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# Oligodeoxynucleotides Differentially Modulate Activation of TLR7 and TLR8 by Imidazoquinolines

Keith K. B. Gorden, Xiaohong Qiu, John J. L. Battiste, Paul P. D. Wightman, John P. Vasilakos, and Sefik S. Alkan<sup>1</sup>

Among the 11 human TLRs, a subfamily TLR7, TLR8, and TLR9 display similarities in structure and endosomal localization. Natural agonists consisting of nucleic acids, such as ssRNA or DNA with CpG motifs, activate the innate immune cells through these TLRs. Immune response modifiers (IRMs) of imidazoquinoline class compounds 3M-001, 3M-002, and 3M-003 have been shown to activate the innate immune system via TLR7, TLR8, and TLR7/8, respectively. In looking at the effect of the agonists of the TLR7, TLR8, and TLR9 on the activation of NF- $\kappa$ B of transfected HEK cells, we discovered that some oligodeoxynucleotides (ODNs) could modulate imidazoquinoline effects in a negative or positive manner. In this study we demonstrate that poly(T) ODNs can inhibit TLR7 and enhance TLR8 signaling events involving NF- $\kappa$ B activation in HEK cells and cytokine production (IFN- $\alpha$ , TNF, and IL-12) by human primary PBMC. In contrast, TLR3 agonist poly(I:C) does not affect imidazoquinoline-induced responses. The modulation of TLR7 and TLR8 responses is independent of CpG motifs or the nature of the ODN backbone structure. Furthermore, we show that to be an effective modulator, the ODNs need to be in the cell at the same time with either of the TLR7 or TLR8 agonist. We have also demonstrated that there is a physical interaction between IRMs and ODNs. The cross-talk between ODNs, IRMs, and TLR7 and TLR8 uncovered by this study may have practical implications in the field of microbial infections, vaccination, and tumor therapy. *The Journal of Immunology*, 2006, 177: 8164–8170.

As part of the survival strategy, organisms have developed pathogen-sensing systems that detect the presence of microbial signature structures or pathogen-associated molecular patterns. TLRs are one of the main sensors of the innate immune system and consist of 11 functional receptors that are mostly expressed on the cell surface. These TLRs recognize bacterial products such as lipid ligands (TLR1/2, TLR2/6 (1) and TLR4 (2, 3)) and protein ligands (TLR5 (4) and TLR11 mouse only). Four of them (TLR3, TLR7, TLR8, and TLR9), however, are expressed in the endosomal/lysosomal compartments and each of these TLRs recognize nucleotide-containing molecules (5–8) (also see Refs. 9–14). Due to their strategic localization, these TLRs are well poised to recognize RNA or DNA molecules that become available after the coat of an intracellular microbe (e.g., a virus) is removed within the infected cell. Thus, TLR3 senses dsRNA (15) while TLR7 and TLR8 recognize ssRNA (16, 17). TLR9 is known to detect unmethylated DNA carrying CpG motifs (18, 19). The detection of such nucleotide-containing molecules by the TLRs activates the NF- $\kappa$ B pathway, and several inflammatory cytokines are released to send the message to other cells that there is an “infection” (13, 20, 21).

One of the major questions in the TLR field is how this restricted family of TLRs recognizes the wide spectrum of pathogen-associated molecular patterns that exist. Early studies suggested that the innate immune system increases the pathogen recognition repertoire by allowing cooperation between TLRs. For example,

TLR2 has been shown to cooperate with either TLR1 and TLR6 (22). Recent emerging data suggest that there exists a different kind of interaction between endoplasmic TLRs as well. For example, studies have shown that in directly activated plasmacytoid dendritic cells (pDC)<sup>2</sup> and in B cells by CpG oligodeoxynucleotide (ODN), TLR9 expression is rapidly decreased as compared with TLR7, which was up-regulated in pDC and down-regulated in B cells (23). Also, a study suggested that there are strong relationships within the TLR7, TLR8, and TLR9 subfamily (24). Another study showed in human pDCs that, upon stimulation with CpG, TLR9 traffics to tubular lysosomal compartments, together with TLR7 (25). There is evidence that although they use distinct regulatory elements, TLR3 and TLR7 colocalize near the phagosomes (26).

