Immunostimulatory RNA oligonucleotides trigger an antigen-specific cytotoxic T-cell and IgG2a response

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Single-stranded RNA oligonucleotides containing an immunostimulatory motif (immunostimulatory RNA [isRNA]) are potent inducers of interferon-α via the Toll-like receptor 7. We investigated the effect of isRNA on the development of an immune response. We show that isRNA activates dendritic cells and induces production of Th1-type cytokines both in vitro and in vivo. Cytokine production led to bystander activation of T and B cells. We further demonstrate that isRNA triggers the generation of antigen-specific cytotoxic T cells and of an IgG2a-biased antibody response to antigen in a sequence-dependent manner. In summary, we provide evidence for the first time that isRNA oligonucleotides can simultaneously activate the innate and adaptive arms of the immune system. (Blood. 2007; 109:2953-2960)

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Introduction

Recognition of pathogens by the innate immune system is mediated through pattern-recognition receptors that recognize distinct microbial components.[1] Nucleic acids from pathogens are recognized by several classes of receptors, including Toll-like receptors (TLRs) and cytoplasmic receptors. Microbial DNA, in particular DNA rich in unmethylated CpG motifs, is detected by TLR9 in the endosome.[2,3] Long double-stranded RNA (> 30 nucleotides), a replicatory intermediate for some viruses, is detected by TLR3, by the serine-threonine kinase PKR, and by the cytoplasmic helicase proteins RIG-I and MDA5.[4-9] The 5′-triphosphate end of RNA generated by viral polymerases directly binds to RIG-I.[10] Single-stranded RNA (ssRNA) from ssRNA viruses has been shown to be detected through TLR7 and TLR8.[11,12] Furthermore, we have recently described double-stranded, short interfering RNA (siRNA) molecules that interact with TLR7 in a sequence-specific manner to induce IFN-α production in dendritic cells (DCs).[13] The stimulatory activity on DCs was also observed with the corresponding single-stranded RNA oligoribonucleotides (ORNs).

DCs are professional antigen-presenting cells that express a variety of pattern-recognition receptors. Exposure of DCs to ligands for these receptors, such as microbial nucleic acids, activates intracellular signaling cascades that rapidly induce the expression of a variety of genes involved in maturation and migration of DCs.[1] Mature DCs can directly interact with immune effector cells such as cytotoxic T lymphocytes (CTLs), a process that is essential for the induction of protective immunity against infectious diseases and tumors. In addition, mature, activated DCs also represent a critical source of IL-12 and IFN-α, 2 key cytokines in the driving of both innate and Th1-dependent acquired immune responses.[14-16]

The immune-activating effects of TLR ligands have prompted their use in vaccine formulations. It is well established that binding of synthetic CpG oligodeoxynucleotides (ODNs) to TLR9 both enhances the generation of an innate immune response and promotes protective Th1-type immunity in animal models.[17] In humans, clinical studies have demonstrated a potential for CpG ODNs as adjuvants in antiviral vaccination.[18,19] Furthermore, CpG ODNs combined with a peptide antigen promote CD8^{+} T-cell responses to tumor antigens in patients with melanoma.[20] However, TLR9 displays a restricted expression pattern in humans, where this receptor is expressed on B cells and plasmacytoid DCs but not, as is the case in mice, on professional antigen-presenting cells that are crucial for the induction of immunity to viral and tumor antigens.[21]

Here we characterized the in vitro and in vivo immunostimulating potential of synthetic single-stranded ORNs that activate TLR7. We show for the first time that ORNs can be used to trigger an immune response to a model antigen in a sequence-dependent manner. This immunostimulatory effect led to the generation of antigen-specific, cytotoxic T cells and antibodies of the IgG2a isotype in the context of a Th1-type immune response. We therefore demonstrate that immunostimulatory RNA can at the same time drive both an innate immune response and an adaptive response to antigen.

Materials and methods

Mice

Female Balb/c and C57BL/6 mice were purchased from Harlan-Winkelmann (Borchen, Germany). Mice were 5 to 12 weeks of age at the onset of experiments. Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

Reagents

The 20-mer ORNs 9.2dr (5′-UGUCCUUCAAUGUCCUUCAA-3′) and PolyA in both the unmodified phosphodiester and fully phosphorothioated (PTO) forms and the ORNs TLR4.1s (5′-UACUUAGACUACUACCUCG-3′), 9.2as (5′-UUGAAGGACAGGUUAAGCU-3′), and MyD88s (5′-CAGACAAACUAUCGACUGAtt-3′) in fully PTO form were from CureVac (Tübingen, Germany). The PTO-modified CpG oligodeoxyribonucleotide 1826 (5′-TCCATGACGTTCCTGACGTT-3′) was obtained from the Coley Pharmaceutical Group (Langenfeld, Germany). For flow cytometry analysis, cells were stained with anti–mouse B220-PE, CD3-APC, CD4-PE, CD8-PerCP, CD11b-PerCP, CD11c-APC, CD69-FITC, and isotype controls (BD Biosciences, Heidelberg, Germany). Chicken egg ovalbumin (OVA) was purchased from Sigma-Aldrich (St Louis, MO).

