



Educational Resources

Technical Note: Introduction to Spectral Flow Cytometry



SONY



## CpG RNA: Identification of Novel Single-Stranded RNA That Stimulates Human CD14<sup>+</sup>CD11c<sup>+</sup> Monocytes

This information is current as of July 23, 2020.

Takahiro Sugiyama, Mayda Gursel, Fumihiko Takeshita, Cevayir Coban, Jacqueline Conover, Tsuneyasu Kaisho, Shizuo Akira, Dennis M. Klinman and Ken J. Ishii

*J Immunol* 2005; 174:2273-2279; ;  
doi: 10.4049/jimmunol.174.4.2273  
<http://www.jimmunol.org/content/174/4/2273>

**References** This article **cites 34 articles**, 15 of which you can access for free at:  
<http://www.jimmunol.org/content/174/4/2273.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days\*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

\*average

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:  
<http://jimmunol.org/subscription>

**Permissions** Submit copyright permission requests at:  
<http://www.aai.org/About/Publications/JI/copyright.html>

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:  
<http://jimmunol.org/alerts>

*The Journal of Immunology* is published twice each month by  
The American Association of Immunologists, Inc.,  
1451 Rockville Pike, Suite 650, Rockville, MD 20852  
Copyright © 2005 by The American Association of  
Immunologists. All rights reserved.  
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# CpG RNA: Identification of Novel Single-Stranded RNA That Stimulates Human CD14<sup>+</sup>CD11c<sup>+</sup> Monocytes

Takahiro Sugiyama,<sup>\*†</sup> Mayda Gursel,<sup>\*</sup> Fumihiko Takeshita,<sup>‡</sup> Cevayir Coban,<sup>§¶</sup> Jacqueline Conover,<sup>\*</sup> Tsuneyasu Kaisho,<sup>†</sup> Shizuo Akira,<sup>§¶</sup> Dennis M. Klinman,<sup>\*</sup> and Ken J. Ishii<sup>1\*§¶</sup>

Synthetic immunostimulatory nucleic acids such as CpG DNA are being harnessed therapeutically as vaccine adjuvants, anti-cancer or antiallergic agents. Efforts to identify nucleic acid-based agents capable of more specifically modulating the immune system are being developed. The current study identifies a novel class of single-stranded oligoribonucleotides (ORN) containing unmethylated CpG motifs and a poly(G) run at the 3' end (CpG ORN) that directly stimulate human CD14<sup>+</sup>CD11c<sup>+</sup> monocytes but not dendritic cells or B cells. CpG ORN activate NF- $\kappa$ B and p38 MAPK, resulting in IL-6 and IL-12 production and costimulatory molecule up-regulation but not IFN $\alpha$ . Methylation of cytosine at the 5' portion in core CpG motif abrogates such activation. TLR3, 7, 8, or 9 alone did not confer response to CpG ORN, in contrast to previously reported respective nucleic acid ligands. These data suggest that CpG ORN represent a novel class of synthetic immunostimulatory nucleic acids with distinct target cells, receptors, and functions from that of previously known immunomodulatory nucleic acids. *The Journal of Immunology*, 2005, 174: 2273–2279.

The innate immune system recognizes specific sequence motifs expressed in the DNA and/or RNA of various pathogens (1–4). For example, recognition of CpG motifs in DNA or dsRNA molecules triggers a strong proinflammatory response via TLRs (5, 6). The immunostimulatory activity of CpG DNA is being harnessed therapeutically to prevent or treat infectious diseases, allergy, cancer, and/or autoimmune diseases (7, 8).

Unmethylated CpG motifs are recognized via TLR9 expressed on certain immune cells (such as B cells, plasmacytoid dendritic cells (pDC),<sup>2</sup> and macrophages) (4, 7). CpG DNA is taken up via an endo/phagocytosis pathway controlled by PI3K (9) and interact with intracellular TLR9 (10). The interaction of CpG DNA with TLR9 triggers the recruitment of the MyD88 adaptor molecule, followed by the activation of IL-1R-associated kinase, TNFR-associated factor 6, and I $\kappa$ B kinase, culminating in the nuclear translocation of NF- $\kappa$ B (5, 11, 12). Such activation by CpG DNA results in the production of proinflammatory cytokines (such as IL-6, IL-12, and IFN $\alpha$ / $\gamma$ ), chemokines (such as MCP-1, IFN- $\gamma$ -inducible protein 10, MIP-1 $\alpha$ / $\beta$ ), and Igs (reviewed in Refs. 4 and 7). Recent studies suggest that CpG oligodeoxynucleotides (ODN) are categorized at least into two groups in both a structural and functional

manner (7, 8): one is K (also known as B type) CpG ODN consisted of all phosphorothioate backbone with multiple TCG motifs that preferentially stimulate B cells to proliferate and secrete IL-6 and IgM, and the other is type D CpG ODN (also known as A type) with mixed backbone with one palindromic CpG motifs plus poly(G) run at the 3' end that stimulates pDC to secrete large amount of IFN $\alpha$ , followed by myeloid DC maturation and NK cell activation. These CpG ODN optimized for humans, in contrast to those for mice, are not able to induce a robust amount of IL-12. Since IL-12 is considered to play a critical role in CpG-mediated efficacy in vivo, there is room to improve and/or modify such humanized CpG-based immunotherapeutic nucleic acids.

dsRNA, such as that found during intermediate stages of viral infection, is recognized by the innate immune system through TLR3. dsRNA is a potent stimulator of human monocytes and myeloid DC, inducing the secretion of both IL-12 and IFN $\alpha$  (6, 13). More recently, ssRNA was shown to activate pDC in mice and myeloid DC in humans to produce IFN $\alpha$  and IL-12, respectively (14–17). However, whether the immunomodulatory activity of RNA is sequence specific has not been fully investigated.