The exact mechanism of RNA recognition by the endoplasmic TLRs is not known. In an attempt to uncover possible interactions between TLR3, TLR7, TLR8, and TLR9 at the ligand level we used 3M Pharmaceuticals' synthetic TLR7 and TLR8 agonist molecules, the purine analogue imidazoquinolines that are collectively called immune response modifiers (IRMs). These molecules can be divided into three categories: TLR7 selective, TLR8 selective, and TLR7/8 mixed compounds. HEK293 cells do not express TLR7 and cannot respond to TLR agonists unless transfected with a human TLR7 construct. Similarly, HEK cells transfected with TLR8 only respond to TLR8 selective compounds. Recently, we have demonstrated that the TLR7 selective compounds (e.g., imiquimod or 3M-001) preferentially activate pDC and B cells and cause secretion of type I IFNs and Ab production, respectively. Conversely, structurally similar TLR8 selective agonists (e.g., 3M-002) preferentially stimulate myeloid DCs to produce inflammatory cytokines such as TNF, IL-12, and others (27).

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<sup>2</sup> Abbreviations used in this paper: pDC, plasmacytoid dendritic cell; ODN, oligodeoxynucleotide; IRM, immune response modifier; NMR, nuclear magnetic resonance.

In this study examining the effect of the agonists of the TLR7, TLR8, and TLR9 subfamily on the activation of NF- $\kappa$ B of transfected HEK cells, we have discovered that ODNs can modulate IRM effects. In this study, we demonstrate that poly(dT) ODNs but not poly(dG) ODNs inhibit TLR7 and enhance TLR8 signaling events involving NF- $\kappa$ B activation in HEK cells and human primary cells. However, TLR3 agonist did not affect imidazoquinoline-induced responses. We find that the modulation of TLR7 and TLR8 responses is independent of CpG motifs and the nature of ODN backbone.

## Materials and Methods

### Reagents and media

Small molecule imidazoquinoline TLR7, TLR8, and TLR7/8 agonists (3M-001, 3M-002, 3M-003, 3M-043) were prepared by 3M Pharmaceuticals and previously described (27). 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. Oligonucleotides (CpG2006 TCGTCGTT TGTGCGTTTGTGCGTT, GpC2006 TGCTGCTTTGTGCTTTTGTGCTT, K23 TCGAGCGTTCTC, and the phosphorothioate and phosphodiester versions of poly(A), poly(T), poly(G), and poly(C) oligomers) were obtained from Invitrogen Life Technologies. Recombinant human TLR7 (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 (27). The day before transfection, HEK293 cells (CRL-1573; American Type Culture Collection) were seeded in six-well plates (BD Biosciences) at  $4 \times 10^5$  cells/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 complete RPMI medium (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 0.5 ml in 48-well, flat-bottom plates. The cells were treated for 18–24 h with various TLR agonists as shown in figures. Monocyte-derived DC were generated and treated with various TLR agonists as previously described (27).

### Determination of secreted cytokines

TNF- $\alpha$  protein levels were measured from tissue culture supernatants by ORIGIN 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-labeled (BV-TAG; BioVeris) 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- $\alpha$  (part no. AHC3419) Ab pairs were obtained from BioSource International. Ab anti-TNF- $\alpha$  (part no. AHC3419) was biotin labeled. Ab anti-TNF- $\alpha$  (part no. AHC3712) was 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- $\alpha$  were generated using rTNF- $\alpha$  (catalogue no. 210-TA; R&D Systems). The minimum level of detection for the TNF- $\alpha$  and IL-12p40/70 ORIGIN assays was 40 pg/ml.

IFN- $\alpha$  protein levels were measured from tissue culture supernatants using the MultiSpecies IFN- $\alpha$  ELISA (PBL Biomedical Laboratories). The minimum level of detection for IFN- $\alpha$  was 20 pg/ml.

### Nuclear magnetic resonance (NMR) spectroscopy

All samples for NMR spectroscopy were made from stock solutions of 50 mM 3M-043 in DMSO-d<sub>6</sub> and 2 mM DNA oligomers (10mer phosphorothioate homopolymers) in D<sub>2</sub>O. Buffer conditions were 15 mM deuterated sodium acetate (pH 8 or pH 5) and 50 mM NaCl. The final concentrations of 3M-043 and DNA oligomers were 100 mM (final DMSO concentration 0.2%). Samples were run on a Bruker Avance 700 MHz NMR spectrometer equipped with a TXI inverse detection probe and analyzed by monitoring the chemical shift of 3M-043 aromatic <sup>1</sup>H resonances with and without ODNs.