Bone marrow cell culture and generation of DCs

Bone marrow cells were harvested from murine femur and tibia and erythrocytes were lysed with ammonium chloride buffer (BD Biosciences). For bone marrow cell cultures, cells from Balb/c mice were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 μg/mL streptomycin, and 1 IU/mL penicillin (complete RPMI). To prepare bone marrow-derived DCs (BMDCs), bone marrow cells from C57BL/6 mice were cultured in complete RPMI supplemented with 20 ng/mL GM-CSF and 20 ng/mL IL-4 (Tebu Bio, Offenbach, Germany; DC medium). On day 7, loosely adherent cells were harvested and washed. DCs (CD11c$^+$ cells) generally represented 70% of the preparation. DCs were enriched by magnetic cell sorting after labeling with anti-CD11c microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) (CD11c$^+$ cells above 95%). Bone marrow cells were activated with 5 μg/mL ORN complexed with N-[1-(2,3-dioleoyloxy)propyl]-N, N, N-trimethylammonium methyl-sulfate (DOTAP; Roche, Germany) according to the manufacturer's instructions or with 6 μg/mL CpG ODN 1826 or 1 μg/mL LPS. BMDCs were activated with 10 μg/mL ORN or 1 μg/mL CpG ODN complexed with DOTAP or 1 μg/mL LPS. For coculture experiments, BMDCs were stimulated with ORN or CpG ODN complexed with DOTAP for 6 hours, then washed and cocultured with splenocytes (2×10^5 BMDCs with 2×10^5 splenocytes) in 96-well U-bottom plates for an additional 18 hours. Supernatants were harvested for detection of cytokines by enzyme-linked immunosorbent assay (ELISA). For analysis of activation markers, cells were stained with fluorochrome-coupled monoclonal antibodies (mAbs) and analyzed by flow cytometry. Data were acquired on a FACSCalibur (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Quantification of cytokines in serum and supernatant

Cytokine concentrations were determined by ELISA for IL-6 (BioSource, Solingen, Germany) and IL-12p40 (BD Biosciences) according to the manufacturer's protocol. IFN-α was measured according to the following protocol: rat monoclonal antibody to mouse IFN-α (clone RMMA-1) was used as the capture antibody, rabbit polyclonal antibody to mouse IFN-α for detection (both from PBL Biomedical Laboratories, Piscataway, NJ) together with HRP-conjugated donkey antibody to rabbit IgG as the secondary reagent (Jackson ImmunoLaboratories, Bar Harbor, ME). Recombinant mouse IFN-α (PBL Biomedical Laboratories) was used as standard (IFN-α concentration in IU/mL).

Immunostimulation in vivo and immunization

For in vivo immunostimulation, 100 μg DOTAP was incubated with 20 μg oligonucleotides and 20 μL HBS for 20 minutes and injected intravenously into the retro-orbital plexus. Single-cell suspensions were prepared from spleen and lymph nodes 18 hours after injection and treated with ammonium chloride buffer to lyse erythrocytes. For analysis of activation markers, cells were stained with fluorochrome-coupled mAbs and analyzed by flow cytometry. Blood was obtained by retro-orbital puncture at the indicated time points. Serum was prepared by centrifugation and stored at −20°C. For immunization, 20 to 50 μg OVA was injected subcutaneously together with oligonucleotides complexed to DOTAP on day 0, day 14, and

day 21. The OVA-specific T-cell response was determined on day 21 and serum antibodies on day 28.

OVA-specific immune response

Serum antibodies to OVA were determined by ELISA; 96-well plates were coated overnight with 10 μg/mL OVA in PBS and blocked 1 hour with 1% BSA in PBS. After incubation of serum samples for 1 hour, plates were washed with PBS/1% Tween 20 and goat anti–mouse IgG, IgG1, or IgG2a conjugated to HRP (Southern Biotechnology Laboratories, Birmingham, AL) was added at 1 μg/mL for 1 hour. Plates were again washed and ELISA was developed by o-phenylenediamine (Sigma-Aldrich). The reaction was stopped by 1 M H_2SO_4 and optical density (OD) was read by photometer at 450 nm. For the detection of OVA-specific CD8$^+$ T cells, freshly isolated splenocytes were lysed by ammonium chloride buffer and stained with H-2k^b-OVA$_{257-264}$-PE pentamers (Proimmune, Oxford, United Kingdom) and anti-CD8. For the detection of intracellular IFN-γ, cells were restimulated for 1 hour with 5 μg/mL OVA$_{257-264}$ peptide, brefeldin A was added at a concentration of 1 μg/mL, and cells were incubated for a further 4 hours. Cells were stained with anti-CD8, then fixed with 2% paraformaldehyde and treated with permeabilizing solution (0.5% bovine serum albumin, 0.5% saponin, 0.02% sodium azide in PBS). The fixed cells were stained with FITC-conjugated anti–IFN-γ antibody (BD Biosciences) for 25 minutes. The percentage of CD8$^+$ T cells expressing IFN-γ was determined by flow cytometry.

