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CUTTING EDGE

Cutting Edge: Species-Specific TLR9-Mediated Recognition of CpG and Non-CpG Phosphorothioate-Modified Oligonucleotides[1]

Tara L. Roberts,[*†] Matthew J. Sweet,*[*†‡] David A. Hume,*[*†§] and Katryn J. Stacey*[2*†‡]

***Different DNA motifs are required for optimal stimulation of mouse and human immune cells by CpG oligodeoxynucleotides (ODN). These species differences presumably reflect sequence differences in TLR9, the CpG DNA receptor. In this study, we show that this sequence specificity is restricted to phosphorothioate (PS)-modified ODN and is not observed when a natural phosphodiester backbone is used. Thus, human and mouse cells have not evolved to recognize different CpG motifs in natural DNA. Nonoptimal PS-ODN (i.e., mouse CpG motif on human cells and vice versa) gave delayed and less sustained phosphorylation of p38 MAPK than optimal motifs. When the CpG dinucleotide was inverted to GC in each ODN, some residual activity of the PS-ODN was retained in a species-specific, TLR-9-dependent manner. Thus, TLR9 may be responsible for mediating many published CpG-independent responses to PS-ODN. The Journal of Immunology,* 2005, 174: 605–608.**

Bacterial DNA is recognized by cells of the innate immune system by the presence of unmethylated CG dinucleotides in particular sequence contexts (CpG motifs) (1, 2). Cellular responses to CpG DNA require initial internalization of the DNA followed by endosomal acidification (3–5). The CpG motif is then recognized and signaling is initiated via TLR9 (6).

Responses to bacterial DNA are mimicked by oligodeoxynucleotides (ODN)[3] containing a CpG motif. Immunostimulatory ODN have potential applications in cancer and allergy therapy and as vaccine adjuvants (7). In these in vivo applications nuclease-resistant phosphorothioate (PS)-ODN are used. In the PS modification, one of the nonbridging oxygens in the backbone is replaced by sulfur. Much of the work examining the immune response to CpG DNA has used only PS-ODN. PS-ODN have been reported to have CpG-independent effects on B cell proliferation, Ab production and IL-6 production (8–10), and macrophage chemotaxis (11) and in vivo to induce splenomegaly and infiltration of tissues by mononuclear cells (9). The mechanisms responsible for non-CpG-related effects of PS-ODN have not been characterized.

Mice and humans respond preferentially to different CpG-containing PS-ODN (12, 13). The optimal mouse motif is purine-purine-C-G-pyrimidine-pyrimidine; GACGTT and AACGTT are most commonly used. For humans, GTCGTT in multiple copies, separated by TT dinucleotides is optimal. HEK293 cells transfected with human TLR9 respond preferentially to the human motif, but when transfected with mouse TLR9 are more sensitive to the mouse motif (14–16). This suggests that TLR9 itself directly interacts with DNA and mediates species-specific binding. These studies were performed exclusively using PS-ODN. Direct CpG-dependent interaction of PO-ODN with TLR9 has recently been confirmed (17). However, the binding affinity of TLR9 for phosphodiester (PO)-ODN of different sequences did not correlate with the established species-specific responses to PS-ODN. In this article, we show that species-specificity for recognition of stimulatory sequences is restricted to PS-ODN and that TLR9 can mediate responses to non-CpG PS-ODN.

Materials and Methods

Cells and mice

The mouse macrophage cell line RAW264.7 and human B cell line RPMI8226 were purchased from the American Type Tissue Culture Collection. WEHI231 mouse B cells were a gift from D. Tarlinton (Walter and Eliza Hall Institute, Melbourne, Australia). Medium used was RPMI 1640 with 10% FCS, 2 mM L-glutamine, 20 U/ml penicillin, and 20 μg/ml streptomycin. TLR9 knockout mice, obtained from Shizuo Akira (Research Institute for Microbial Diseases), were bred from heterozygotes and wild-type siblings were used as controls. To obtain splenocytes, spleens were homogenized through a tea strainer and a single-cell suspension generated by passage through an 18-gauge needle. PBMCs were obtained from fresh human blood and collected into heparinized tubes. Ficoll-Paque (Amersham Biosciences) solution was layered under whole blood diluted 1/4 with saline and samples centrifuged at 400 $\times$ *g* for 15–20 min. PBMCs at the interface were removed and washed twice with PBS with centrifugation at 500 $\times$ *g* for 10 min at 10°C. PBMCs were used immediately.

