labeled secondary Ab. The ORI-TAG labels based on ruthenium tris-bipyridyl chemistry were used, producing light after being stimulated with an electrical potential. The intensity of light emitted from the ORI-TAG-labeled cytokine was directly proportional to the cytokine concentration in the sample.

TNF- $\alpha$  (AHC3419 and AHC3712) and IL-12p40/70 (AHC7129 and AHC8122) Ab pairs were obtained from BioSource International. Abs AHC3419 (anti-TNF- $\alpha$ ) and AHC7129 (anti-IL-12) were biotin labeled. Abs AHC3712 (anti-TNF- $\alpha$ ) and AHC8122 (anti-IL-12) were ORI-TAG labeled. The assay was performed by first incubating the biotin-labeled Abs with M-280 streptavidin Dynabeads (DynaL Biotech). Then, tissue culture supernatants, biotin-labeled Ab/streptavidin beads, and ORI-TAG-labeled Abs were coincubated in 96-well microtiter plates for 2.5 h at room temperature. The plates were read using the IGEN M8 workstation, and the data were analyzed with SoftMax Pro software (Molecular Devices). Standard curves for TNF- $\alpha$  and IL-12p40/70 were generated using rTNF- $\alpha$  (210-TA; R&D Systems) and rIL-12p70 (219-IL; R&D Systems), respectively. The minimum level of detection for the TNF- $\alpha$  and IL-12p40/70 Origen assays is 40 pg/ml.

IFN- $\alpha$  protein levels were measured from tissue culture supernatants using the MultiSpecies IFN- $\alpha$  ELISA (Peska Biomedical Laboratories).



**FIGURE 1.** Small molecule imidazoquinolines activate NF- $\kappa$ B through human TLR7, TLR8, or both TLR7 and TLR8 in HEK293 cells. HEK293 cells cultured at  $4 \times 10^5$  cells/well in 3 ml were cotransfected with human TLR7 or human TLR8 and an NF- $\kappa$ B-luciferase reporter construct. After 18–24 h, the cells were stimulated with various concentrations of four small molecule imidazoquinolines (3M-001, 3M-002, 3M-003, and 3M-006). After an additional 15 h, luciferase production was measured as described in *Materials and Methods*. The data are representative of three separate experiments and are expressed as fold change relative to vehicle (DMSO) control.



**FIGURE 2.** Differential production of IFN- $\alpha$ , TNF- $\alpha$ , and IL-12 from human PBMC stimulated with immune response modifiers (IRMs). Human PBMC cultured at  $2 \times 10^6$ /ml in 0.25 ml were stimulated for 24 h with various concentrations of 3M-001, 3M-002, 3M-003, and 3M-006. Secreted IFN- $\alpha$ , TNF- $\alpha$ , and IL-12p40/70 were analyzed from the supernatants by ELISA or ORIGEN. The data are expressed as picograms per milliliter and are representative of nine experiments from nine separate donors. The minimum levels of detection for IFN- $\alpha$ , TNF- $\alpha$ , and IL-12p40/70 are 20, 40, and 40 pg/ml, respectively.

The minimum level of detection for IFN- $\alpha$  is 20 pg/ml. All other cytokines and chemokines were detected using SearchLight Proteome arrays (Pierce). All cytokine and chemokine results are expressed in picograms per milliliter. The limit for all cytokines and chemokines detected by proteome array is 40 pg/ml.

#### Identification of cells expressing IL-12 by flow cytometry

Whole blood was stimulated with various TLR agonists for 5 h in the presence of 10  $\mu$ g/ml brefeldin A (Sigma-Aldrich). Cells were harvested and stained for 30 min with a non-DC lineage Ab mixture (FITC), HLA-DR (PerCP), and CD11c (allophycocyanin). Blood cells were then treated with FACS lyse and Perm2 buffers (BD Biosciences) as directed for intracellular staining. Finally, cells were stained with anti-IL-12-PE for 30 min, washed, and resuspended for flow cytometry. Analysis for cytokine production by peripheral blood DC was performed by gating on lin-(FITC) and HLA-DR<sup>+</sup> (PerCP) cells. This population was further monitored for the expression of CD11c (allophycocyanin) and IL-12 (PE).

## Results

#### Identification of TLR7- and TLR8-selective agonists

Several small molecules, structurally similar to imiquimod and resiquimod, that induce cytokines from human PBMC were evaluated for their capacity to activate NF- $\kappa$ B in cells transfected with various human TLRs. HEK293 cells were cotransfected with a NF- $\kappa$ B-luciferase reporter construct and human TLR7 or human TLR8. The transfected HEK293 cells were stimulated with imidazoquinoline molecules at 0.03–30  $\mu$ M. Three types of TLR agonists were identified based on their ability to activate NF- $\kappa$ B in cells transfected with either TLR7 or TLR8. One group activated NF- $\kappa$ B in cells transfected with TLR7, but not with TLR8 (TLR7 agonists); another group activated NF- $\kappa$ B in cells transfected with TLR8, but not with TLR7 (TLR8 agonists); and a third group activated NF- $\kappa$ B in cells transfected with either TLR7 or TLR8 (TLR7/8 agonists). Representative examples of the three groups of small molecule TLR agonists are shown in Fig. 1. The TLR7 agonist, 3M-001, preferentially activates NF- $\kappa$ B through TLR7 at

$\sim 3 \mu$ M. The TLR8 agonist, 3M-002, preferentially activates NF- $\kappa$ B through TLR8 at  $\sim 3 \mu$ M. The TLR7/8 agonist, 3M-003, activates NF- $\kappa$ B through TLR7 and TLR8 with effective concentrations at 0.3 and 3  $\mu$ M, respectively. Note that significant NF- $\kappa$ B activation refers in this study to at least a 2-fold increase in luciferase production relative to the vehicle control or inactive analog control, and the increase in luciferase production is concentration dependent. 3M-006, an analog of the other small molecule TLR agonists, does not activate NF- $\kappa$ B in HEK cells transfected with either TLR7 or TLR8; therefore, it will be referred to as an inactive analog.