In vivo cytotoxicity assay

Targets were prepared from C57BL/6 splenocytes. The suspension was divided into 2 populations, pulsed or unpulsed with 100 μg/mL OVA$_{257-264}$ for 1 hour at 37°C, washed extensively, and labeled with a high concentration (15 μM) or with a low concentration (1.5 μM) of CFSE (Molecular Probes, Eugene, OR), respectively. Peptide-pulsed CFSE^{high} cells and unpulsed CFSE^{low} cells were mixed and a total of 10^7 CFSE-labeled cells were injected intravenously into immunized mice. After 16 hours, splenocytes were isolated and analyzed by flow cytometry to detect the CFSE-labeled target cells. Specific lysis was calculated by the following formula: specific lysis (%) = 100% × [1-(CFSE^{high} cells/CFSE^{low} cells)/(CFSE^{high} cells in naive mice/CFSE^{low} cells in naive mice)].

Statistics

All data are presented as mean ± SEM and were analyzed by unpaired, one-way analysis of variance (ANOVA) with the Newman-Keuls multiple-comparison test. Significance was set at a $P < .05$. Statistical analysis were performed using SPSS software (SPSS, Chicago, IL).

Results

PTO RNA oligonucleotides activate DCs more efficiently than unmodified oligonucleotides in a sequence-dependent manner

We evaluated the immunostimulatory effects of the 20-mer ORN sequence 9.2dr on the activation of bone marrow cells. Freshly isolated bone marrow cells were stimulated with either the unmodified or fully PTO ORN 9.2dr. IL-12p40, IL-6, IFN-α, and IL-10 were assessed in the culture supernatants. Both IL-12 and IFN-α production were induced by unmodified 9.2dr. The PTO modification of the ORN backbone led to higher cytokine production and in addition to the production of IL-6 (Figure 1A). IL-10 was not induced by ORNs. Cytokine production was sequence-dependent since no cytokines were detected after stimulation with a PolyA ORN of the same length in either unmodified or PTO form. Furthermore, the sequences TLR4.1, TLR9.2as, and MyD88 589 described previously[13] did not result in detectable cytokine levels (data not shown). All cytokines were also induced by CpG ODN 1826 or LPS used as positive controls. To assess the effect of ORNs
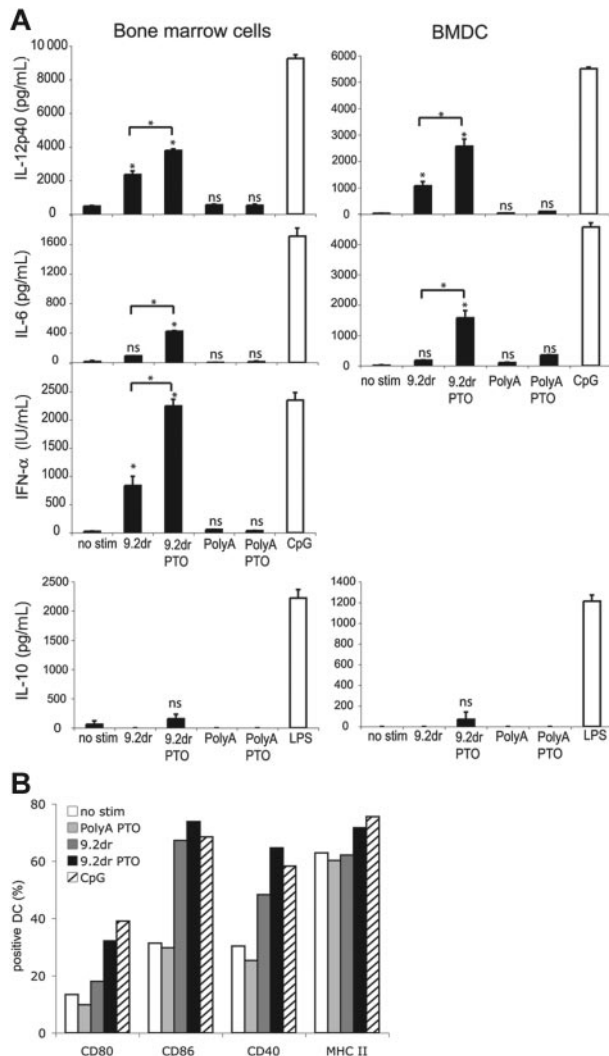
Figure 1. Both unmodified and PTO RNA oligonucleotides stimulate bone marrow cells and DCs in a sequence-dependent manner. Murine bone marrow cells or BMDCs were activated for 24 hours with ORNs 9.2dr, 9.2dr PTO, PolyA, or PolyA PTO complexed with DOTAP or with CpG ODN 1826 or LPS. (A) Supernatants were analyzed by ELISA for IL-12p40, IL-6, IFN-α, and IL-10 production. Data show the mean of triplicate samples ± SEM and are representative of at least 2 independent experiments. *P < .001; ns indicates not significant. The asterisk without brackets indicates comparison to unstimulated sample. (B) Surface expression of the activation markers CD80, CD86, CD40, and MHC-II on DCs was measured by fluorescence-activated cell sorting (FACS) after activation of bone marrow cells with ORNs or CpG ODN for 24 hours. Data are expressed as percent of CD11c$^+$ cells expressing the respective activation marker and are representative of 2 independent experiments.