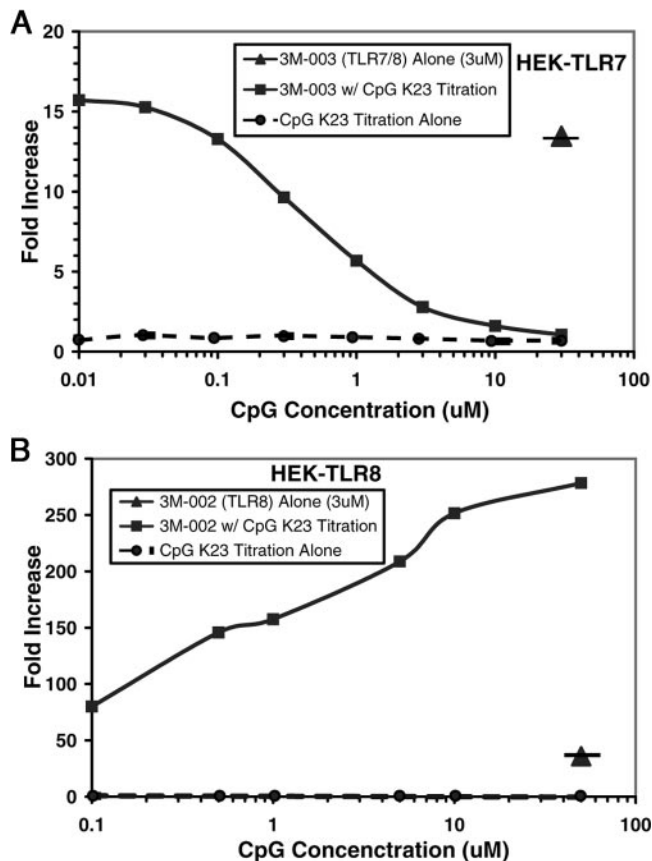
## Results

### CpG oligonucleotides modulate imidazoquinoline activation of TLR7 and TLR8

To investigate the interactions between TLR7, TLR8, and TLR9, we stimulated TLR7 or TLR8 transfected HEK cells with imidazoquinoline compounds alone and in the presence of a titrated amount of CpG oligonucleotides (Fig. 1). Addition of CpG to the TLR7 transfected cells was able to inhibit the activation of an NF- $\kappa$ B luciferase reporter construct by imidazoquinolines in a dose-dependent manner. When the CpGs were titrated into the imidazoquinoline stimulated TLR8 transfected HEK cells, however, the CpG induced a dose-dependent enhancement of NF- $\kappa$ B activation that was more potent than previously seen in any of our experiments. It is important to note that the CpG oligonucleotides alone did not activate NF- $\kappa$ B in either TLR7 or TLR8 transfected cells. These effects were limited to TLR7 and TLR8 as the activation of an NF- $\kappa$ B luciferase reporter by LPS in TLR4/MD-2 transfected HEK cells and by FltC in TLR5 transfected cells were unaffected by various doses of CpG oligonucleotides (data not shown). The converse experiment in which TLR9 transfected HEK cells were stimulated with CpG ODN and an imidazoquinoline compound titration showed no effect on the activation of an NF- $\kappa$ B luciferase reporter construct (data not shown).

ssRNA has been previously identified as a natural agonist for both TLR7 and TLR8, and we looked to see whether the ODN modulation of TLR7 and TLR8 responses was specific to 3M Pharmaceuticals' small molecule agonists or whether ODN would also affect ssRNA agonists as well. The ssRNA sequences ssRNA8A and ssRNA40 were identified as TLR7- and TLR8-specific agonists, respectively (16). In TLR7 transfected HEK cells a titration of CpG ODN inhibited the activation of an NF- $\kappa$ B reporter construct by ssRNA8A administered by DOTAP (*N*-1-(2,3-dioleoyloxy)propyl-*N,N,N*-trimethylammonium methylsulfate), similar to its inhibition of small molecule TLR7 agonists. When ssRNA40 was used against the same CpG ODN titration in TLR8 transfected HEK cells, NF- $\kappa$ B activation was inhibited as well, contrary to the ODN enhancement of NF- $\kappa$ B with TLR8 small molecule agonists (data not shown). This result could be explained by a competition for the DOTAP transfection reagent between the TLR active ssRNA and the inert ODN, the