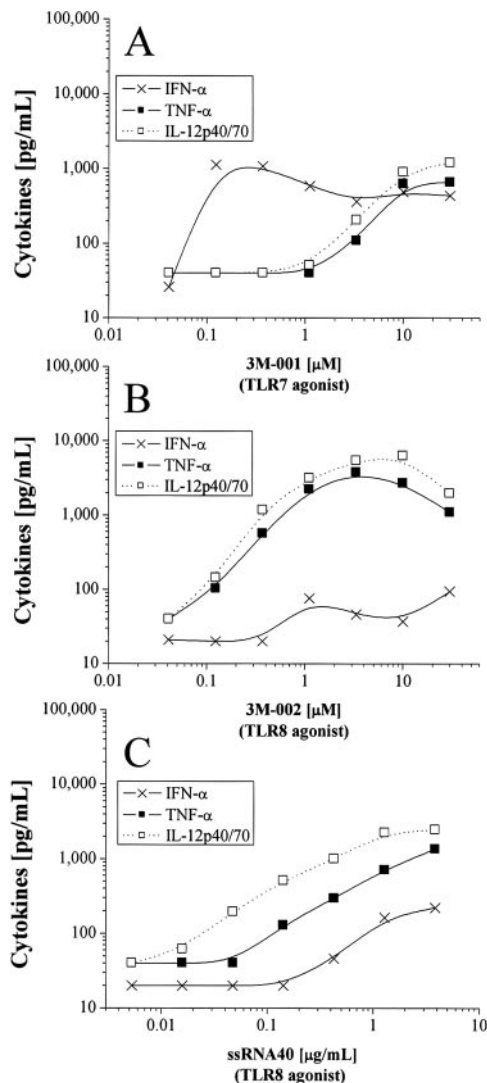
#### IFN- $\alpha$ , TNF- $\alpha$ , and IL-12 production from human PBMC stimulated with TLR7 and TLR8 agonists

To define the roles of TLR7 and TLR8 in cytokine production from primary immune cells, representative examples of TLR7, TLR8, and TLR7/8 agonists were used to stimulate various populations of human innate immune cells. First, human PBMC were stimulated with various concentrations of TLR agonists. After 24 h, IFN- $\alpha$ , TNF- $\alpha$ , and IL-12 production was assessed (Fig. 2). The TLR7, TLR8, and TLR7/8 agonists induce IFN- $\alpha$ , TNF- $\alpha$ , and IL-12 from human PBMC. These three cytokines were evaluated because they broadly represent cytokines produced by innate immune cells and are known to be induced by TLR agonists. The TLR7 agonist preferentially induces IFN- $\alpha$  secretion (Fig. 2A). Production of IFN- $\alpha$  is seen at 0.03  $\mu$ M, and  $\sim 100$  times more 3M-001 is required to induce any TNF- $\alpha$  and IL-12. The TLR8 agonist induces TNF- $\alpha$  and IL-12 at 0.3  $\mu$ M, and  $\sim 3$ -times more 3M-002 is required to induce IFN- $\alpha$  production (Fig. 2B). The TLR7 agonist induces  $\sim 5$ –10 times more IFN- $\alpha$  than the TLR8 agonist. In contrast, the TLR8 agonist induces  $\sim 10$  times more TNF- $\alpha$  and IL-12 than the TLR7 agonist. The TLR7/8 agonist 3M-003 induces IFN- $\alpha$  at 0.03  $\mu$ M and TNF- $\alpha$ , and IL-12 at 0.1  $\mu$ M

(Fig. 2C). The magnitude of the response for these three cytokines is similar to the TLR8 agonist profile. The key difference between the TLR7/8 and TLR8 agonist cytokine profile is that IFN- $\alpha$  is induced at lower concentrations than TNF- $\alpha$  or IL-12 by 3M-003 TLR7/8 agonist. An inactive imidazoquinoline molecule, 3M-006, prepared in a similar fashion to 3M-001, 3M-002, and 3M003, did not induce cytokines at concentrations up to 30  $\mu$ M (Fig. 2D).

#### *TLR8 agonist ssRNA and 3M-002 induce similar cytokine profiles from human PBMC*

The putative natural ligand for TLR8, ssRNA (ssRNA40), has been shown to induce cytokines from human PBMC (20). A comparison regarding cytokine production was made between the TLR8 agonist ssRNA40 and 3M-002 (Fig. 3). Similar to Fig. 2, the TLR7-selective agonist 3M-001 induces IFN- $\alpha$  at submicromolar



**FIGURE 3.** The TLR8 agonist ssRNA and 3M-002 induce similar cytokine profiles from human PBMC. Human PBMC cultured at  $2 \times 10^6$ /ml in 0.25 ml were stimulated for 24 h with various concentrations of 3M-001, 3M-002, and ssRNA40 complexed with DOTAP. Secreted IFN- $\alpha$ , TNF- $\alpha$ , and IL-12p40/70 were analyzed from the supernatants by ELISA or ORIGIN. The data are expressed as picograms per milliliter and are representative of nine experiments from three separate donors. The minimum levels of detection for IFN- $\alpha$ , TNF- $\alpha$ , and IL-12p40/70 are 20, 40, and 40 pg/ml, respectively. Note that DOTAP or DMSO (small molecule diluent) did not induce the production of cytokines from human PBMC (our unpublished observations).