on DCs, CD11c$^+$ DCs from a 7-day bone marrow culture were stimulated with ORNs. IL-12p40 was detected in the supernatant of 9.2dr-stimulated and 9.2dr PTO-stimulated DCs, whereas only 9.2dr PTO induced IL-6 production (Figure 1A). Neither IL-4 nor IL-10 production was detected after stimulation with ORNs (Figure 1A and data not shown). PolyA ORN did not induce cytokine production in DCs.

Surface expression of the activation markers MHC-II, CD80, CD86, and CD40 was measured on CD11c$^+$ DCs after stimulation of bone marrow cells with ORNs or CpG ODN (Figure 1B). Increased surface expression of CD86 and CD40 was seen after stimulation with 9.2dr and of all activation markers after stimulation with 9.2dr PTO. The increase in expression of the activation markers by 9.2dr PTO was similar to that seen with CpG ODN.

To assess the effect of 9.2dr-activated DCs on immune effector cells, purified BMDCs were activated with the PTO ORN 9.2dr or PolyA, washed, and cocultured with splenocytes. The early activation marker CD69 was up-regulated on CD3$^+$ and CD8$^+$ T cells as well as on B cells after coculture with 9.2dr PTO-activated DCs (Figure 2A). In contrast, DCs incubated with the PolyA PTO ORN did not activate splenocytes. Furthermore, splenocytes cocultured with 9.2dr PTO-activated DCs produced the effector cytokine IFN-γ. Similar levels of IFN-γ were induced in splenocytes by CpG ODN 1826-activated DCs. In contrast, no IFN-γ was detected in the supernatant of cocultures with PolyA PTO-cultured DC (Figure 2B).

PTO RNA oligonucleotides activate innate immunity and induce Th1-type cytokines in vivo

To characterize the immune effect of ORN in vivo, C57BL/6 mice were injected intravenously with either unmodified or PTO ORNs complexed with DOTAP. As shown in Figure 3A-B, 9.2dr PTO application resulted in a strong up-regulation of the early activation molecule CD69 on the surface of splenic CD4$^+$ and CD8$^+$ T cells as well as on B cells. CD69 expression was up-regulated on a small proportion of splenocytes in mice injected with the unmodified 9.2dr ORN. The increase was, however, below the level of significance. No increase in CD69 expression was seen in mice injected with either form of PolyA. CD69 was also up-regulated on
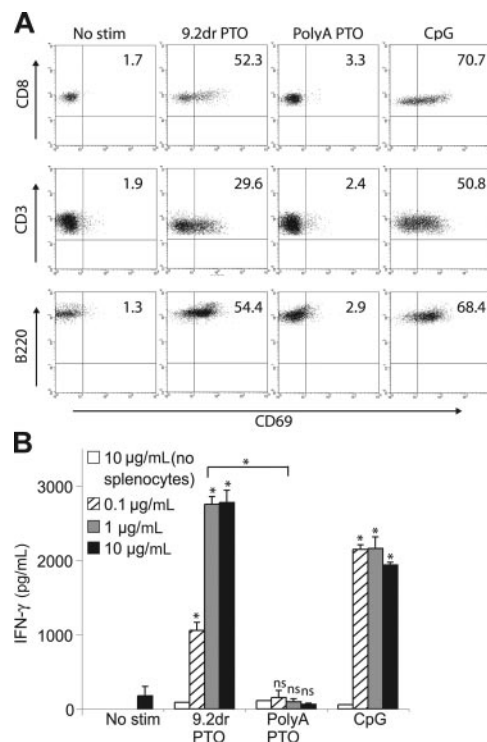