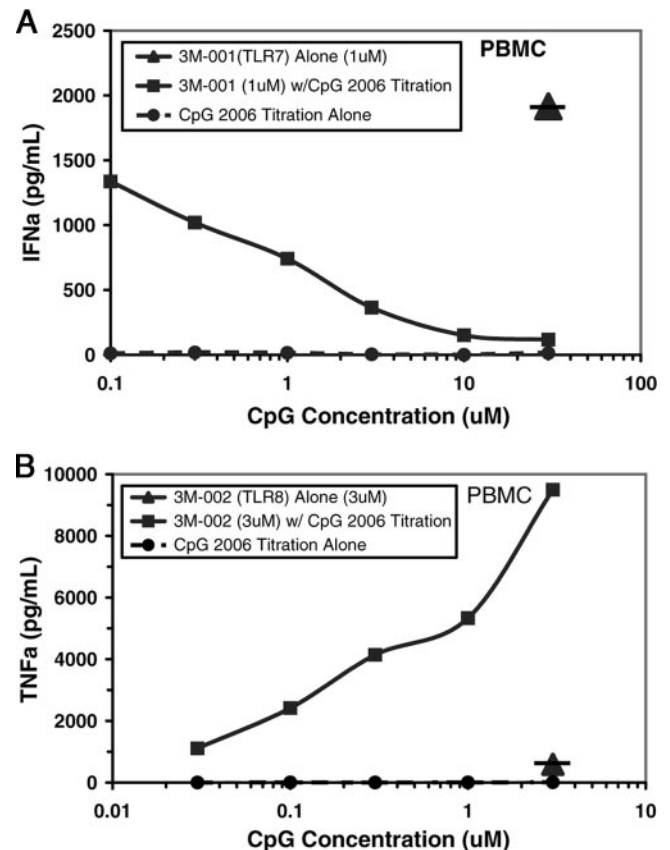


**FIGURE 1.** CpG oligonucleotides modulate Irm activation of TLR7 and TLR8. HEK293 cells were transfected by Eugene 6 with a plasmid expressing either human TLR7 (A) or human TLR8 (B) and an NF- $\kappa$ B luciferase reporter construct. One day after transfection, the cells were stimulated with the TLR7/8 agonist (3M-003) or TLR8 agonist (3M-002) at 3  $\mu\text{M}$  alone or in combination with K23 CpG ODN at the indicated concentrations and incubated overnight before assaying for luciferase activity.

increase in ODN preventing the ssRNA from access to the endosomal TLRs.

#### ODNs modulate Irm-induced cytokine production in PBMC

Previously, we have described imidazoquinoline compounds (IRMs) that are able to selectively activate TLR7 or TLR8 in human PBMCs (27). The compound 3M-001 selectively activates TLR7 and is a potent inducer of IFN- $\alpha$  and IFN-regulated proteins from human PBMC. The compound 3M-002 selectively activates TLR8 and induces TNF- $\alpha$ , IL-12, and other proinflammatory cytokines from human PBMC. Using these as TLR7 and TLR8 agonists and using IFN- $\alpha$  and TNF- $\alpha$  or IL-12 as markers for TLR7 and TLR8 activation, respectively, we examined primary PBMC cells to see whether the modulation observed with CpG oligonucleotides in transfected HEK cells would also affect cytokine induction. To eliminate the possible confounding contribution of CpG-stimulated cytokines in these experiments, we used a B cell-type CpG oligonucleotide (CpG2006) that induces B cell proliferation but does not stimulate detectable levels of IFN- $\alpha$ , TNF- $\alpha$ , or IL-12 from PBMCs. We titrated CpG oligonucleotides against TLR7 selective (3M-001) and TLR8 selective (3M-002) IRMs in PBMCs and assayed for IFN- $\alpha$  (TLR7), and TNF- $\alpha$  (TLR8), respectively (Fig. 2). As in the HEK system, the CpG oligonucleotides were able to inhibit TLR7 activation as measured by the



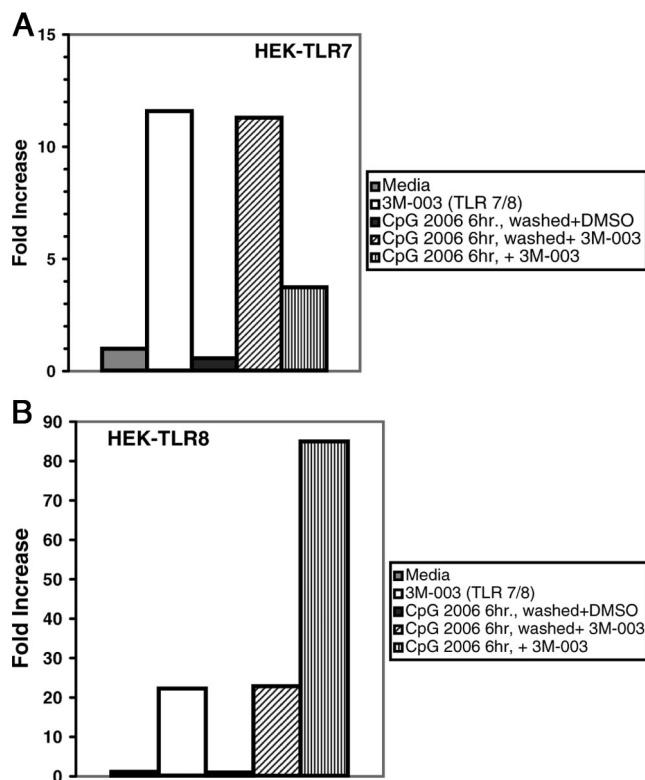
**FIGURE 2.** ODNs modulate Irm-induced cytokine production in PBMC. Human PBMC were plated in a 96-well plate and stimulated with 1  $\mu\text{M}$  TLR7 selective (A) imidazoquinoline compound 3M-001 or 3  $\mu\text{M}$  TLR8 selective 3M-002 alone (B), TLR7 or TLR8 selective imidazoquinoline in combination with a titration of CpG ODN2006, or the CpG2006 titration alone. The cells were incubated overnight and assayed for IFN- $\alpha$  and TNF- $\alpha$  by immunoassay.