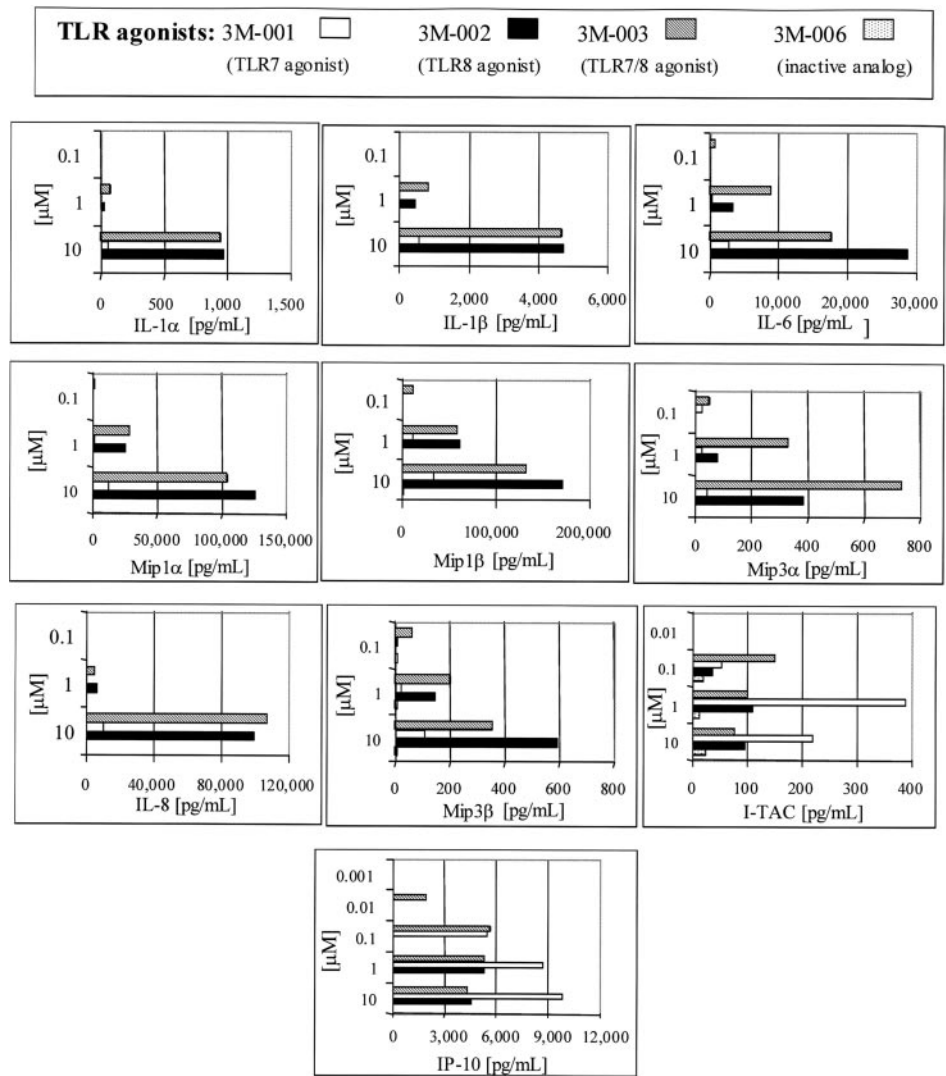
concentrations; TNF- $\alpha$  and IL-12 are induced at higher concentrations of 3M-001 (3–30  $\mu$ M). In contrast, lower concentrations of ssRNA40 and 3M-002 are required to TNF- $\alpha$  and IL-12 than IFN- $\alpha$ . The levels of IFN- $\alpha$  induced by ssRNA40 and 3M-002 are lower than the levels of TNF- $\alpha$  and IL-12 across the entire concentration-response curve. Note that the concentrations of ssRNA40 used in Fig. 3 are similar to those previously reported to induce IFN- $\alpha$ , TNF- $\alpha$ , and IL-12 from human PBMC (20). Overall, the profile of IFN- $\alpha$ , TNF- $\alpha$ , and IL-12 induced by the natural TLR8 agonist ssRNA40 is similar to the small molecule TLR8 agonist 3M-002.

#### *Differential cytokine production from human PBMC stimulated with TLR7 and TLR8 agonists*

In addition to TNF- $\alpha$ , IL-12, and IFN- $\alpha$ , other cytokines known to be produced after TLR stimulation were evaluated from human PBMC after stimulation with TLR7, TLR8, and TLR7/8 agonists (Fig. 4). Specifically, cytokines considered to be induced via an NF- $\kappa$ B-dependent mechanism, such as IL-1 and IL-8, and cytokines known to be associated with type I IFN induction, such as IFN- $\gamma$ -inducible protein-10, were also evaluated. The TLR8 agonist induces IL-1 $\alpha/\beta$ , IL-6, IL-8, MIP-1 $\alpha\beta$ , and MIP-3 $\alpha\beta$ . All these cytokines are associated with inflammatory responses, particularly those thought to require activation of NF- $\kappa$ B. The TLR7 agonist induces much lower levels of these cytokines. In contrast, the TLR7 agonist effectively induces IP-10 and IFN-inducible T cell  $\alpha$  chemoattractant (I-TAC); these cytokines are IFN-inducible cytokines. The TLR8 agonist induces these IFN-inducible cytokines to a lesser degree. The TLR7/8 agonist induces both proinflammatory and IFN-inducible cytokines; however, the levels of IP-10 and I-TAC are lower than the levels induced by the TLR7 agonist. The combined results in Figs. 2–4 indicate that the TLR7 agonist more effectively induces IFN- $\alpha$  and IFN-regulated cytokines, whereas TLR8 agonists more effectively induce proinflammatory cytokines.

#### *Plasmacytoid DC are required for IFN- $\alpha$ production from human PBMC stimulated with TLR7 and TLR8 agonists*

To determine the cell types within the PBMC population required for IFN- $\alpha$ , TNF- $\alpha$ , and IL-12 production after stimulation with the TLR7- and TLR8-selective agonists, monocytes and pDC were removed by immunomagnetic bead selection before stimulation with 3M-001 and 3M-002. Previously, the TLR7/8 agonist resiquimod was shown to require pDC for IFN- $\alpha$  production and monocytes for TNF- $\alpha$  production from human PBMC (22, 26). Similarly, both TLR7- and TLR8-selective agonists lose the ability to induce IFN- $\alpha$  from pDC-deficient PBMC (Fig. 5). In contrast, depletion of monocytes has a slight positive effect on IFN- $\alpha$  production. Monocyte depletion does appear to affect low levels of TNF- $\alpha$  production by 3M-001-stimulated PBMC. However, depletion of monocytes results in a 10- to 25-fold reduction in TNF- $\alpha$  production from TLR8 agonist 3M-002-stimulated PBMC. Interestingly, depletion of pDC or monocytes from PBMC minimally affects IL-12 production. It is possible that some low level of non-depleted monocytes is responsible for the high level of IL-12 produced in the PBMC culture or that other populations of cells either by themselves or in combination with monocytes produce substantial levels of IL-12. Blood mDC were shown to produce IL-12 in response to resiquimod (10, 27). Myeloid DC (BDCA-1 $^+$ ) were depleted from PBMC in a manner similar to pDC and monocyte depletion as described above, and the levels of IFN- $\alpha$ , TNF- $\alpha$ , and IL-12 were not changed relative to the undepleted controls (J. P. Vasilakos, unpublished observations), indicating that mDC are not solely responsible for the IL-12 produced by the immune response



**FIGURE 4.** Differential production of proinflammatory and IFN-induced cytokines from human PBMC stimulated with IRMs. Human PBMC ( $2 \times 10^6$ /ml in 0.5 ml) were stimulated for 24 h with various concentrations of 3M-001, 3M-002, 3M-003, and 3M-006. Secreted IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-3 $\alpha$ , MIP-3 $\beta$ , IP-10, and I-TAC were analyzed from the supernatants by proteome array multiplex analysis. The data are expressed as picograms per milliliter and are representative of three experiments from three separate donors. The minimum level of detection for each cytokine is 40 pg/ml.

modifier-stimulated PBMC (J. P. Vasilakos, unpublished observations). Overall, these data indicate that both TLR7 and TLR8 agonists induce IFN- $\alpha$  from human PBMC in a pDC-dependent fashion. In addition, monocyte stimulation by the TLR8 agonist contributes to the high levels of TNF- $\alpha$  from the PBMC cultures.