Figure 2. ORN-stimulated DCs activate splenocytes to produce IFN-γ. BMDCs were stimulated with ORN 9.2dr PTO, PolyA PTO, or CpG ODN 1826 (A: 1 μg/mL; B: 0.1, 1, or 10 μg/mL) complexed with DOTAP for 6 hours, then washed and cocultured with naive splenocytes. (A) After an additional 18 hours of culture, the activation status of the splenic T-cell (CD3$^+$ and CD4$^+$) and B-cell (B220$^+$, CD11c$^-$) populations was determined by measuring expression of the early activation marker CD69. Representative data from one of 3 experiments are gated on CD3$^+$, CD8$^+$ T cells or B cells, respectively. Numbers indicate the percent of gated lymphocytes that are CD69$^+$. (B) IFN-γ production by splenocytes after 18 hours of coculture was measured in the supernatant by ELISA. Data show the mean of triplicate samples ± SEM and are representative of 3 independent experiments. *P < .001; ns indicates not significant. The asterisk without brackets indicates comparison to unstimulated sample.
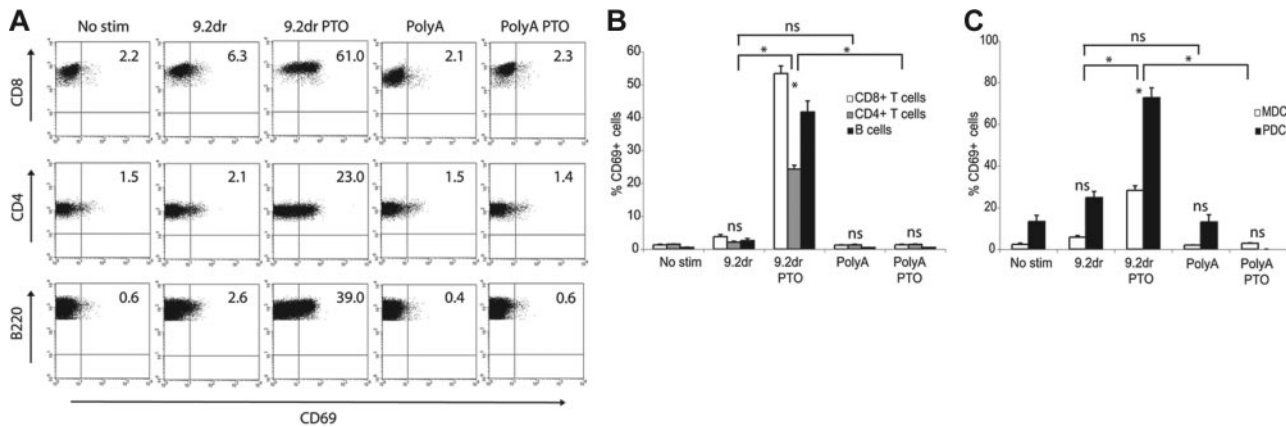
Figure 3. PTO-modified immunostimulatory ORNs activate lymphocytes and DCs in vivo. Mice were injected intravenously with 20 μg ORN 9.2dr, 9.2dr PTO, PolyA, or PolyA PTO and splenocytes were isolated 18 hours after injection. CD69 expression was examined on CD4+ and CD8+ T cells and on B cells (B220+, CD11c−) (A,B) and on MDCs (CD11c+, CD11b+, B220−; MDC) and PDCs (CD11c+, CD11b−, B220+; PDC) (C). (A) Representative data from one of 5 experiments are gated on CD8+, CD4+ T cells or B cells, respectively. Numbers indicate the percent of gated lymphocytes that are CD69+. (B-C) Data show the mean of individual mice in one experiment (PolyA: n = 3, all other groups: n = 4) ± SEM. Results are representative of 5 independent experiments. *P < .001; ns indicates not significant. The asterisk without brackets indicates comparison to unstimulated group. The significance level was the same for all cell populations analyzed.

both myeloid DC (MDC) and plasmacytoid DC (PDC) subpopulations in the spleen of mice treated with 9.2dr PTO, indicating in vivo activation of DCs. Neither the unmodified 9.2dr ORN nor the PolyA ORN significantly increased CD69 expression on DCs. As positive control, CD69 expression was measured in mice injected with CpG ODN (CD8+ T cells, 77%; CD4+ T cells, 40%; B cells, 51%; MDCs, 19%; PDCs, 54%).