The present work examines whether ssRNA have sequence-specific immunomodulatory activity. Results indicate that ssRNA oligonucleotides expressing unmethylated CpG motifs and a poly(G) tail (CpG oligoribonucleotides (ORN)) stimulate CD14<sup>+</sup>CD11c<sup>+</sup> monocytes to produce large amounts of IL-12. This activity is distinct from that of DNA-based CpG ODN. CpG ORN also stimulates human PBMC to activate NF- $\kappa$ B and p38 MAPK and to secrete IL-6, similar to the effect of K-type CpG ODN. Activation of cells by CpG ORN was not mediated through either TLR3, 7/8, or 9, the known receptors for dsRNA, ssRNA, or dsRNA, respectively. The activity of CpG ORN was blocked by inhibitors of PI3K. These data suggest that CpG ORN represent novel immunostimulatory agents stimulating human monocytes to produce IL-12 that is distinct from previously known immunostimulatory nucleic acids.

\*Section of Retroviral Immunology, Division of Viral Products, Center for Biologics, Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892; <sup>†</sup>Laboratory for Host Defense, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan; <sup>‡</sup>Department of Molecular Biodefense Research, School of Medicine, Yokohama City University, Yokohama, Japan; <sup>§</sup>Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Osaka University, Osaka, Japan; and <sup>¶</sup>Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Received for publication August 4, 2004. Accepted for publication December 2, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Address correspondence and reprint requests to Dr. Ken J. Ishii, Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail address: kenishii@biken.osaka-u.ac.jp

<sup>2</sup> Abbreviations used in this paper: pDC, plasmacytoid dendritic cell; ODN, oligodeoxynucleotide; ORN, oligoribonucleotide; 2-OME, 2'-O-methyl.

## Materials and Methods

### Cells and reagents

Normal human PBMC and elutriated monocytes were obtained from the Department of Transfusion Medicine, National Institutes of Health (Bethesda, MD). The purity of elutriated monocytes were 75–90%. Myeloid dendritic cells and pDC were obtained from human monocytes as previously described (18). PBMC were separated by Ficol-Hypaque density gradient centrifugation as previously described (19). To obtain myeloid dendritic cells, monocytes were cultured for 7 days in the presence of rIL-4 and GM-CSF as previously described (20). In some experiments, pDC were depleted by anti-BDCA2 Ab using a MACS column according to manufacturer's protocol (Miltenyi Biotec). Cells were cultured in complete medium (RPMI 1640 with 5% FBS) in the presence of various stimuli for 48–72 h at a concentration of  $2.5 \times 10^6$  cells/ml. HEK-293 cells were purchased from American Type Culture Collection and maintained in DMEM with 10% FBS as described elsewhere (9). Human TLR3 cDNA was a kind gift from Dr. R. Medzhitov (Yale University) and cDNA for human TLR7 and 8 were kind gifts from Dr. B. Beutler (The Scripps Research Institute, La Jolla, CA). TLR3, 7, 8, and 9 were cloned into the pCIneo vector (Invitrogen Life Technologies) and their sequences were confirmed (11). Transfection of HEK-293 cells with these plasmids was performed by using 293 fectin (Invitrogen Life Technologies) according to the manufacturer's recommendation. In some experiments, the PI3K inhibitor wortmannin or the p38 inhibitor SB203580 was used at 1 or 2  $\mu$ M, respectively. LPS, wortmannin, and SB203580 were purchased from Sigma-Aldrich. dsRNA poly(IC) was purchased from Amersham Pharmacia and R848 was purchased from Invivogen. FuGENE6 was purchased from Roche Molecular Biochemicals.

### Synthesis of oligonucleotides

ODN were synthesized at the Center for Biologics Evaluation and Research Core Facility as previously described (21). Some ODN and ORN were purchased from Dharmacon Research. ORN were protected by 2'-bis(acetoxyethoxy)-methyl ether (22) that did not alter their immunostimulatory activity whereas 2'-O-methyl (2-OME) modification of RNA abrogates such effects (data not shown). ORN were synthesized using either phosphodiester or phosphorothioate linkages. All oligonucleotides contained <0.1 endotoxin unit/mg.

### Cytokine ELISA

Cells ( $2.5 \times 10^6$ /ml PBMC or elutriated monocytes and  $5 \times 10^4$ /ml HEK-293 cells) were stimulated for 48 h in vitro (24 h for HEK 293 cells). Cell culture supernatants were analyzed for cytokine content by ELISA as described elsewhere (19). Briefly, 96-well Immulon II plates were coated with anti-human IL-6 (clone mAb206; R&D Systems), IL-8 (clone 6217; R&D Systems), IL-12 p70 (clone 20C2; BD Pharmingen), IFN- $\gamma$  (clone M-700A; Endogen), or IFN $\alpha$  (PBL Biomedical Laboratories) in PBS (pH 7.2) for 5 h. The plates were blocked and washed, and then supernatants were added for 2 h at room temperature. The plates were then washed and treated with biotinylated anti-cytokine Ab (IL-6: clone AHC0963; BioSource International; IL-8: polyclonal; R&D Systems; IL-12: C8.6; BD Pharmingen; IFN- $\gamma$ : clone M-701; Endogen) followed by phosphatase-conjugated avidin and a phosphatase-specific colorimetric substrate. The concentration of cytokines was determined by the standard curve obtained using known amounts of recombinant cytokine. All assays were performed in triplicate.

### Flow cytometric analysis of cell surface molecules and intracellular cytokines

PBMC were stimulated with various ODN or ORN for 24 h. Cells were washed, fixed, and stained with FITC-labeled CD14 or CD19, PE-labeled CD86 or CD83, and Cy3-labeled CD11c for 30 min at room temperature as previously described (18). Stained cells were washed and resuspended in PBS-0.1% BSA/Na $_3$ . Abs against human CD11c and 14, CD83, and CD86 were obtained from BD Pharmingen.

For intracellular cytokine staining, brefeldin A (10  $\mu$ g/ml; Sigma-Aldrich) was added 4 h after stimulation. Cells were washed with 3 mM EDTA-PBS, fixed, and permeabilized by using the FIX and PERM cell permeabilization kit (Caltag Laboratories) as recommended by the manufacturer. Cells were stained with PE-conjugated anti IL-12 Ab (BD Pharmingen) and with FITC anti-CD14 (or CD19) and Cy3 anti-CD11c. After staining, the cells were washed with saponin containing PBS and resuspended in PBS-0.1% BSA/Na $_3$ . One hundred thousand events per sample were analyzed using a FACSort (BD Biosciences). CellQuest software was used for data analysis.