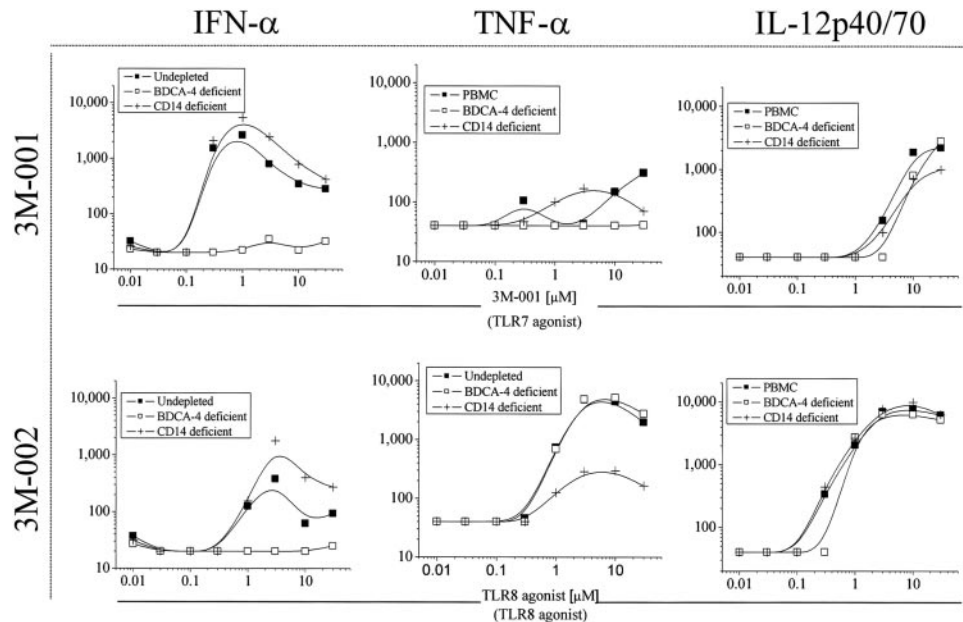
*The TLR8 agonist 3M-002 effectively stimulates cytokine production from mDC, but not pDC*

To identify IL-12-producing cells in blood, intracellular cytokine staining was assessed after stimulation of whole blood with various TLR agonists in the presence of brefeldin A, which inhibits the secretion of cytokines, but does not affect their transcription or translation. First, DC were identified by flow cytometry as lineage<sup>-</sup>, HLA-DR<sup>+</sup>, which constitute ~0.5% of the blood cells (Fig. 6A). Myeloid and pDC were further differentiated as CD11c<sup>+</sup> and CD11c<sup>-</sup>, respectively. The TLR4, TLR7, TLR8, and TLR7/8 agonists induced IL-12 from the mDC, but not the pDC. TLR8 and TLR7/8 agonists induced the highest percentage of IL-12-producing mDC (~30%). Approximately 14% of the mDC produced IL-12 in response to the TLR4 agonist, and 9% of the mDC produced IL-12 after stimulation with the TLR7 agonist. The TLR9 agonist and the vehicle control did not stimulate IL-12 production. In addition, the IL-12 fluorescence intensity of the 3M-002- and 3M-003-treated groups was about half an order of magnitude

greater than the fluorescence intensity of the 3M-001-stimulated group. The results indicate that activation of TLR8 in mDC results in IL-12 production, and the TLR8-selective agonist more effectively induces IL-12-producing cells than the TLR7-selective agonist. The results in Fig. 6A are consistent with those in Fig. 2, where 10  $\mu$ M 3M-001, 3M-002, and 3M-003 induce detectable levels of IL-12 secretion from human PBMC. The results in Fig. 2 show ~10-fold greater IL-12 production from PBMC stimulated via TLR8, and the results in Fig. 6A show about a half-log increase in IL-12 fluorescence intensity in PBMC stimulated via TLR8.

The cell types within the PBMC culture responsible for the production of IFN- $\alpha$ , TNF- $\alpha$ , and IL-12 after stimulation with TLR7 and TLR8 agonists were also evaluated by enriching for various cell types by positive selection. CD123<sup>high</sup>/HLA-DR<sup>+</sup>/lin<sup>-</sup> pDC and CD11c<sup>+</sup>/HLA-DR<sup>+</sup>/lin<sup>-</sup> mDC were cultured independently and stimulated with various TLR agonists. Plasmacytoid DC (BDCA-4 isolated) produced IFN- $\alpha$  in response to TLR7, TLR7/8, and TLR9 agonists (Fig. 6B). TLR4 and TLR8 agonists did not induce IFN- $\alpha$  from pDC-enriched cultures. IL-12 was not produced by pDC in response to any of the agonists under the indicated conditions. Similar findings were seen with BDCA-2 isolated pDC (J. P. Vasilakos, unpublished observations). Myeloid DC (BDCA-1 isolated) produced IL-12 in response to TLR4, TLR8, and TLR7/8 agonists. TLR7 and TLR9 agonists did not induce the

**FIGURE 5.** Plasmacytoid DC are required for IFN- $\alpha$  production from human PBMC stimulated with TLR7 and TLR8 agonists. Human plasmacytoid DC and monocytes were depleted from human PBMC with BDCA-4 and CD14 microbeads, respectively. Cells were cultured at  $1 \times 10^6$ /ml in 0.2 ml and stimulated with various concentrations of 3M-001 or 3M-002 for 24 h. Secreted IFN- $\alpha$ , TNF- $\alpha$ , and IL-12p40/70 were measured from the culture supernatants via ELISA or ORIGEN. The minimum levels of detection for IFN- $\alpha$ , TNF- $\alpha$ , and IL-12p40/70 are 20, 40, and 40 pg/ml, respectively. The data are presented in picograms per milliliter from one donor, representative of four donors.



secretion of IL-12 from mDC-enriched cultures. IFN- $\alpha$  is not produced by mDC in response to any of the agonists under the indicated conditions. TNF- $\alpha$  was produced by both isolated pDC and mDC after stimulation with TLR7/8 agonist (Fig. 6C). The TLR7 and TLR9 agonists induced TNF- $\alpha$  production from isolated pDC, and the TLR4 and TLR8 agonists induced TNF- $\alpha$  secretion from isolated mDC.