Oligonucleotides

PS- and PO-ODN were obtained from Geneworks. PS-ODN used were: 1668, TCCATGACGTTCCTGATGCT (1); 2006, TCGTCGTTTTGTCGTTTTGTCGTT (12); 1826, TCCATGACGTTCCTGACGTT (18); 1668-GC, TCCATGAGCTTCCTGATGCT; 2006-GC, TGCTGCTTTTGTGCTTT

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[3] Abbreviations used in this paper: ODN, oligodeoxynucleotide; PS, phosphorothioate; PO, phosphodiester; PS-ODN, PS-modified ODN; PO-ODN, PO-modified ODN.

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TGTGCTT. The PO-ODN versions of 1668 and 2006 are designated 1668-O and 2006-O.

ELAM promoter assay

The mouse macrophage cell line RAW264.7 stably transfected with the human NF-κB-responsive ELAM promoter driving expression of enhanced GFP, termed ELAM9, has been described (19). ELAM9 cells were treated with indicated amount of DNA for 4–6 h before harvesting for analysis of GFP expression by flow cytometry as described previously (19).

ELISA

Two hundred fifty thousand cells per well were plated in 250 μl of complete medium and treated with DNA for 24 h. ELISA was performed using paired Abs against human and mouse IL-6 (BD Pharmingen) as described previously (20).

Western blotting

Cells were lysed in boiling 66 mM Tris-HCl (pH 7.4) plus 2% SDS with 1 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM sodium molybdate, and 1 mM sodium vanadate as phosphatase inhibitors. Western blotting was performed as described elsewhere (21), and p38 or its activated form phosphorylated on threonine 180 and tyrosine 182 were detected using Abs from Cell Signaling Technology.

Results and Discussion

Species specificity is restricted to PS-ODN

We examined mouse and human motifs as both PS- and PO-ODN in activation of human and mouse cells. 1668 and 2006 are PS-ODN containing the optimal mouse and human motifs, respectively. 1668 was more active than 2006 in assays of activation of a mouse macrophage cell line, mouse WEHI231 B cells and mouse splenocytes (Fig. 1, *A–C*). The converse was true for IL-6 production from human PBMCs where the human motif in 2006 had superior activity at low concentrations, although both sequences gave the same response above 1 μM (Fig. 1*D*). Unexpectedly, the corresponding PO-ODN, 1668-O and 2006-O had identical activity on all cells (Fig. 1). The measured IL-6 produced by splenocytes and PBMCs in response to PO-ODN was very low. However, in four separate experiments on PBMCs, IL-6 levels in response to PO-ODN were at least four times the detection limit and there was no difference between the activities of mouse and human motifs.

The fact that species-specific CpG responses are restricted to PS-ODN has not been widely appreciated. A recent report showed that mouse TLR9-transfected HEK293 cells gave a similar response to 2006-O and 1668-O at a single high concentration of 10 μM (17). In our experience, assessment of the potency of CpG motifs requires analysis at a range of concentrations, since poorly activating sequences such as cytosine-methylated CpG motifs can attain the same level of activation as optimal motifs if the concentration is high enough (result not shown).

TLR9 recognition of DNA

Binding data in Biacore studies has clearly shown CpG-specific binding of PO-ODN to TLR9 (17). Binding to a PO version of 1668 was much more rapid and reached a higher level than its GC inversion. Interestingly, TLR9 bound only to single stranded molecules, suggesting that DNA strand separation in the endosome is an obligatory step for recognition of CpG motifs within dsDNA. Binding was dependent on acid pH, consistent with the requirement for endosomal maturation in responses to CpG DNA. In contrast to expectations, mouse TLR9 interacted more strongly with PO-ODN containing human motifs, such as 2006-O, than 1668-O (17). Our results here show clearly in a number of different cell types that these two PO-ODN have indistinguishable activity (Fig. 1). There could be a number of explanations for the discrepancy between TLR9 binding and activity. It cannot be assumed that the measured rate of ODN binding to TLR9 in vitro is the sole determinant of TLR9 activation. An affinity for TLR9 is an obvious prerequisite for activity, but an antagonist may well have higher affinity. It is likely that the conformation adopted by the ODN upon TLR9 binding and ability to induce conformational