induction of IFN- $\alpha$  and enhance TLR8 activation as measured by TNF- $\alpha$ .

To eliminate the possibility of an unknown CpG-induced factor that could provide an enhancement of TLR8 stimulation and proinflammatory cytokine secretion, we looked at the stimulation of monocyte-derived DC subset not expressing TLR9. When CpG oligonucleotides were titrated along with the addition of the TLR8 selective agonist 3M-002, monocyte-derived DC had a dose-dependent enhancement in the production of IL-12 and TNF- $\alpha$  (data not shown), which correlates with NF- $\kappa$ B activation in TLR8 transfected HEK cells (Fig. 1).

#### Effective modulation of TLRs requires simultaneous presence of ODNs and TLR agonists

To determine whether the modulation of Irm activity was due to a direct interaction between the ODN and the compounds or whether the action of the ODN is upon the function of the cell, such as a modulation of cellular uptake mechanisms, we repeated the experiments in the transfected HEK system with some modifications. In these experiments, TLR7 or TLR8 transfected HEK cells were incubated with CpG ODN for 6 h. After the initial incubation, the CpG ODN containing media was removed, the cells were washed, and imidazoquinoline-containing media was added for overnight incubation. The CpG ODN and imidazoquinoline had to be present simultaneously for the modulation of NF- $\kappa$ B activation to occur, in the case of both TLR7 inhibition (Fig. 3A)



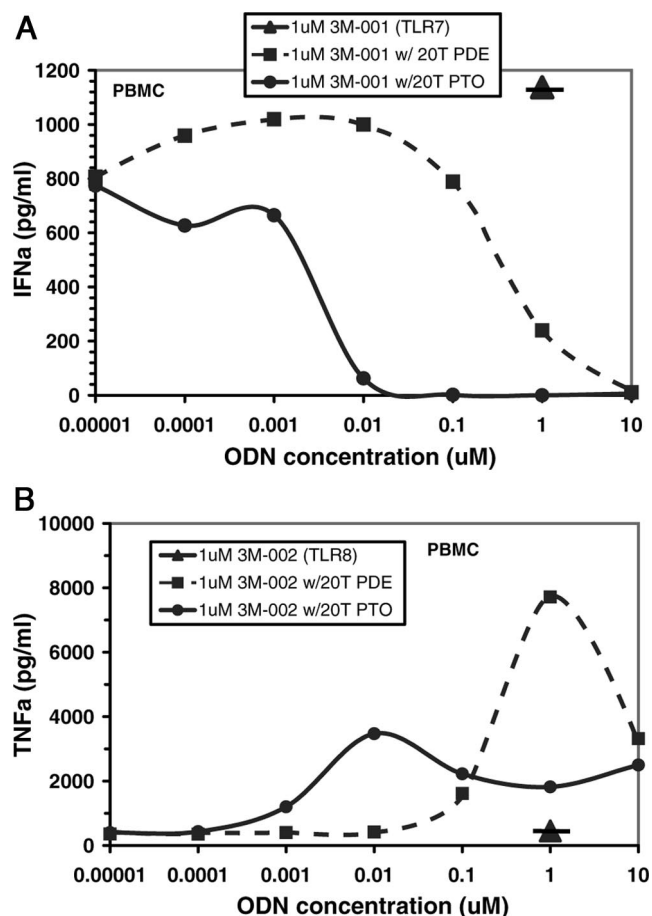
**FIGURE 3.** For effective modulation requires simultaneous presence of ODNs and TLR agonists. HEK293 cells were transfected by Fugene 6 with a plasmid expressing either human TLR7 (A) or human TLR8 (B) and an NF- $\kappa$ B Luciferase reporter construct. One day after transfection the cells were initially incubated with TLR7/8 agonist (3M-003), CpG2006, or media for 6 h. After the incubation, the medium from the CpG2006-containing groups was removed and the cells were washed twice with PBS before adding fresh media containing either 3M-003 or an equivalent volume of DMSO as a control. The cells were then incubated overnight and assayed for luciferase activity.

and TLR8 enhancement (Fig. 3B). This effect was the same in experiments in which the addition of CpG and IRM were reversed, with initial IRM incubation followed by washing and an overnight CpG ODN incubation (data not shown). From these results we concluded that IRM and ODN needed to be together to be effective.