In the serum of ORN-injected mice, IL-12p40 was increased 2 hours after injection with 9.2dr and 9.2dr PTO and the levels remained high at 6 hours after injection (Figure 4). At 22 hours after injection, IL-12p40 had returned to baseline in mice injected with unmodified 9.2dr but was still above baseline in mice treated with 9.2dr PTO. At no time point was IL-12p40 increased in mice injected with either form of PolyA. An increase in serum IL-6 was seen at the earliest time point of 2 hours in mice injected with 9.2dr PTO. IL-6 was no longer detectable in serum 6 hours after injection. IFN-α serum levels were increased after 2 hours and 6 hours in mice treated with 9.2dr PTO. No increase in either IL-6 or IFN-α was detected in mice injected with unmodified 9.2dr ORN or with PolyA ORN at any time point. As positive control, serum cytokines were measured in mice injected with CpG (IL-12p40, 2450 pg/mL; IL-6, 1390 pg/mL; IFN-α, 1015 U/mL).

PTO RNA oligonucleotides induce an antigen-specific IgG2a immune response

To evaluate the effect of immunostimulatory ORNs on the development of an immune response to antigen, mice were immunized with OVA together with PTO ORNs twice at a 14-day interval. Seven days after the second immunization, serum levels of OVA-specific antibodies were measured. In mice immunized with OVA and 9.2dr PTO, levels of OVA-specific IgG were increased compared to mice immunized with either OVA alone or OVA and PolyA (Figure 5). OVA-specific IgG1 was increased in all groups having received OVA, so that no difference between groups treated with OVA alone or OVA with ORNs was detected. In contrast, an increase in OVA-specific antibodies of the IgG2a isotype, indicative of a Th1-type response, was seen in mice immunized with OVA together with 9.2dr PTO compared to mice immunized with OVA alone. No increase was seen in mice treated with OVA and

PolyA ORN. Assessment of total serum IgG1 and IgG2a revealed no change in the treated groups compared to control mice.

PTO RNA oligonucleotides trigger the induction of antigen-specific cytotoxic T cells

OVA-specific CD8 T cells were assessed in mice immunized with OVA. Whereas a small increase in pentamer-positive OVA-specific
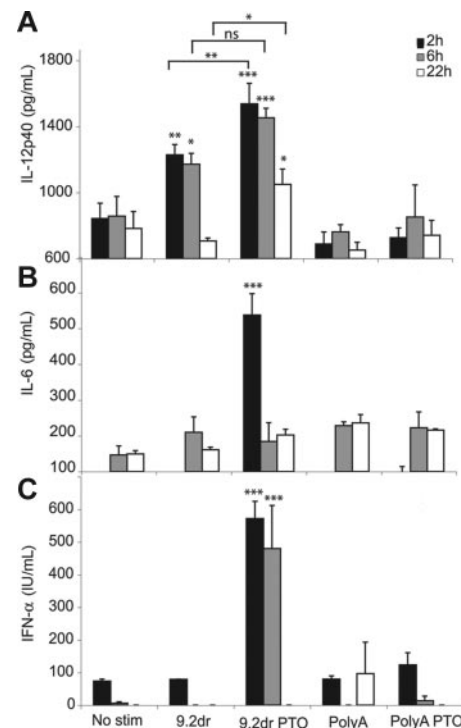

Figure 4. Immunostimulatory ORNs induce production of Th1-type cytokines in vivo. Mice were injected as described in Figure 3 with ORN 9.2dr, 9.2dr PTO, PolyA, or PolyA PTO. Blood samples were taken 2, 6, and 22 hours after injection and concentrations of (A) IL-12p40, (B) IL-6, and (C) IFN-α were measured in serum by ELISA. Data show the mean values of individual mice (PolyA: n = 3, all other groups: n = 4) ± SEM. Results are representative of 2 independent experiments. *P < .05; **P < .01; ***P < .001; ns indicates not significant. The asterisk without brackets indicates comparison with unstimulated group.
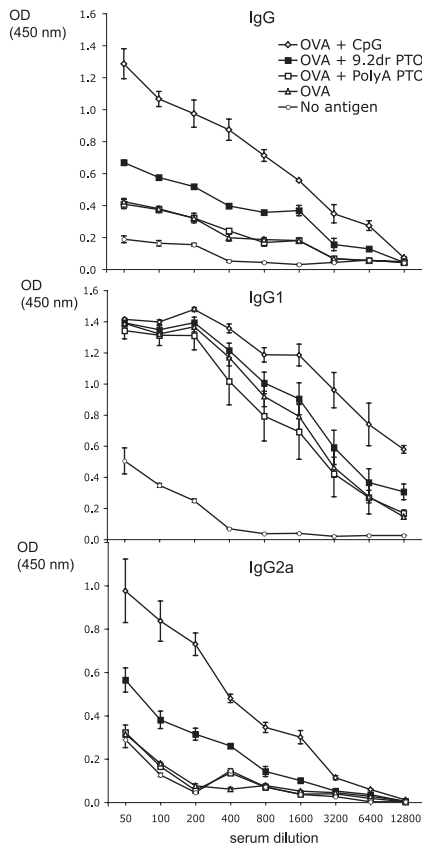
Figure 5. Immunostimulatory ORNs induce specific antibodies after immunization with OVA. Mice were immunized 3 times with 20 µg OVA together with ORN 9.2dr PTO, PolyA PTO, or CpG ODN subcutaneously. One week after the third immunization, OVA-specific IgG, IgG1, and IgG2a were measured in serial dilutions of serum by ELISA. Data show the mean values of individual mice (n = 5) ± SEM. Results are representative of 2 independent experiments.