To determine whether the blood DC subsets affect the stimulatory capacity of each other by the small molecule TLR agonists, the pDC- and mDC-enriched populations were also cocultured and stimulated with the same TLR agonists (Fig. 6D). Similar to individually cultured pDC and mDC, mixed blood DC produce IFN- $\alpha$  in response to TLR7, TLR7/8, and TLR9 agonists. TLR4 and TLR8 agonists do not induce IFN- $\alpha$  from the pDC/mDC mixed culture. From the same culture, secreted IL-12 is induced by TLR4, TLR7/8, and TLR8 agonists. TLR7 and TLR9 agonists do not induce IL-12 from the blood DC cultured under the indicated conditions. The experiments indicate that mixing pDC and mDC do not affect their cytokine response patterns to TLR7 and TLR8 agonists.

*The TLR8 agonist 3M-002, but not the TLR7 agonist, effectively stimulates cytokine production from monocytes and monocyte-derived DC (Mo-DC)*

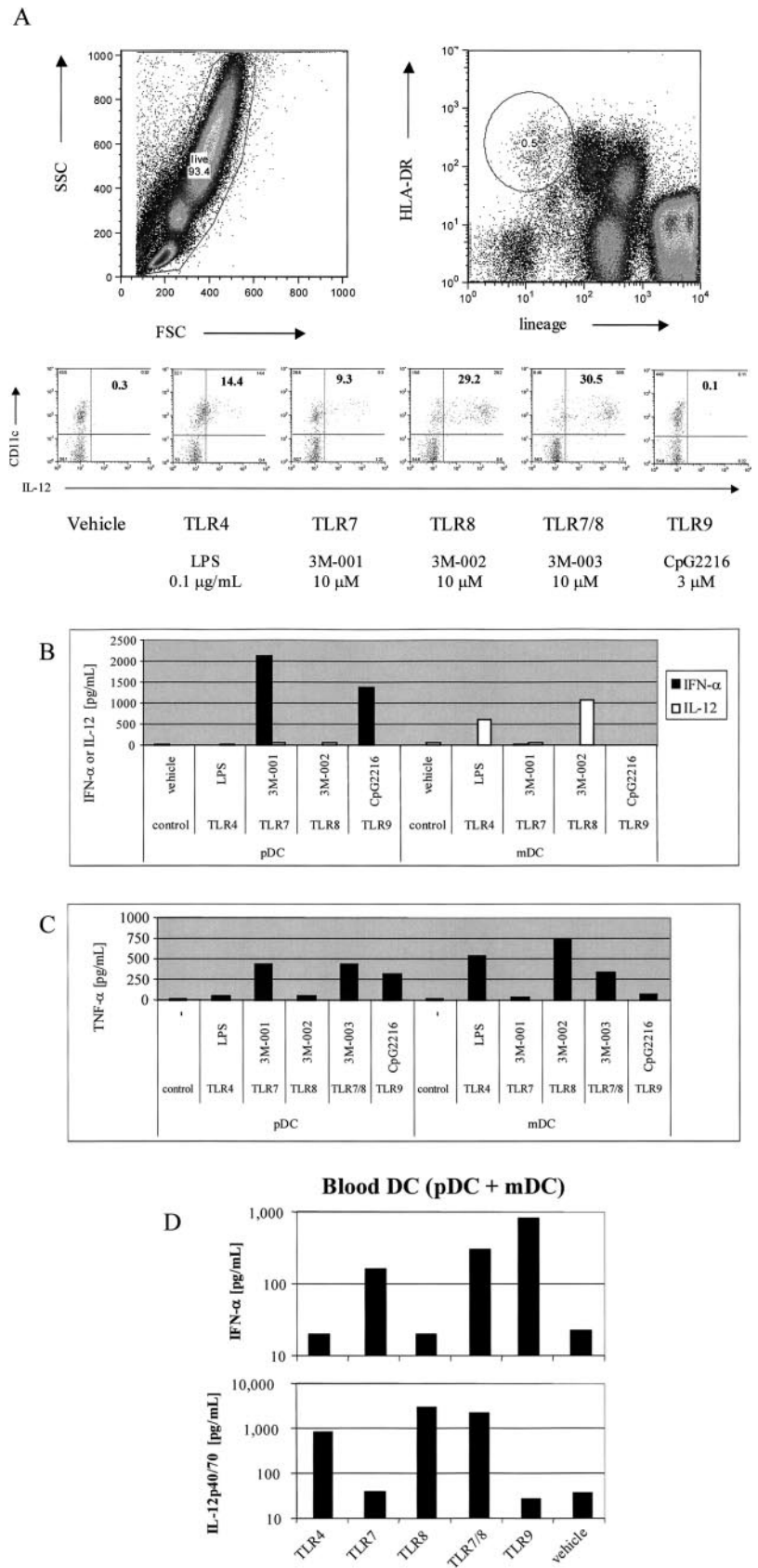
Monocytes express TLR7 and TLR8, whereas Mo-DC express mainly TLR8 (9, 28). Cytokine production from these cells was determined after stimulation with TLR7- and TLR8-selective agonists (Fig. 7). Monocytes were isolated by positive selection with CD14 magnetic beads. After stimulation with TLR7, TLR8, and TLR7/8 agonists, monocytes secreted TNF- $\alpha$  and IL-12. However, TLR8 and TLR7/8 agonists more effectively induced TNF- $\alpha$  and IL-12 from the monocytes than the TLR7 agonist. Both TLR8 and TLR7/8 agonists induced TNF- $\alpha$  and IL-12 at  $\sim 0.1 \mu\text{M}$ ; 30–100 times more TLR7 agonist was required to induce the same cytokines from monocytes. The TLR8 and TLR7/8 agonists induced  $\sim 10$ - to 100-fold more secreted TNF- $\alpha$  and IL-12 from the monocytes than the TLR7 agonists. In additional studies, monocytes prepared as shown in Fig. 6 produced TNF- $\alpha$  and IL-12 in response to TLR2 and TLR4 agonists, but not in response to TLR9 agonists (J. P. Vasilakos, unpublished observations).