### Western blot analysis

Elutriated monocytes stimulated with CpG ORN or its controls were harvested, washed with ice-cold PBS, and lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM NaF, 15 mM *p*-nitrophenyl phosphate disodium hexahydrate, 2 mM Na $_3$ VO $_4$ , 1% Nonidet P-40, 0.1% SDS, 1% deoxycholate, 0.025% Na $_3$ N, 1 mM DTT, and protease inhibitor mixture; Sigma-Aldrich). Whole cell extracts were separated on SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Bio-Rad) using a NuPAGE system according to the manufacturer's protocol (Invitrogen Life Technologies). The membrane was blocked by 5% skim milk and blotted with specific Abs. The membrane-bound Abs were visualized with HRP-conjugated Ab to rabbit IgG using the LumiGLO reagent (Cell Signaling Technology). Anti-phosphor-p38, p38, phosphor-I $\kappa$ B, and I $\kappa$ B were purchased from Cell Signaling Technology.

### Statistical analysis

One-way ANOVA was used to compare groups. Values are expressed as mean  $\pm$  SD. A  $p < 0.05$  was considered to be significant.

## Results

### Activity of synthetic RNA containing immunostimulatory CpG motifs

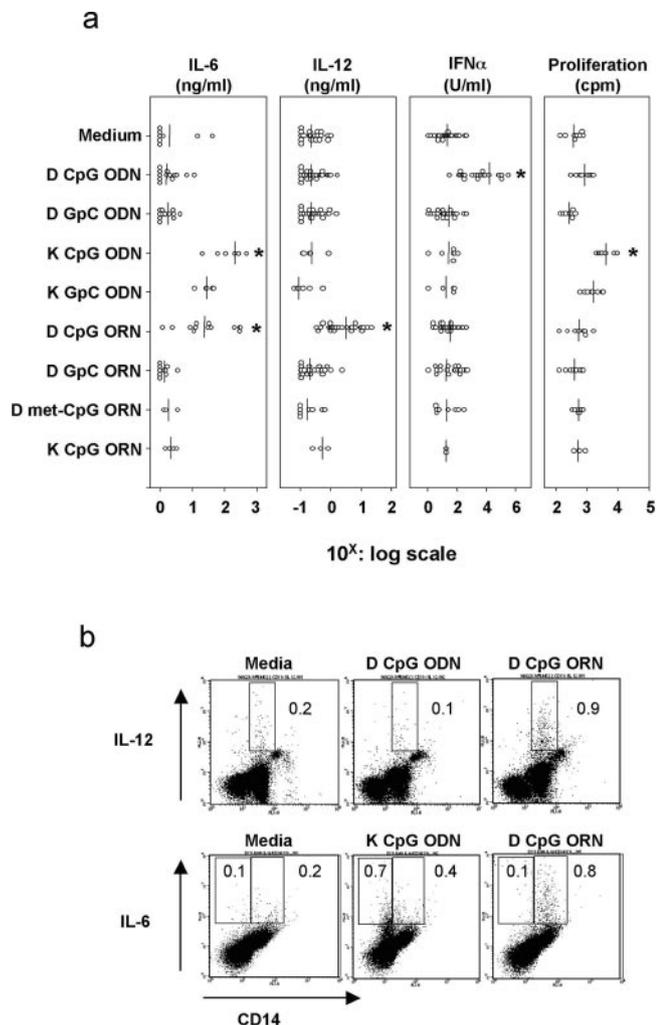
Previous studies established that synthetic DNA-based ODN expressing CpG motifs were immunostimulatory (4, 7). This work examines whether ssRNA oligonucleotides expressing CpG motifs could also activate cells of the immune system. ORN were synthesized using phosphorothioate bases to increase their resistance to nuclease digestion. Initial studies compared the activity of D-type CpG ODN (previously shown to stimulate pDC to produce IFN $\alpha$ ) and K-type CpG ODN (previously shown to stimulate B cells to produce IL-6 and proliferate) to their RNA analogues (sequences shown in Table I). Consistent with previous studies, D-type CpG ODN triggered a significant increase in IFN $\alpha$  production by human PBMC, whereas K-type CpG ODN stimulated IL-6 production and proliferation ( $p < 0.01$  for both effects, Fig. 1).

The immunomodulatory properties of D-type CpG ORN, RNA-version of D type CpG ODN, were quite distinct from those of either D- or K-type ODN. D-type CpG ORN uniquely induced the production of IL-12 in addition to IL-6 ( $p < 0.01$ ), yet had no effect on IFN $\alpha$  production and proliferation (Fig. 1*a*). These immunostimulatory effects were abrogated when the CpG dinucleotides were replaced with GpC (control ORN (GpC)), or 5'-methylated C and G (control ORN (methyl C)) (Fig. 1*a*). None of the K-type CpG ORN was immunostimulatory (Fig. 1*a* and data not shown).

Table I. Sequences of CpG DNA and CpG RNA<sup>a</sup>

DNA sequence	
D-type CpG ODN (D35)	GGtgcatcgcgatgcaggggGG
D-type control ODN (D122)	GGtgcatcgcgatgcaggggGG
K-type CpG ODN (K3)	ATCGACTCTCCGAGCGTTCTC
K-type control ODN (K10)	ATCGACTCTGCAGGCTTCTC
RNA sequence	
D-type CpG ORN (R35)	GGUGCAUCGAUGCAGGGGGG
D-type control ORN (R36, methylated C)	GGUGCAU <sup>5M</sup> CGAUGCAGGGGGG
D-type control ORN (R37, GpC)	GGUGCAUGCAGGGGGG
Control ORN (R38, poly(A)tail)	GGUGCAUCGAUCAAAAAAA
R35 poly(U) (R39, poly-U tail)	GGUGCAUCGAUCAUUUUUU
R35 poly(C) (R40, poly(C) tail)	GGUGCAUCGAUCACCCCCC
K-type CpG ORN (R3)	AUCGACUCUCGAGCGUUCUC

<sup>a</sup> Nucleotides with uppercase character are phosphorothioate backbones, and those with lowercase letters are phosphodiester backbone. Underlines are active CpG motifs of ODN or ORN.