CD8$^+$ T cells was detected in mice treated with OVA alone or with OVA together with PolyA PTO, over 4% of OVA-specific CD8$^+$ T cells were detected in mice having received OVA together with 9.2dr PTO (Figure 6A,C). Splenocytes from immunized mice were restimulated with the MHC-I–restricted peptide OVA$_{257-264}$ and assessed for IFN-γ production (Figure 6B,D). An increase in IFN-γ–producing CD8$^+$ T cells was seen after restimulation in mice immunized with OVA and 9.2dr PTO compared to mice immunized with either OVA alone or OVA and PolyA. Mice immunized with OVA together with CpG ODN were used as positive control.

An in vivo cytotoxicity assay was performed to assess the functionality of the OVA-specific CD8$^+$ T cells. Immunized mice were injected with CFSE-stained splenocytes from naive mice preincubated with OVA$_{257-264}$ peptide. Unloaded splenocytes stained with a lower CFSE concentration served as reference. While low levels of specific lysis were detected in mice immunized with OVA alone or with OVA and PolyA, the OVA-specific lysis was increased to 60% in mice immunized with 9.2dr PTO (Figure 7).

Discussion

ssRNA and ssRNA viruses such as vesicular stomatitis virus and influenza virus are recognized by immune cells through TLR7 and TLR8,[11,12,22] but specific sequence motifs responsible for viral RNA recognition have not been described. We have recently characterized an immunostimulatory RNA sequence consisting of 9 bases (GUCCUUCAA), which needs, however, to be part of a longer oligonucleotide to become active.[13] The TLR7 dependence of the immunostimulatory activity of this RNA sequence was demonstrated in vitro and in vivo using TLR7-deficient mice. The sequence used in the present study, 9.2dr, contains the 9-mer immunostimulatory motif twice, separated by a uridine base. In this study we characterized the potential of 9.2dr to stimulate the innate immune system and describe for the first time the ability of ORNs
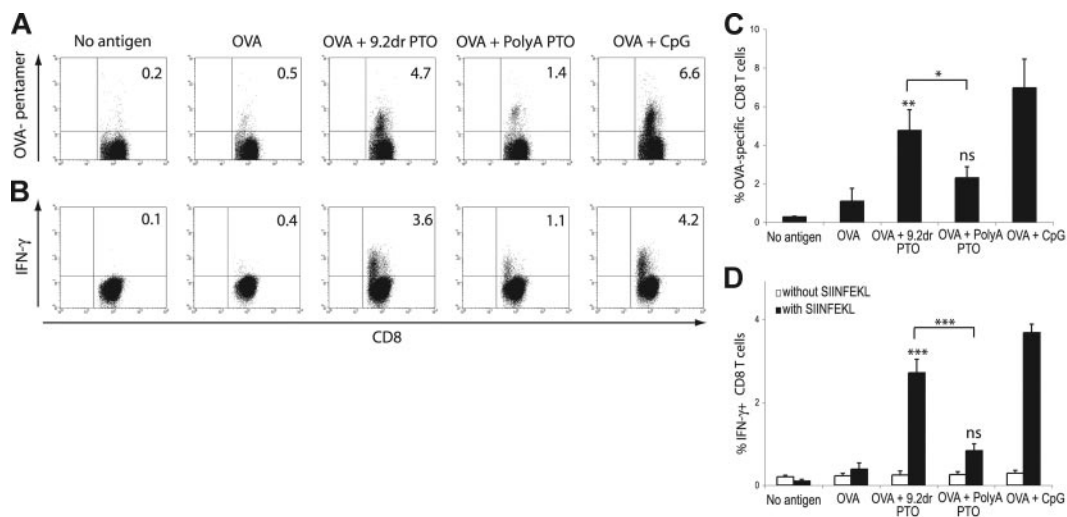