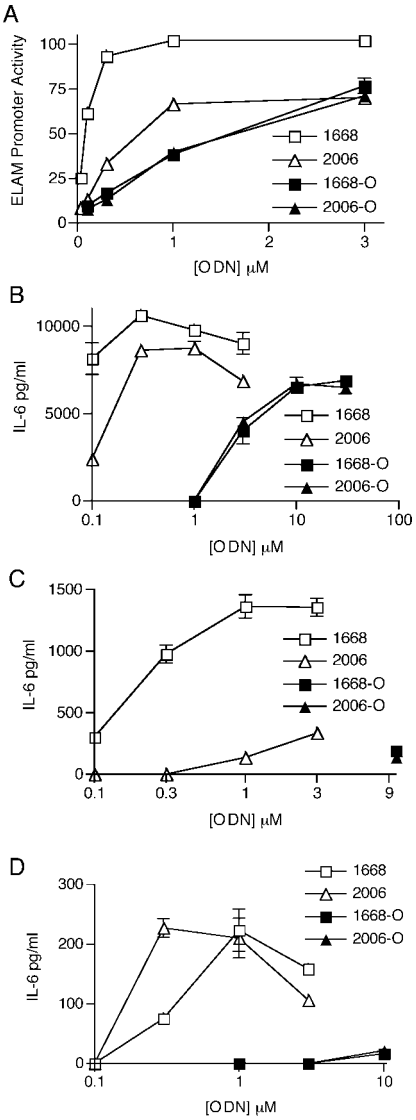

FIGURE 1. Species specificity for CpG motif recognition is restricted to PS-ODN. 1668 contains the mouse motif and 2006 efficiently activates human cells. They were tested as PS and PO versions on mouse and human cells. *A,* Activation of the ELAM promoter in mouse macrophages stably transfected with an ELAM promoter-GFP construct. Each point is the average of duplicates and error bars show the values obtained. The experiment is representative of four independent assays. *B,* IL-6 production by the mouse B cell line WEHI231. Each point is the average of triplicates and error bars show the SD. The experiment is representative of two independent assays. *C,* IL-6 production by mouse splenocytes. Splenocytes were isolated from CD1 mice, left for 2 h in culture, and then treated with ODN for 24 h. Each point is the average of triplicates and error bars indicate the SD. The experiment is representative of two independent assays. *D,* IL-6 production by human PBMCs. Each point is the average of triplicates and error bars indicate the SD. The experiment is representative of two independent assays.

change in TLR9 are important. Alternatively, binding conditions in in vitro experiments may not adequately replicate the endosomal environment, other interacting proteins may be required or the pH or chemical environment may not be optimal.

Nonoptimal PS-ODN give delayed and reduced signaling

We previously showed that signaling in response to a PS-ODN was delayed compared with its PO-ODN counterpart (21). More recent results demonstrated that this delay is not seen with PS-ODN 1668, and hence is sequence dependent (result not shown). Mouse and human motifs may therefore vary in the rapidity with which they activate different cell types. We compared the kinetics of induction of p38 phosphorylation in response to mouse and human ODN by Western blotting. In both mouse WEHI231 B cells and the mouse RAW264.7 macrophage cell line, PS-ODN containing the mouse motif (1668 or 1826) induced earlier and more sustained phosphorylation than the human ODN 2006 (Fig. 2, *A* and *B*), consistent with the more effective response to the mouse motif. Conversely, in the human B cell line RPMI8226, 2006 induced phosphorylation earlier than 1668 (Fig. 2*C*). In general, we found signaling in human RPMI8226 cells to be slower than that of mouse cells, even with optimal motifs. In other experiments, no p38 phosphorylation was seen after 30 min of exposure to 2006 (result not shown). This may be related to the apparent lower affinity of human TLR9 for DNA (17).