Monocytes were differentiated in vitro into DC with GM-CSF, IL-4, and TGF- $\beta$  and stimulated with various TLR agonists (Fig. 7). Secreted TNF- $\alpha$  and IL-12 were measured 24 h poststimulation. Similar to monocytes, Mo-DC produced TNF- $\alpha$  and IL-12 in response to TLR8 and TLR7/8 agonists. However, the TLR7 agonist did not induce either of these cytokines from the Mo-DC. The TLR8 agonist was slightly more potent than the TLR7/8 agonist, and the TLR8 agonist induced slightly more TNF- $\alpha$  and IL-12 from the Mo-DC than the TLR7/8 agonist. In separate studies, monocytes produced TNF- $\alpha$  and IL-12 in response to TLR4 agonist, but not to TLR9 agonists (J. P. Vasilakos, unpublished observations). Overall, the results in Fig. 7 demonstrate that monocyte and monocytes differentiated into DC with GM-CSF, IL-4, and TGF- $\beta$  are more effectively activated through TLR8 than through TLR7 stimulation.

A schematic representation of all the results is shown in Fig. 8. Overall the results indicate that despite phylogenetic and structural similarities between TLR7 and TLR8, these TLRs appear to differ functionally with regard to cytokine profiles induced from human PBMC as well as from isolated PBMC cell populations. Furthermore, TLR7 and TLR8 seem to work in a cell-selective fashion, in that TLR7 predominantly functions in pDC and TLR8 appears to function primarily in mDC, monocytes, and Mo-DC.

## Discussion

Phylogenetic analysis shows that TLR7 and TLR8, as well as TLR9 belong to a subfamily of TLRs (29, 30). Constitutively expressed TLR7 and TLR8 stimulate an NF- $\kappa\text{B}$  signaling pathway indirectly, supporting the assertion that these receptors are involved in cellular responses to stimuli that activate innate immunity. Functionally, TLR7 and TLR8 are thought to form a subgroup within the TLR family that recognizes pathogen-associated molecular patterns in endosomal/lysosomal compartments (31). The function of TLR7 has been partially established due to the availability of small molecule TLR7 agonists and TLR7-deficient mice (21). The role of TLR8 is not well established due to the apparent lack of function of murine TLR8 and the lack of TLR8-selective agonists (23). Recently, two guanosine- and uridine-rich ssRNA sequences, RNA40 and RNA42, were shown to activate NF- $\kappa\text{B}$  in HEK cells transfected with human TLR8, but not with



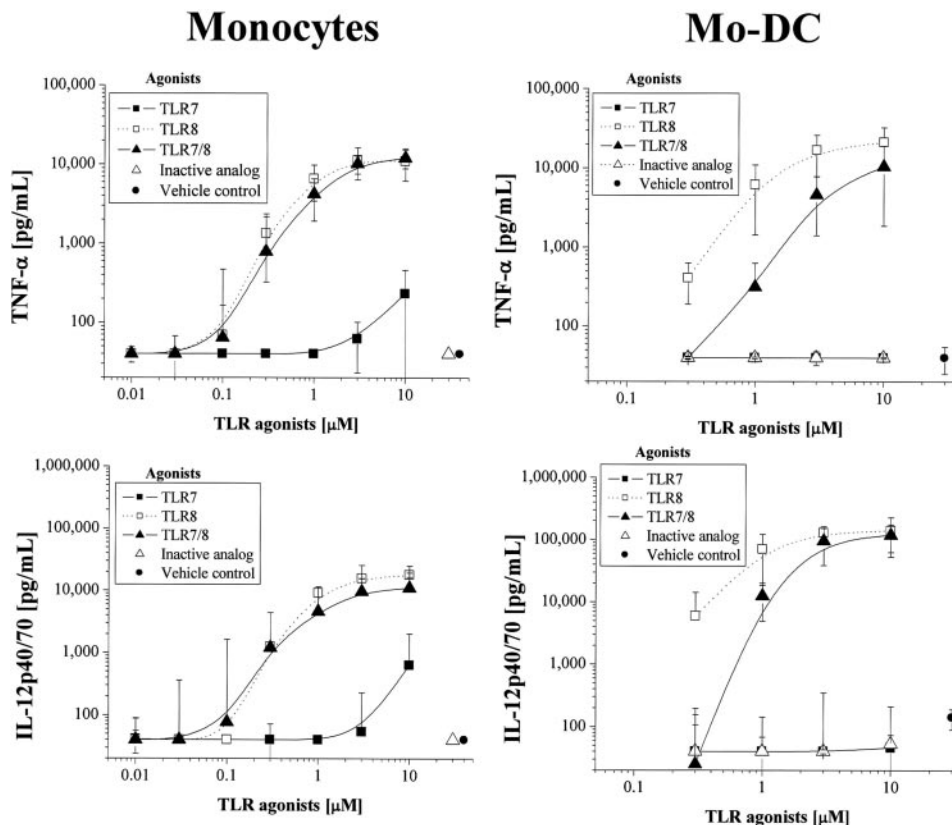
**FIGURE 6.** TLR7 and TLR8 agonists preferentially induce cytokine production from pDC and mDC, respectively. *A*, Whole blood was stimulated for 5 h with 10  $\mu$ M 3M-001, 3M-002, or 3M-003; 0.1  $\mu$ g/ml LPS; or 3  $\mu$ M CpG2216 or was treated with vehicle control (DMSO) (vehicle) in the presence of 10  $\mu$ g/ml brefeldin A, which was added for 5 h. The cells were stained with fluorochrome-labeled Abs specific for HLA-DR, a mixture of non-DC lineage markers (FITC; including CD3, CD16, CD19, CD20, CD14, and CD56), HLA-DR (PerCP), CD11c (allophycocyanin), and intracellular IL-12p40/70 (PE). FITC, CD11c-allophycocyanin, and intracellular IL-12 (from blood DC) were evaluated from in  $lin^{-}$ , HLA-DR $^{+}$  gated dendritic cells. The values in the upper right quadrants represent the percentage of  $lin^{-}$ , HLA-DR $^{+}$  cells expressing both CD11c and IL-12. The data presented are from a single experiment and are representative of two experiments from two separate donors. *B*, Blood-derived pDC and mDC were enriched from human PBMC by positive selection with BDCA-4 and BDCA-1 immunomagnetic beads, respectively. Each population of cells was cultured separately at  $1 \times 10^6$ /ml in 0.25 ml and stimulated for 24 h with 3  $\mu$ M TLR7, TLR8, or TLR7/8 agonists. The cells were also stimulated with 0.1  $\mu$ g/ml LPS or 3  $\mu$ M CpG2216. Secreted IFN- $\alpha$ , TNF- $\alpha$ , and IL-12 were analyzed from the supernatants by ELISA or ORIGEN. The data are expressed as picograms per milliliter and are representative of two experiments from two separate donors. The minimum levels of detection for IFN- $\alpha$ , TNF- $\alpha$ , and IL-12 are 20, 40, and 40 pg/ml, respectively. *C*, Similar to *B*, except mDC and pDC were cocultured before stimulation with the various TLR agonists. The data shown in Fig. 5 are representative of three experiments from three separate donors.