To determine whether the CpG motif was necessary for ODN modulation of IRM function, we repeated the TLR7 and TLR8 HEK experiments with an ODN similar to CpG2006 with the modification that the CpG residues were reversed to GpC residues. We observed that the activation of NF- $\kappa$ B through TLR7 was still inhibited and the GpC version of 2006 was more potent at enhancing TLR8-mediated activation of NF- $\kappa$ B (data not shown). When we tested 20mer oligonucleotides consisting of pure poly(A), poly(T), poly(G), or poly(C) ODNs titrated against IRMs in the transfected HEK system, we found that with the exception of poly(G), all sequences were capable of inhibiting TLR7 activation (data not shown).

#### Phosphorothioate modification of ODN backbone

An initial observation that was made when working in the transfected HEK system was that the ODNs with phosphorothioate-modified structures were able to enhance TLR8 activation or inhibit TLR7 activation, whereas the ODNs with a phosphodiesterase backbone were unable to modulate IRM function. To confirm this dependence in the modulation, we used cytokine pro-

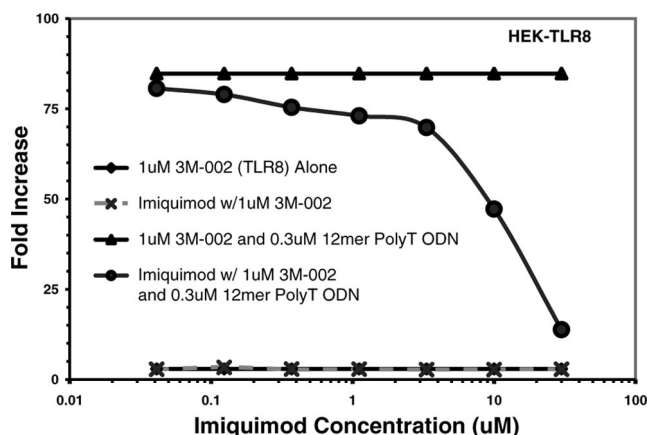


**FIGURE 4.** ODNs with phosphorothioate (PTO) and phosphodiesterase (PDO) backbones. Phosphorothioate and phosphodiesterase versions of poly(T) 20mers human PBMC were plated in a 96-well plate and stimulated with 1  $\mu$ M TLR7 selective imidazoquinoline compound 3M-001 (A) or 3  $\mu$ M TLR8 selective 3M-002 (B) alone, TLR7 or TLR8 selective imidazoquinoline in combination with a titration of phosphorothioate or phosphodiesterase poly(T) ODN. The cells were incubated overnight and assayed for TNF- $\alpha$  and IFN- $\alpha$ .

duction from PBMCs stimulated with IRMs. Phosphorothioate versions of poly(T) 20mers were titrated against the TLR7 selective 3M-001 and the TLR8 selective 3M-002. In these experiments, the phosphorothioate oligonucleotides were nearly 100-fold more potent at modulating TLR7 (IFN- $\alpha$ ) and TLR8 (IL-12) in the PBMCs than was previously observed in the HEK system. The phosphodiesterase form of poly(T), while less potent, was very active at modulation of both of the TLR-related cytokines IFN- $\alpha$  and TNF (Fig. 4). From these results we conclude that the ODN modulation of IRM activity is independent of the backbone structure of the oligonucleotide and that the HEK dependence on the phosphorothioate form of oligonucleotides may be an artifact peculiar to that cell line.

#### Kinetics of TLR7 inhibition vs TLR8 enhancement

After analysis of dose-response curves in the phosphorothioate vs phosphodiesterase titrations in the PBMC, it was apparent that the enhancement of TLR8 activation mirrored the inhibition of TLR7 activation, with similar minimum effective doses and peaks of activity. This observation along with the similar core structure of imidazoquinolines suggested that the same event causing TLR7 inhibition might be responsible for TLR8 enhancement. With this