The relative activity of mouse and human motifs in cells from each species was thus a result of more rapid and more sustained signaling in response to the preferred motif (Fig. 2). We have found no difference in the rate of uptake or the intracellular trafficking of fluorescently labeled 2006 and 1668 that could explain the observed differences in activity (result not shown). The delayed signaling in response to nonoptimal motifs is likely to be due to either a reduced affinity for TLR9 or a structural conformation of bound ODN which is not optimal for TLR9 activation.

PS modification may constrain TLR9 binding

The PS modification replaces oxygen in the PO backbone with the larger sulfur atom. This may restrict the conformations available to PS-ODN within a putative DNA binding groove on TLR9. Conformations adopted to accommodate the sulfur atom may not allow certain base sequences to fit well within the binding site. Consistent with this, the sequence requirements for activity of PS-ODN are known to be more stringent than for PO-ODN (7). Some active PO-ODN become inactive when PS modified (12). Elucidation of the basis of species-specific PS-ODN recognition requires greater understanding of the structural basis for CpG DNA interaction with TLR9.

GC ODN also show species-specific activity

The inversion of the CpGs within 2006 and 1668 to GC did not completely prevent their activity, and the residual activity of GC inversions retained the species specificity of the parent ODN (Fig. 3). On mouse WEHI231 B cells (Fig. 3*A*) and RAW264.7 macrophage-like cells (result not shown), the GC inversion of 1668 had readily detectable activity, while 2006-GC was inactive. In human cells, the sequence specificity was less absolute; both GC inversions had activity on the RPMI8226 human B cell line, although 2006-GC activity was detectable at lower concentrations (Fig. 3*B*). The response to 2006-GC reached a maximum and then declined with increasing concentration. The phenomenon of "self inhibition" by PS-ODN at higher concentrations has been observed before (21) and is sequence dependent because it is more pronounced with

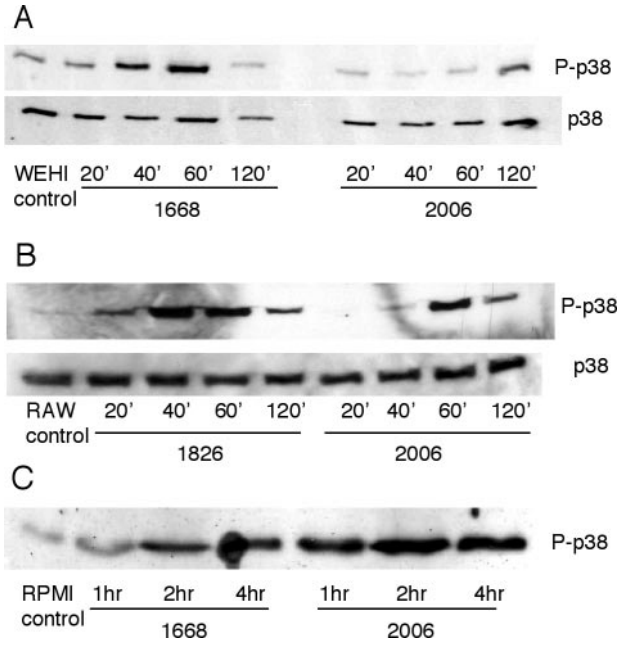

FIGURE 2. Nonoptimal PS-ODN give delayed signaling. Western blots were performed to detect the phosphorylated form of p38 at various times after the addition of PS-ODN. *A,* WEHI231 mouse B cells were treated with 1 μM 1668 or 2006. Blotting for total p38 was used for loading control. *B,* RAW264.7 mouse macrophage cells were treated with 1 μM mouse (1826) or human (2006) ODN. Blotting for total p38 was used for loading control. *C,* RPMI8226 human B cells were treated with 1 μM 1668 or 2006. Protein assay was used to ensure equal amounts of protein were loaded.