**FIGURE 1.** Stimulation of human PBMC by CpG ORN. Human PBMC were stimulated *in vitro* with synthetic ssDNA or ssRNA containing K- or D-type CpG motifs for 48 h. *a*, The production of IL-6, IL-12, and IFN $\alpha$  and proliferation were measured by ELISA as described in *Materials and Methods*. Each open circle represents results from a single donor, while the bar represents the averages (geometric mean) of all values (\*,  $p < 0.01$  vs medium). *b*, Cell surface expression of CD14 and intracellular cytokine production (IL-6 and IL-12) were measured by flow cytometry as described in *Materials and Methods*. Numbers show the percentage of cells in the gated area. Results are representative of two individual experiments.

#### Monocytes are directly responsive to RNA-based CpG

The cell type(s) responding to D-type CpG ORN was determined by monitoring intracytoplasmic cytokine production by various stimulated cell types. As seen in Fig. 1*b*, the majority of cells that responded to D CpG ORN by secreting IL-12 and IL-6 were CD14<sup>+</sup> monocytes, while IL-6 production by K-type CpG ODN derived from the CD14<sup>-</sup> fraction of PBMC. To further explore the response of monocytes to D-type CpG ORN, their expression of surface markers was examined. As seen in Table II, CD11c<sup>+</sup>CD14<sup>+</sup> monocytes responded to D-type CpG ORN by up-regulating expression of CD83 and CD86. ORN lacking the critical CpG motif had no such effect. Elutriated monocytes were then stimulated with CpG ORN (Fig. 2*a*). Consistent with data obtained from unfractionated PBMC, D type but not control ORN stimulated CD14<sup>+</sup>CD11c<sup>+</sup> monocytes to produce significant amounts of IL-12 (Fig. 2*a*). When these elutriated monocytes were analyzed for critical signaling molecules for IL-12, IL-6, and co-

Table II. CpG RNA increases costimulatory molecule or activation marker expression on immune cells<sup>a</sup>

	CD11c/CD83 (MFI) <sup>b</sup>	CD11c/CD86 (MFI)
D-type CpG ORN (R35)	6.1 ± 2.9*	6.3 ± 2.4*
D-type control ORN (R37, GpC)	1.4 ± 0.8	1.6 ± 0.8
	CD14/CD83 (MFI)	CD14/CD86 (MFI)
D-type CpG ORN (R35)	3.4 ± 1.2*	3.2 ± 0.9*
D-type control ORN (R37, GpC)	1.1 ± 0.1	1.1 ± 0.1

<sup>a</sup> Fold increase (media = 1, \*  $P < 0.05$ .)

<sup>b</sup> MFI, mean fluorescence intensity.

stimulatory markers up-regulation, such as NF- $\kappa$ B and p38 MAPK activation (1), CpG ORN but not control ORN (C methylated) induce I $\kappa$ B phosphorylation, its following degradation and p38 phosphorylation within 60 min (Fig. 2*b*). These data indicate that CD14<sup>+</sup>CD11c<sup>+</sup> monocytes were major responder cell types in PBMC to activate NF- $\kappa$ B and p38 MAPK, resulting in production of IL-12 and IL-6 and up-regulation of costimulatory markers.

Previous studies showed that DNA-based CpG ODN act on B cells and pDC rather than monocytes. However, factors produced by pDC may indirectly stimulate monocytes in the same cultures (23). We therefore examined whether CpG ORN were acting directly on monocytes or through pDC intermediates. PBMC were elutriated to generate highly enriched monocyte preparations, and these preparations were further purified by specific depletion of pDC. Consistent with previous reports, elimination of pDC abrogated the ability of D-type ODN to stimulate the rest of the cells to produce IFN $\alpha$  (Fig. 2*c*). In contrast, removal of pDC had no impact on the ability of D-type CpG ORN to stimulate highly purified monocytes to produce IL-12 or IL-6 (Fig. 2*c*). These findings suggest that unlike their CpG-containing DNA counterparts, CpG-containing ORN act directly on monocytes.

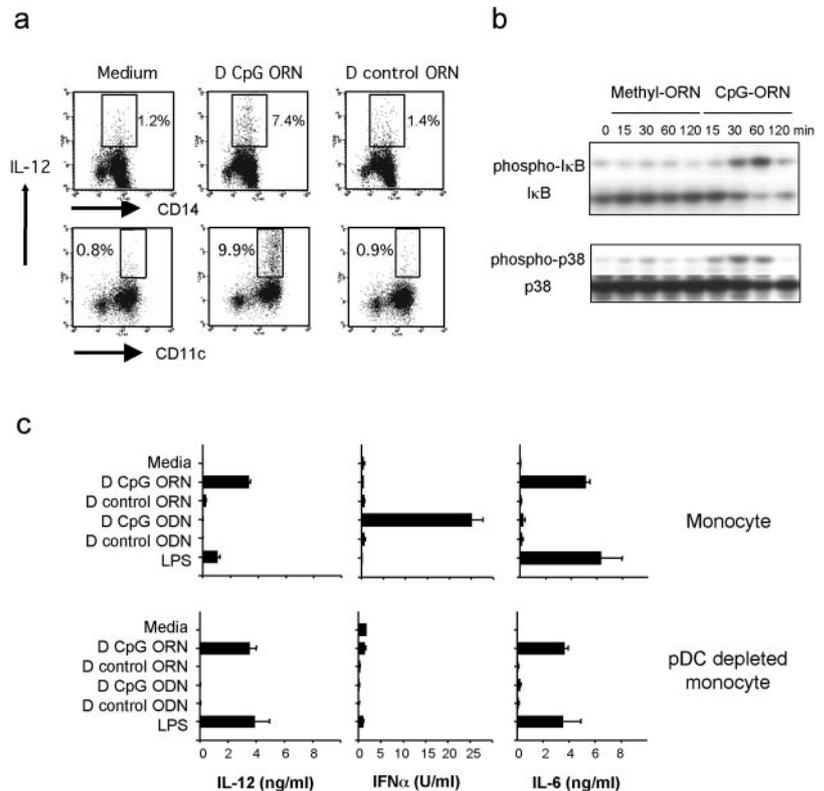
#### Immunostimulatory RNA requires an unmethylated CpG motif and a 3' poly(G) tail

The influence of sequence and structure on the immunostimulatory activity of D-type ORN was examined. Stimulation was abrogated when the central CG was flipped to GC or the C methylated (Fig. 3, *a* and *b*). Stimulation was also reduced by substitutions in the 5' or 3' region immediately flanking the CG dinucleotides. Specifically, optimal stimulation was elicited by a 3' AU and reduced by replacements by AA or UA. The 5' AU was less critical to D-type CpG ORN stimulation (Fig. 3*b* and data not shown).