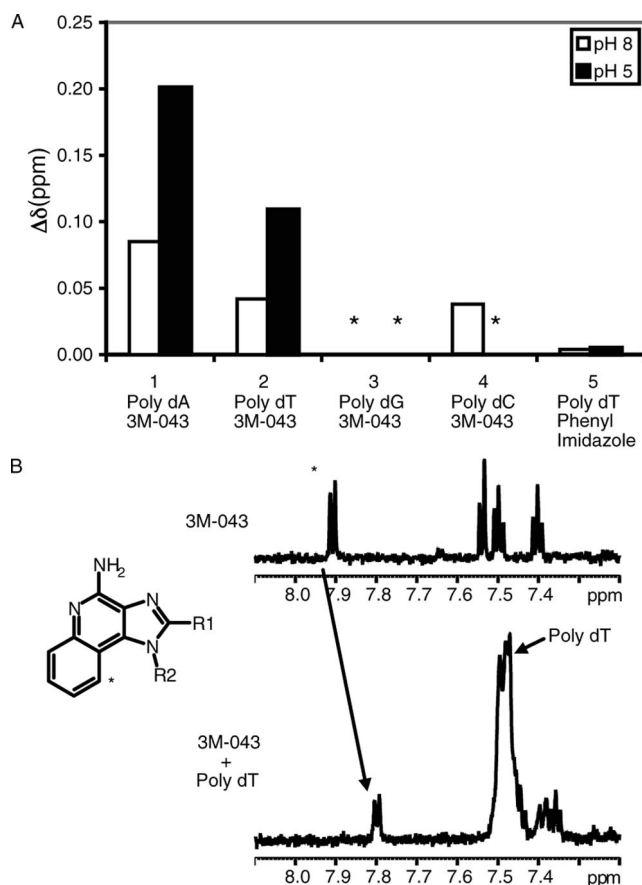


**FIGURE 5.** Kinetics of TLR7 inhibition vs TLR8 enhancement. HEK293 cells, transfected with a plasmid expressing human TLR8 and an NF- $\kappa$ B luciferase reporter construct, were stimulated with the TLR8 selective imidazoquinoline 3M-002 at 1  $\mu$ M alone or in combination with 0.3  $\mu$ M poly(T) ODN. To these groups a titration of the TLR7 selective imiquimod was added along with an imiquimod titration alone control. The cells were incubated overnight before assaying for luciferase activity.

in mind, we set out to determine whether a TLR7 selective compound could compete with a TLR8 selective compound for interaction with an ODN and eliminate the ODN enhancement of TLR8 activation. For the TLR7 selective agonist we used imiquimod, a compound unable to stimulate NF- $\kappa$ B in TLR8 transfected HEK cells, in a titration against 3M-002 (TLR8) with and without a 12mer poly(T) ODN. Titrating with imiquimod showed a dose-dependent inhibition of poly(T) enhancement of TLR8 activation of NF- $\kappa$ B (Fig. 5). This response suggested that the two TLR7 and TLR8 agonists might be able to bind ODN in the same fashion via their common core structures.

#### Physical interaction between ODNs and imidazoquinolines

NMR studies were done to directly test whether there is a physical association between the DNA oligomers and IRM (imidazoquinoline) compounds. For these studies, the phosphorothioate oligomers poly(A), poly(T), poly(G), and poly(C) ODNs were used to assess whether there was any sequence specificity to the interaction because large differences were observed in the activation of HEK reporter constructs with these different oligomers. The interaction was assayed by chemical shift perturbation analysis of the imidazoquinoline NMR resonances in the presence and absence of DNA oligomers. The difference in chemical shift for an aromatic resonance of the compound 3M-043 (a structural analogue of 3M-001) is shown in Fig. 6. Clear changes in chemical shift were observed indicating an interaction between 3M-043 and DNA oligomers. In addition, there appeared to be strong pH dependence to the interaction with a higher affinity at low pH. Lower pH was evaluated because it more closely matches the environment of the endosome where the TLR7, TLR8, and TLR9 subfamily are localized. Imidazoquinolines have a pKa of  $\sim 7$ ; therefore, they are positively charged at pH 5. To test whether the interaction is purely nonspecific electrostatics with the DNA backbone, a positively charged aromatic molecule with a similar pKa of  $\sim 7$  (phenyl imidazole) was used as a control. Almost no chemical shift perturbation was observed for phenyl imidazole, and there was very little pH dependence. Thus, the observed changes with imidazoquinolines are more specific, yet may still involve an electrostatic interaction with the thio-phosphate backbone of the oligomers (nearly identical results were obtained with phosphodiesterase poly(T) ODNs and other IRMs 3M-001 and 3M-002 (data not shown)).



**FIGURE 6.** Interaction of IRM with DNA homopolymers. *A*, Results of chemical shift perturbation analysis for 3M-043 and 10mer DNA phosphorothioate oligomers poly(A), poly(T), poly(G), and poly(C) ODNs (100  $\mu$ M concentration each). The y-axis is the change in chemical shift of one aromatic resonance of 3M-043 or phenyl imidazole upon addition of DNA oligomer. Data are the chemical shift perturbations at pH 8 and pH 5, respectively. No perturbation (\*) could be measured due to competitive broadening of IRM resonance in the presence of DNA. *B*, Image is NMR spectrum of 3M-043 alone (*top*) and in the presence of DNA oligomer (*bottom*). The resonance described in *A* is on the spectrum and structure (\*). The structure shows the core of the imidazoquinolines that typically have varying substitutions at R<sub>1</sub> and R<sub>2</sub>.