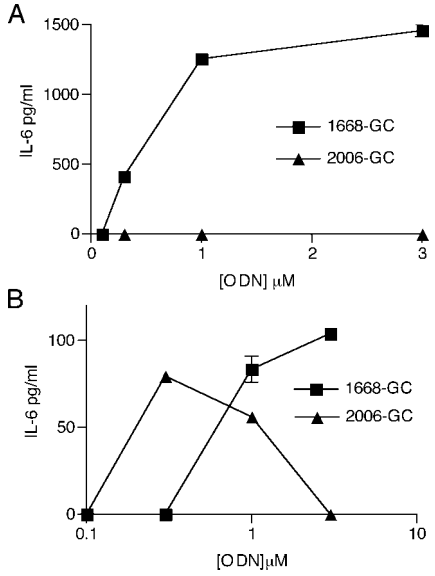

FIGURE 3. GC inversions of mouse and human CpG PS-ODN show residual species-specific activity. *A,* IL-6 induced in mouse WEHI231 B cells. Each point is the average of triplicates and error bars show the SD. The experiment is representative of two independent assays. *B,* IL-6 induced in human RPMI8226 B cells. Each point is the average of triplicates and error bars show the SD. The experiment is representative of two independent assays.

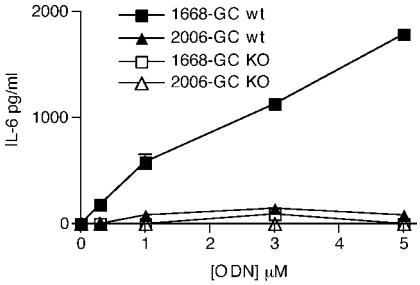

FIGURE 4. TLR9 is required for the response to non-CpG-containing ODN. Splenocytes from TLR9$^{-/-}$ knockout mice and wild-type littermates were left overnight in culture and then treated with various concentrations of human and mouse GC-inversion PS-ODN (2006-GC and 1668-GC, respectively). Each point is the average of duplicates and error bars indicate the values obtained. The experiment is representative of two independent assays.

certain PS-ODN. CpG-free PS-ODN rich in thymidine residues can activate human B cells (22, 23). The T-rich nature of 2006 may be important for its high activity on human cells and may be responsible for responses to 2006-GC at low concentrations. Human cells showed less discrimination between CpG and GC ODN than mouse cells did. In human RPMI8226 cells, induction of IL-6 by 2006-GC was 15% of the response to 2006 (result not shown). For activation of the ELAM promoter in a mouse macrophage cell line or IL-6 induction in mouse WEHI231 cells, responses to 1668-GC reached <5% of the value for 1668 (result not shown).

GC ODN activity is dependent on TLR9

Given that the species specificity for CpG PS-ODN is mediated by TLR9 (14), TLR9 is likely to be required for the residual species-specific response to the GC inverted ODN. To confirm this, splenocytes were isolated from TLR9 knockout mice and wild-type littermates. Wild-type splenocytes produced IL-6 in response to 1668-GC but not 2006-GC (Fig. 4). Splenocytes from TLR9$^{-/-}$ mice had no response to non-CpG PS-ODN (Fig. 4) or CpG ODN (result not shown). Thus, the induction of IL-6 in response to GC PS-ODN also requires the presence of TLR9. A recent article has shown similar TLR9 dependence for Ab production in response to non-CpG PS-ODN (10). Responses to non-CpG PS-ODN are not just sequence-independent responses to the PS backbone as some sequences are inactive. It is likely that many of the reported immunostimulatory responses to non-CpG PS-ODN of various sequences (8, 9, 11, 22, 23) are mediated through TLR9.

Conclusion

Species specificity for CpG motif recognition is a necessary concern in therapy, where PS-ODN are used, and is also a useful tool for studying TLR9-mediated activation. However, our work here suggests that human and mouse TLR9 have not evolved to recognize different CpG motifs in natural DNA. Species specificity for CpG motifs is not relevant to the in vivo response to infection nor to plasmid-based DNA vaccination, since in both cases the DNA presented to the immune system would have the native PO backbone. There would consequently be no expectation that mouse and human viruses or other pathogens have differentially suppressed "mouse" and "human" CpG motifs in their genomes.

Inversion of the CpG dinucleotide in mouse and human PS-ODN left residual TLR9-dependent, species-specific responses. Thus, many reported immunostimulatory responses to non-CpG PS-ODN may also be mediated through TLR9. For applications such as antisense therapy, which use PS-ODN, avoiding immune stimulation may not be as simple as just avoiding CpG motifs. CpG-independent immunostimulatory activity of PS-ODN will probably depend on the ability of the PS-ODN to fit within the binding pocket of TLR9 and partially mimic the structure adopted by efficient CpG-containing ligands.

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