Whereas D-type CpG ODN require that the bases flanking the central CpG motif form a palindrome (19), no such structural requirement was observed for D-type CpG ORN (Fig. 3*a*). However, both D-type ODN and ORN required a poly(G) run at their 3' end to mediate maximal immune stimulation (19) (Fig. 3*b*).

The effect of the nucleotide backbone on immunostimulatory activity of CpG ORN was then examined. D-type CpG ODN are optimally active when both termini are composed of phosphorothioate linkers and the central region is phosphodiester (19). In contrast, pure phosphorothioate CpG ORN were strongly stimulatory (Fig. 3*c*). Indeed, the level of immune stimulation fell as phosphodiester linkages were added to the central region. This may

**FIGURE 2.** CpG ORN directly stimulate CD14<sup>+</sup> CD11c<sup>+</sup> monocytes to lead NF- $\kappa$ B and p38 MAPK activation and production of IL-6 and IL-12. Human elutriated monocytes were stimulated with CpG ORN or control GpC ORN for 24 h. *a*, Cells were monitored for surface expression of CD14 and CD11c and for intracytoplasmic levels of IL-12. *b*, Elutriated monocytes were stimulated for 15–120 min as indicated, then whole cell lysates were analyzed for phosphorylation of I $\kappa$ B and p38 proteins by Western blot analysis as described in *Materials and Methods*. *c*, Elutriated monocytes were depleted of pDC by using anti-BDCA2 Ab-conjugated magnetic beads as described in *Materials and Methods*. Elutriated monocytes were stimulated with CpG ODN, ORN, or LPS for 48 h. Levels of IL-6, IL-12, and IFN $\alpha$  in culture supernatants were measured by ELISA.



reflect increased sensitivity to nuclease degradation (rather than any effect on structure) since pure phosphodiester CpG ORN were stimulatory if introduced directly into cells by transfection (Fig. 3*d*).

These findings suggest that structural requirements for immunologically active D-type ORN are distinct in terms of base, backbone, and flanking sequences to those for D-type ODN, with the exception that both require the presence of an unmethylated CpG motif and a 3' poly(G) tail (19).

#### *TLR3, 7, 8, or 9 do not confer recognition of CpG ORN*

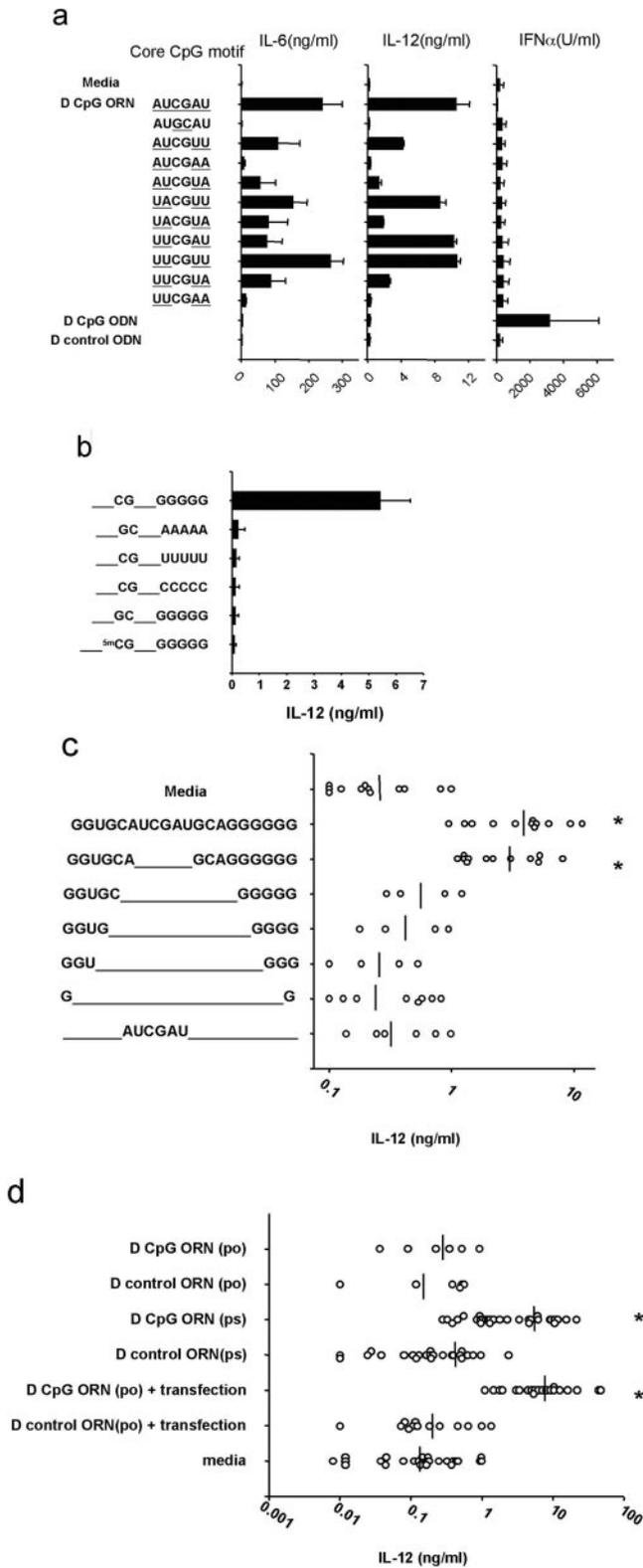
TLRs 3, 7, 8, and 9 have been shown to recognize distinct structures expressed by nucleic acids (or their analogues) (3). Thus, transfection of a cell line lacking these genes (such as HEK-293) with genes encoding these specific receptors confers responsiveness to the relevant TLR ligand (6, 11, 24). The ability of CpG ORN to interact with these TLRs was examined by transfecting HEK-293 cells with TLR3, 7, 8, or 9. Although such TLR transfections resulted in successful recognition of respective known ligands, none of the transfected cells responded to D-type ORN (Fig. 4*a*). Neither simultaneous transfection of TLR and CpG ORN, nor subsequent transfection of CpG ORN 24 h after TLR transfections conferred the recognition of CpG ORN (Fig. 4*b* and data not shown). These data suggest that CpG ORN does not utilize TLR3, 7, 8, or 9 alone, which is distinct from dsRNA, ssRNA, or CpG DNA. PI3K play an important role in mediating the uptake and shuttling of CpG ODN into TLR9 containing endo/phagosomes to initiate CpG DNA-induced immune activation (9). In addition, CpG DNA-induced signaling was shown to activate p38 MAPK (25). To determine whether these molecules also contributed to the recognition, uptake or signaling induced by D-type ORN, inhibitors for PI3K and p38 MAPK were added to D-type ORN-stimulated cell cultures. PI3K inhibit the activation of PBMC by CpG ORN as well as CpG ODN, but not dsRNA or