human TLR7, thereby implicating ssRNA as a natural ligand for human TLR8 (20).  
 Most studies evaluating the function of human TLR7 have used the TLR7/8 agonist R-848 (resiquimod) as a tool to dissect cellular

function. Because the promiscuous activity of resiquimod does not easily allow for differentiation of TLR7- or TLR8-specific responses, it is possible that some of the effects of resiquimod considered to be TLR7-driven are actually TLR8-driven. To further



**FIGURE 7.** TLR8 agonist more effectively induces the secretion of TNF- $\alpha$  and IL-12 from human monocytes and monocyte-derived DC than TLR7 agonist. Human monocytes were enriched from PBMC by positive selection with CD14 immunomagnetic beads. Mo-DC were differentiated in vitro from GM-CSF-, IL-4-, and TGF- $\beta$ 1-treated monocytes. Monocytes and Mo-DC cells were cultured at  $5 \times 10^5$ /ml in 0.2 ml and stimulated for 24 h with various concentrations of 3M-001, 3M-002, 3M-003, and 3M-006. Secreted TNF- $\alpha$  and IL-12p40/70 were measured from culture supernatants by ORIGEN. The data are expressed as picograms per milliliter (median  $\pm$  SD, six experiments from six separate donors). The minimum levels of detection for TNF- $\alpha$  and IL-12p40/70 are 40 and 20 pg/ml, respectively.



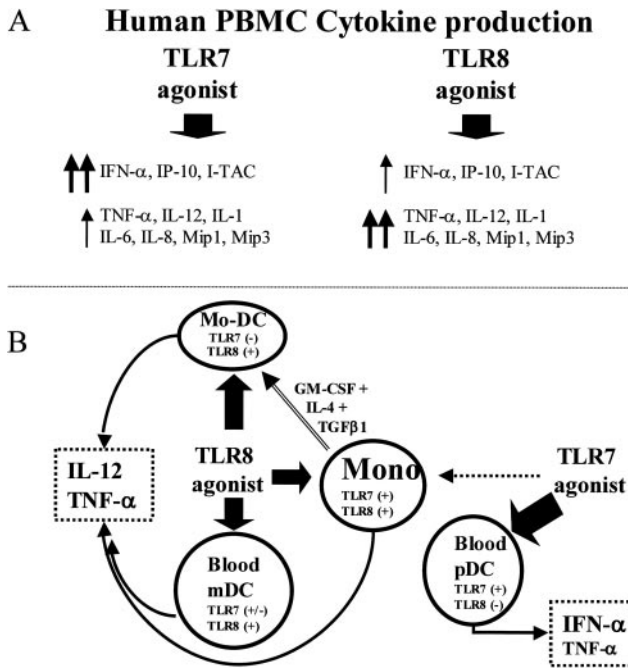
elucidate the function of TLR8 and to further differentiate the functions of human TLR7 and TLR8 from each other, it is essential to use agonists that selectively activate TLR7 or TLR8 on defined populations of innate immune cells. Therefore, using agonists selective for TLR7 and TLR8, we evaluated cytokine production from human PBMC or isolated monocytes, monocyte-derived DC, blood mDC, and blood pDC after stimulation with appropriate TLR agonists.

In this study we define TLR7- and TLR8-selective agonists by their ability to activate NF- $\kappa$ B in HEK293 cells genetically reconstituted with either TLR7 or TLR8. The major caveat in this study is that not all TLR-driven events are NF- $\kappa$ B dependent. Indeed, CpG2216 is very effective at inducing IFN- $\alpha$  from pDC, but is comparatively poor at inducing NF- $\kappa$ B activation in HEK cells transfected with TLR9 (K. B. Gorden, unpublished observations). Therefore, the term selective in this study is strictly used in the context of NF- $\kappa$ B activation in transfected HEK293 cells. Nevertheless, the results demonstrate that TLR7 and TLR8 agonists differentially induce cytokine production from isolated human innate immune cells. Both TLR7 and TLR8 agonists can induce IFN- $\alpha$ , TNF- $\alpha$ , and IL-12 production from PBMC. However, the TLR7 agonist 3M-001 more effectively induces IFN- $\alpha$ , and the TLR8 agonist 3M-002 more effectively induces TNF- $\alpha$  and IL-12. Concordant with robust IFN- $\alpha$  production, cytokines IP-10 and I-TAC are more effectively generated from TLR7-stimulated PBMC. Although the precise mechanism of IP-10 and I-TAC induction after 3M-001 stimulation is unknown, both IP-10 and I-TAC can be induced by the combination of IFN- $\alpha$  and TLR5 stimulation in PBMC (32). These results are consistent with findings showing that CpG oligodeoxynucleotides induce IFN- $\alpha$  from pDC, which subsequently induces IP-10 from monocytes (33). Similarly, high levels of TNF- $\alpha$  and IL-12 production from TLR8-activated PBMC are associated with high levels of IL-1, IL-6, IL-8, and other cytokines considered, in general, as proinflammatory cyto-

kines. Therefore, it seems reasonable that high levels of proinflammatory cytokines requiring NF- $\kappa$ B activation are the most effectively induced cytokines stimulated by the TLR8 agonist.