An important question is whether the imidazoquinoline compounds interact preferentially with the different homopolymer DNA sequences that might explain their differences in activity. A detailed comparison of the affinity of oligomers for imidazoquinolines is complicated by the fact that complete broadening of the imidazoquinoline resonances was observed under some conditions. Although the broadening is indicative of an interaction, no quantitative chemical shift perturbation value could be obtained (e.g., for poly(G) ODN) (Fig. 6). Note that the presence of extreme broadening tended to correlate with formation of higher order DNA structures. Nevertheless, all of the experiments indicate the ability of the different homopolymers to bind to imidazoquinolines with roughly similar affinity. In addition, poly(A) ODN, if anything, appears to bind slightly tighter to imidazoquinolines than poly(T), yet poly(T) has a much higher biological activity. Therefore, we conclude the observed differences in biological activity are not strictly due to affinity for imidazoquinoline compounds.

#### Discussion

The innate immune system recognizes a wide range of pathogen-associated molecules with a limited set of germline-encoded



recognition molecules such as TLRs, RIG-I, and NODs (28). TLR3, TLR7, TLR8, and TLR9 recognize oligonucleotide molecules if they are present within the endosomal/lysosomal compartments. Of these oligonucleotides, ssRNAs are the natural agonist for the TLR7 and TLR8. In addition, small purine-like molecules such as imiquimod, resiquimod, and loxoribine are agonists for TLR7 and TLR8 (7). Imiquimod (R-837) is approved for the treatment of actinic keratosis, basal cell carcinoma, and genital warts (29, 30). Some case reports indicate that imiquimod might be beneficial for melanomas (30). It is therefore prudent to study the mechanism of action of these drugs.

We show that when IRMs and poly(T) are exposed to the cell simultaneously, TLR7 responses are impaired while TLR8-mediated functions are enhanced. Similar phenomenon occurred between ssRNA and poly(T) (data not shown) suggesting that these interactions might have physiological relevance. The precise mechanism of action of the IRM and poly(T) combination is not known. It is possible that when target cells such as pDCs (27) are exposed to IRM together with poly(T), they interact with each other and combine in the endosomal compartment in a manner that is not optimal for interaction with TLR7 and cause the observed reduction of IRM-induced IFN- $\alpha$ . This response could be due to changes in the kinetics of endosomal/lysosomal trafficking of TLR7-agonist complex. We think that the same poly(T) and IRM combination is favored for TLR8 or related accessory factors.

The requirement for simultaneous presentation of IRMs and poly(T) suggested that a direct interaction between ODNs and imidazoquinolines might be responsible for both the TLR7 inhibition and TLR8 enhancement. NMR data presented in this study provided evidence for a direct interaction between the DNA and IRMs in vitro. This interaction is expected to be stronger within an acidic endosomal environment in which both of these components concentrate.

We noted that poly(T) formed no higher order structure and shows the highest degree of modulation of imidazoquinoline activity through TLR8. Oligomer biological activity may correlate with a DNA sequence that has significant "single-stranded" character and may be critical for recognition of the complex by a receptor. If these assumptions are correct, a TLR7 selective compound unable to stimulate NF- $\kappa$ B in TLR8 transfected HEK cells would compete with a TLR8 selective compound for ODN interaction and eliminate ODN enhancement of TLR8 activation. Indeed, titrating in imiquimod showed a dose-dependent inhibition of poly(T) enhancement of TLR8 activation of NF- $\kappa$ B. This suggested that the two IRM compounds might be able to bind ODN in the same fashion via their common core structures.

Taken together our results indicate existence of a physical interaction between small molecule IRMs and poly(T) ODN, and this Imidazoquinoline-ODN complex suppresses TLR7 and enhances TLR8 responses. These results may have relevance to recent reports demonstrating that certain CpG ODNs can suppress immune responses (31–33). In addition, while this manuscript was under review, a study has reported similar findings to our own in the modulation of human TLR7 and TLR8 by T-rich phosphorothioate ODN and R848 (34). How TLR7, TLR8, and TLR9 discriminate between stimulatory and suppressive structures remains to be elucidated and could involve additional accessory signaling elements. Further studies in progress, such as ODN effects on intracellular localization and trafficking using the system described in this study, may provide some answers and show the utility in treating diseases via TLR modulation.

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## Disclosures

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