LPS, consistent with previous reports (data not shown and Ref. 9). The p38 inhibitor SB203580 also blocked the activation of PBMC by CpG ORN as well as the uptake of CpG ORN into the endo/phagosome (data not shown). These data suggest that CpG ORN-induced monocyte activation requires endo/phagocytosis of CpG ORN, controlled by PI3K and possibly by p38 MAPK, resulting in the production of proinflammatory cytokines such as IL-6 and IL-12.

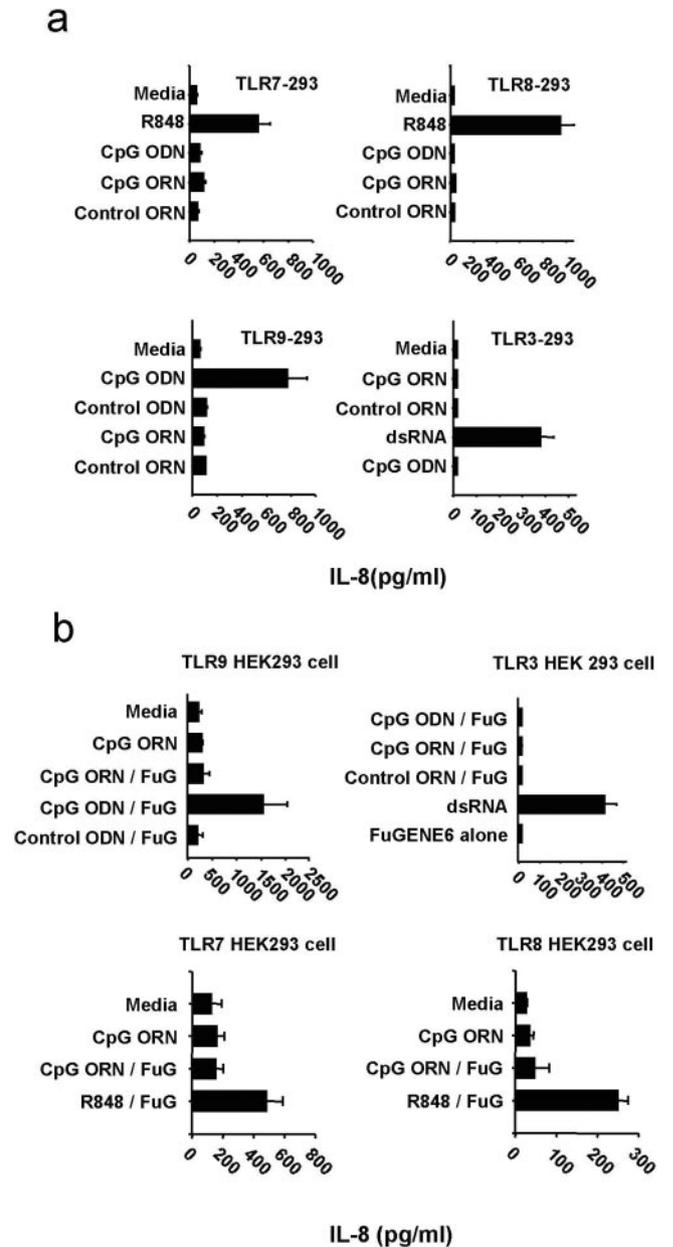
#### **Discussion**

The host's innate immune system recognizes and responds to DNA and RNA expressed by infectious pathogens. Accumulating evidence suggests that most but not all of this recognition is mediated by TLRs. For example, TLR9 is critical for the recognition of single-stranded CpG DNA (4, 7), while dsDNA up-regulates MHC-related genes through TLR-independent pathways (K. J. Ishii, unpublished data), which was independent of the existence of CpG motifs within DNA sequences (26, 27). Similarly, dsRNA stimulates immune and nonimmune cells via both TLR3 and protein kinase, dsRNA dependent (6, 28). ssRNA was recently shown to stimulate dendritic cells via TLR7 or 8 (14–17). Heil et al. (15) demonstrated that GU-rich ssRNA stimulated pDC via TLR7 in mice and possibly TLR8 in humans. Diebold et al. (14) reveal that the nucleotides preference of ssRNA-mediated pDC activation was attributed to poly(U) but not poly(G), poly(C), or poly(A). Koski et al. (16) have shown that human myeloid dendritic cells are activated by uridine-rich ssRNA derived from bacteria without the poly(A) tail but not from vertebrate mRNA containing the poly(A) tail (16).