It is intriguing that the TLR8 agonist 3M-002 induces IFN- $\alpha$  from human PBMC in a pDC-dependent manner (Fig. 5), but isolated pDC do not produce IFN- $\alpha$  after stimulation with the TLR8 agonist (Fig. 6, B and D). The reason for this discrepancy is unknown. One possibility is that the positive selection procedure for pDC using magnetic microbeads may diminish the ability of pDC to produce IFN- $\alpha$ , particularly if the TLR8 agonist activates pDC weakly through a TLR7-dependent mechanism. Because TLR7, TLR8, and TLR9 are structurally similar, it is possible that the TLR8 agonist used in this study can directly induce IFN- $\alpha$  from pDC through TLR7, but to a lesser extent than the more effective IFN- $\alpha$ -inducing molecule 3M-001. Another possibility is that the TLR8 agonist may induce IFN- $\alpha$  production from pDC in human PBMC culture in an indirect manner, where pDC are required for IFN- $\alpha$  production, but additional cell types in the culture may stimulate pDC to produce IFN- $\alpha$ . Finally, it is possible that efficient stimulation of IFN- $\alpha$  from pDC is independent of NF- $\kappa$ B stimulation through TLR7. This hypothesis is based on results using the very effective IFN- $\alpha$  inducer CpG2216, which is thought to be a human TLR9 agonist, but poorly activates NF- $\kappa$ B through TLR9 in transfected HEK293 cells (K. B. Gorden, unpublished observations). Perhaps IFN- $\alpha$  induction from pDC is inhibited by efficient NF- $\kappa$ B activation through the TLR pathway, in that the most effective NF- $\kappa$ B activators may be the least effective IFN- $\alpha$  inducers and vice versa. Studies are underway to resolve this discrepancy.

Results using two different ssRNA TLR8 agonists show that TLR8 activation results in TNF- $\alpha$ , IL-6, and IL-12 production from human PBMC (20); these results are consistent with the results reported in this study using the small molecule TLR8 agonist



**FIGURE 8.** The relative effects of TLR7 and TLR8 stimulation on cytokine production from human PBMC and various types of purified cells from human PBMC. *A*, Human PBMC preferentially produce IFN- $\alpha$ , IP-10, and I-TAC in response to stimulation with the TLR7-selective agonist (double arrow). Other cytokines, typically regarded as NF- $\kappa$ B-regulated cytokines, such as TNF- $\alpha$ , and IL-12 are also produced by TLR7-activated PBMC, but to a lesser extent (single arrow). Comparatively, TLR8-selective stimulation of human PBMC preferentially induces NF- $\kappa$ B-regulated cytokines associated with inflammation, such as TNF- $\alpha$  and IL-12 (double arrows). IFN- $\alpha$ , IP-10, and I-TAC are also induced by the TLR8-selective agonist, but to a lesser extent (single arrow). *B*, TLR7 and TLR8 agonists differentially stimulate human innate immune cells. Plasmacytoid DC are the primary cells stimulated by the TLR7-selective agonist; monocytes are activated to a lesser degree. Therefore, activation of TLR7 results in preferential activation of pDC and IFN- $\alpha$  production. In contrast, mDC, monocytes, and Mo-DC (GM-CSF/IL-4/TGF- $\beta$ ) are preferentially activated through TLR8, cells that predominantly produce proinflammatory cytokines. The TLR7 and TLR8 mRNA expression levels reported for human innate immune cells are consistent with the differential effects of TLR7 and TLR8 activation (9–12, 28, 34–36). +, Expression of the indicated TLR; –, no detectable expression of the indicated TLR;  $\pm$ , some studies have measured the indicated TLR and others did not detect measurable expression.

3M-002. Interestingly, one of the ssRNA sequences, RNA40, induces IFN- $\alpha$  from PBMC and isolated pDC, but another single-stranded sequence, RNA42, does not. The reported findings suggest that TLR8 activation in itself is not sufficient for IFN- $\alpha$  production from pDC, and that RNA42, like 3M-002, has some non-TLR8 activity responsible for IFN- $\alpha$  induction from pDC. Perhaps ssRNA may be recognized by human TLR7 in pDC, resulting in IFN- $\alpha$  production in an NF- $\kappa$ B-independent manner. Regardless, it is likely that reconstitution studies with human TLR7 in fibroblasts have limitations regarding the IFN- $\alpha$  signaling pathway.

Proinflammatory cytokine induction from monocytes, blood mDC, and Mo-DC are effectively induced with the TLR8 agonist, as shown in this study. The TNF- $\alpha$ - and IL-12-inducing ability of TLR7 and TLR8 agonists is consistent with TLR7 and TLR8 expression patterns in monocytes and Mo-DC. Monocytes produce cytokines in response to both TLR7 and TLR8 agonists, but the cytokine levels induced by the TLR8 agonist are approximately

100 times greater than those induced by the TLR7 agonist. Indeed, the lack of TNF- $\alpha$  or IL-12 production from mDC and Mo-DC after stimulation with the TLR7 agonist indicates that these cells either do not express functional TLR7 or that NF- $\kappa$ B activation in these cells is insufficiently driven through TLR7 with the selected small molecule TLR7 agonist. Also consistent with TLR expression profiles, TLR7 expression decreases  $\sim$ 30-fold after differentiation of monocytes into Mo-DC using GM-CSF, IL-4, and TGF- $\beta$ 1, and the TLR7 agonist loses the ability to induce cytokines from Mo-DC. Although some correlation is shown between TLR7 and TLR8 mRNA expression and direct activation of various cell populations with the TLR7 and TLR8 agonists, the correlation is not exact, in that Mo-DC express slightly more TLR7 than TLR8 mRNA (W. Birmachu and J. A. Hanten, unpublished observations), yet only the TLR8 agonist induces cytokine production from Mo-DC. Therefore, the mRNA levels of TLR7 and TLR8 expressed in monocytes and Mo-DC do not sufficiently explain their responses to the small molecule TLR agonists.

There is some controversy regarding whether human blood mDC express TLR7 (9–12). The results in this study indicate that the TLR7 agonist 3M-001, which activates NF- $\kappa$ B through TLR7, but not through TLR8, in HEK cells and efficiently induces IFN- $\alpha$  from pDC, does not activate mDC. If mDC express TLR7, the results shown in this study suggest that TLR7 is not functional in mDC, at least with regard to TNF- $\alpha$  and IL-12 production. It is possible, however, that the positive selection method of isolating mDC with BDCA-1 microbeads diminishes the responsiveness of the mDC to the TLR7 agonist 3M-001.

In conclusion, the results shown in this study indicate that TLR7 and TLR8 are functionally distinct in human innate immune cells. TLR7 in pDC is functionally associated with the production of IFN- $\alpha$ - and IFN-regulated cytokines, similar to the role of TLR9. TLR8 functions in monocytes and myeloid DC and is involved in the production of proinflammatory cytokines such as TNF- $\alpha$ . These results suggest that TLR8 agonists may be effective at driving strong cell-mediated immune responses or Th1-like immune responses requiring myeloid DC activation, whereas TLR7 agonists may be important in driving Ig production. The TLR7- and TLR8-selective agonists presented in this study can now be used to address this hypothesis as well as to understand the nature of the precise connections between innate and adaptive immunities.

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