The current work evaluated whether ssRNA-containing CpG motifs was immunostimulatory. Results obtained from >20 healthy donors' PBMC demonstrate that CpG ORN can stimulate human PBMC to produce significant amounts of IL-12 and IL-6



**FIGURE 3.** Structural elements required for immunostimulatory activity of CpG ORN. Human elutriated monocytes were stimulated with various ORN or ODN (3  $\mu$ M) for 48 h and the production of IL-12, IFN $\alpha$ , or IL-6 was measured by ELISA. *a*, The hexameric CpG motifs in D-type ORN (R35) were modified as indicated, while the other nucleotide sequences are identical to R35 (see Table I). *b*, The hexameric 3' poly(G) sequences of R35 were replaced with either poly(A), poly(C), or poly(U). -CG- indicates that CG dinucleotides in CpG motifs of R35 are flipped to GC. <sup>5m</sup>CG- indicates that the 5' portion of cytosine in the CG dinucleotides was methylated. The other nucleotide sequences of ORN are identical to R35 (see Table I). *c*, Backbone of R35 (see Table I) was modified.



**FIGURE 4.** Involvement of TLRs in CpG ORN-induced immune responses. HEK-293 cells ( $5 \times 10^4$ ) were transfected with plasmids encoding human TLR3, 7, 8, or 9 (1  $\mu$ g) for 24 h. *a*, The cells were then stimulated with CpG ODN (K3, 1  $\mu$ M), LPS (100 ng/ml), dsRNA (20  $\mu$ g/ml), R848 (1  $\mu$ M), or D-type CpG ORN (R35, 1  $\mu$ M) for 24 h. *b*, The TLR-transfected cells were stimulated with CpG ORN mixed with FuGENE6 (FuG) as indicated. The concentration of IL-8 in culture supernatants was measured by ELISA as described in *Materials and Methods*.

and to induce the up-regulation of costimulatory molecules (Fig. 1 and Table II). This activation requires CpG motifs that are characteristic of D-type CpG ODN. Optimal D-type CpG ODN contain purine-pyrimidine-CG-purine-pyrimidine as a core sequence, and

Phosphorothioate linkages are shown as uppercase letters, while the others are phosphodiester. *d*, Cells were stimulated with D-type CpG ORN (R35) with phosphodiester (po) in the presence or absence of transfection reagent or phosphorothioate (ps) backbone without transfection. *a* and *b*, Average  $\pm$  SD of two elutriated monocytes are shown. *c* and *d*, Each open circle represents individual values (\*,  $p < 0.01$  vs medium).

a poly(G) tail at the 3' end (19). Similarly, an unmethylated CG dinucleotide flanked by AU and a 3' poly(G) tail was required for CpG ORN to induce IL-12 production by monocytes (Fig. 3, *a* and *b*).

Unlike D-type CpG ODN, CpG ORN were active when the backbone was entirely phosphorothioate. This may reflect the ability of phosphorothioate ODN to resist nuclease degradation, since phosphodiester ORN were as stimulatory if transfected directly into target cells (Fig. 3, *c* and *d*). Of considerable interest, both the target population of CpG ORN and the nature of the induced response were quite different from that described for CpG ODN. CpG ORN directly stimulated CD14<sup>+</sup>CD11c<sup>+</sup> monocytes to secrete IL-12, yet had no effect on T cells, B cells, or pDC in the productions of cytokines and chemokines and the up-regulation of MHC and costimulatory molecules as far as we examined (Figs. 1*b* and 2*c*; data not shown). In addition, D-type ORN did not stimulate PBMC or purified pDC to produce IFN $\alpha$ , whereas D-type CpG ODN directly stimulate pDC to produce large amounts of IFN $\alpha$ . Although cells that respond to CpG ORN are quite distinct from those of CpG ODN, CpG ORN directly activate CD14<sup>+</sup>CD11c<sup>+</sup> monocytes and indirectly stimulate NK cells to produce IFN- $\gamma$  mediated through IL-12, to a similar extent as that induced by CpG ODN (Table II and T. Sugiyama and K. J. Ishii, unpublished results).

These distinct immunostimulatory activities of CpG ORN led us to further study whether CpG ORN utilize TLRs. If so, which TLR is involved in CpG ORN-induced immune activation? Previous reports have shown that human monocytes express mRNA of TLR8, but not TLR3, 7, or 9 (29), suggesting that TLR8 might be the receptor for CpG ORN (15, 16). Yet HEK-293 cells transfected with TLR8 did not respond to D-type CpG ORN (Fig. 4). This finding suggests that either TLR8 is not involved in the recognition of CpG ORN or that some coreceptor(s), adaptor(s), and/or signaling molecule(s) is additionally needed for CpG ORN-mediated activation even if TLR(s) is involved (11, 30).

Significance of our finding in physiological conditions is yet unclear. Unmethylated CpG motifs in genome are observed more frequently in bacterial and certain viral DNA than vertebrate DNA, thereby being recognized by the innate immune system via TLR9 (3, 4, 7, 8). In contrast, to our knowledge, 5'-C methylation in RNA has not been reported while methylation of other portions of nucleotides are known (e.g., 2'-O methylation of rRNA and small nuclear RNA directed by small nucleolar RNA, 7'-G cap methylation of mRNA) (31–34). Yet it is possible that RNA, including mRNA, tRNA, rRNA, or a small RNA species such as small nuclear RNA derived from pathogens or damaged hosts, can stimulate the innate immune system if they contain CpG motifs in a similar context to that of CpG ORN. It is of note that ORN were protected by 2'-bis(acetoxyethoxy)-methyl ether (22) that did not alter their immunostimulatory activity while OME modification of RNA abrogates such effects (data not shown). Further studies will clarify the requirement of the precise molecular feature of immunostimulatory RNA including their sequences and resulting higher structures.

In summary, the current work demonstrates that ssRNA-containing CpG motifs directly stimulate human monocytes to activate NF- $\kappa$ B and p38 MAPK, resulting in the productions of IL-6 and IL-12 and up-regulation of costimulatory markers such as CD86. Their activity and mode of recognition is distinct from that of CpG ODN, dsRNA, or GU-rich ssRNA. Additional study is required to determine whether the unique effects of CpG ORN can be used therapeutically, but the importance of IL-12 in protective immune response in infection, allergy, or cancer suggests that the capacity to selectively induce these cytokines may be of benefit in the de-

velopment of vaccine adjuvants, immunoprotective agents for infection, and antiallergic or anticancer agents.

## Acknowledgments

We thank all members of Dr. Klinman's laboratory and Dr. Akira's laboratory for generous support.

## References

- Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2:675.
- Medzhitov, R. 2001. Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* 1:135.
- Wagner, H. 2004. The immunobiology of the TLR9 subfamily. *Trends Immunol.* 25:381.
- Krieg, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 20:709.
- Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740.
- Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF- $\kappa$ B by Toll-like receptor 3. *Nature* 413:732.
- Klinman, D. M. 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat. Rev. Immunol.* 4:249.
- Ishii, K. J., I. Gursel, M. Gursel, and D. M. Klinman. 2004. Immunotherapeutic utility of stimulatory and suppressive oligodeoxynucleotides. *Curr. Opin. Mol. Ther.* 6:166.
- Ishii, K. J., F. Takeshita, I. Gursel, M. Gursel, J. Conover, A. Nussenzweig, and D. M. Klinman. 2002. Potential role of phosphatidylinositol 3 kinase, rather than DNA-dependent protein kinase, in CpG DNA-induced immune activation. *J. Exp. Med.* 196:269.
- Latz, E., A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, and D. T. Golenbock. 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat. Immunol.* 5:190.
- Takeshita, F., C. A. Leifer, I. Gursel, K. J. Ishii, S. Takeshita, M. Gursel, and D. M. Klinman. 2001. Cutting edge: role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. *J. Immunol.* 167:3555.
- Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. USA* 98:9237.
- Kadowaki, N., S. Antonenko, and Y. J. Liu. 2001. Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c-type 2 dendritic cell precursors and CD11c<sup>+</sup> dendritic cells to produce type I IFN. *J. Immunol.* 166:2291.
- Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303:1529.
- Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. *Science* 303:1526.
- Koski, G. K., K. Kariko, S. Xu, D. Weissman, P. A. Cohen, and B. J. Czerniecki. 2004. Cutting edge: innate immune system discriminates between RNA containing bacterial versus eukaryotic structural features that prime for high-level IL-12 secretion by dendritic cells. *J. Immunol.* 172:3989.
- Lund, J. M., L. Alexopoulou, A. Sato, M. Karow, N. C. Adams, N. W. Gale, A. Iwasaki, and R. A. Flavell. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA* 101:5598.
- Gursel, M., D. Verthelyi, I. Gursel, K. J. Ishii, and D. M. Klinman. 2002. Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. *J. Leukocyte Biol.* 71:813.
- Verthelyi, D., K. J. Ishii, M. Gursel, F. Takeshita, and D. M. Klinman. 2001. Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. *J. Immunol.* 166:2372.
- Coban, C., K. J. Ishii, D. J. Sullivan, and N. Kumar. 2002. Purified malaria pigment (hemozoin) enhances dendritic cell maturation and modulates the isotype of antibodies induced by a DNA vaccine. *Infect. Immun.* 70:3939.
- Ishii, K. J., K. Kawakami, I. Gursel, J. Conover, B. H. Joshi, D. M. Klinman, and R. K. Puri. 2003. Antitumor therapy with bacterial DNA and toxin: complete regression of established tumor induced by liposomal CpG oligodeoxynucleotides plus interleukin-13 cytotoxin. *Clin. Cancer Res.* 9:6516.
- Campbell, T. B., C. K. McDonald, and M. Hagen. 1997. The effect of structure in a long target RNA on ribozyme cleavage efficiency. *Nucleic Acids Res.* 25:4985.

23. Gursel, M., D. Verthelyi, and D. M. Klinman. 2002. CpG oligodeoxynucleotides induce human monocytes to mature into functional dendritic cells. *Eur. J. Immunol.* 32:2617.
24. Jurk, M., F. Heil, J. Vollmer, C. Schetter, A. M. Krieg, H. Wagner, G. Lipford, and S. Bauer. 2002. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat. Immunol.* 3:499.
25. Takeshita, F., K. J. Ishii, A. Ueda, Y. Ishigatsubo, and D. M. Klinman. 2000. Positive and negative regulatory elements contribute to CpG oligonucleotide-mediated regulation of human IL-6 gene expression. *Eur. J. Immunol.* 30:108.
26. Ishii, K. J., K. Suzuki, C. Coban, F. Takeshita, Y. Itoh, H. Matoba, L. D. Kohn, and D. M. Klinman. 2001. Genomic DNA released by dying cells induces the maturation of APCs. *J. Immunol.* 167:2602.
27. Suzuki, K., A. Mori, K. J. Ishii, J. Saito, D. S. Singer, D. M. Klinman, P. R. Krause, and L. D. Kohn. 1999. Activation of target-tissue immune-recognition molecules by double-stranded polynucleotides. *Proc. Natl. Acad. Sci. USA* 96:2285.
28. Diebold, S. S., M. Montoya, H. Unger, L. Alexopoulou, P. Roy, L. E. Haswell, A. Al Shamkhani, R. Flavell, P. Borrow, and Reis e Sousa. 2003. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature* 424:324.
29. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of Toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* 168:4531.
30. Verthelyi, D., and R. A. Zeuner. 2003. Differential signaling by CpG DNA in DCs and B cells: not just TLR9. *Trends Immunol.* 24:519.
31. Banerjee, A. K. 1980. 5'-terminal cap structure in eukaryotic messenger ribonucleic acids. *Microbiol. Rev.* 44:175.
32. Bachelier, J. P., and J. Cavaillè. 1997. Guiding ribose methylation of rRNA. *Trends Biochem. Sci.* 22:257.
33. Maden, B. E., and J. M. Hughes. 1997. Eukaryotic ribosomal RNA: the recent excitement in the nucleotide modification problem. *Chromosoma* 105:391.
34. Kiss, T. 2002. Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. *Cell* 109:145.