Figure 6. Immunostimulatory ORNs trigger the production of antigen-specific, IFN-γ–producing CD8 cells. Mice were immunized as described in Figure 5 with 20 µg OVA together with ORN 9.2dr PTO, PolyA PTO, or CpG ODN twice at a 14-day interval. One week after the second immunization, spleen cells were isolated. (A,C) The generation of OVA-specific CTLs was assessed by flow cytometry using H-2k^b-OVA$_{257-264}$ peptide pentamers and an anti-CD8 mAb. (B,D) Splenocytes were restimulated with the OVA$_{257-264}$ peptide for 4 hours and cytoplasmic expression of IFN-γ in CD8$^+$ T cells was examined by flow cytometry. (A-B) Representative data from one of 3 experiments are gated on CD8$^+$ cells. Numbers indicate the percent of CD8$^+$ cells that are OVA-pentamer positive or IFN-γ$^+$. (C-D) Data show the mean values of individual mice (n = 4) ± SEM. Results are representative of 3 independent experiments. *$P < .05$; **$P < .02$; ***$P < .001$; ns indicates not significant. The asterisk without brackets indicates comparison to OVA group.
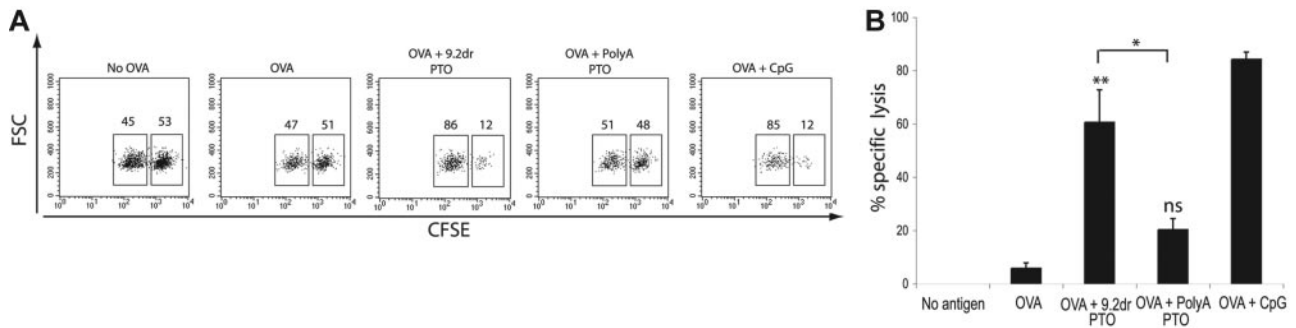
Figure 7. Immunostimulatory ORNs induce antigen-specific cytotoxicity in vivo. Mice were immunized as described in Figure 5 with 50 μg OVA together with ORN 9.2dr PTO, PolyA PTO, or CpG ODN twice at a 14-day interval. One week after the second immunization, spleen cells were isolated. Cytotoxic potential was demonstrated in vivo by specific lysis of labeled splenocytes loaded with OVA$_{257-264}$ peptide compared to unloaded splenocytes. Numbers above boxed areas indicate the percent of CFSE$^+$ cells that are loaded with peptide (CFSE^{high}, right box) or unloaded (CFSE^{low}, left box). (A) Representative data from one of 2 experiments are gated on CFSE$^+$ cells. (B) Data show the mean values of individual mice (OVA, OVA + ORN: n = 5; no OVA, OVA + CpG: n = 3) for one experiment $\pm$ SEM. Results are representative of 2 independent experiments. *$P <$ .01; **$P <$ .001; ns indicates not significant. The asterisk without brackets indicates comparison to OVA group.

to potentiate a Th1-type response to a model antigen in a sequence-dependent manner.

Analysis of the immunostimulatory activity of 9.2dr on the innate immune response showed that this ORN activated both primary bone marrow cultures and purified DCs to produce the proinflammatory cytokines IL-12, IFN-α, and IL-6. Whereas IFN-α was mainly produced by ORN-activated PDCs (data not shown), MDCs produced high amounts of IL-12p40 and IL-6. The immunostimulatory effect of ORNs was also seen in vivo, where injection of 9.2dr resulted in activation of both MDCs and PDCs as well as in a rapid increase in serum concentrations of IL-12, IFN-α, and IL-6. Clearly, the stimulation was sequence-dependent, because a PolyA ORN of the same length showed only minor immunologic activity. The cytokines induced, in particular IL-12 and IFN-α, are critical in the driving of both innate and Th1-type immune responses.[14,16] They stimulate lymphocyte differentiation and activation via the induction of Th1 cytokines such as IFN-γ. Indeed, we observed that after stimulation with ORNs, DCs induced phenotypic activation of T and B lymphocytes in vitro and were potent inducers of IFN-γ production in splenocytes. In vivo, injection of ORNs resulted in increased expression of the activation marker CD69 on B cells and CD4 and CD8 T cells. These effects were detected in the absence of antigen administration. T-cell activation generated without stimulation through the T-cell receptor by antigen is termed bystander activation and has been reported for other TLR ligands. DCs activated by TLR ligands including R848, a ligand for TLR7, play a central role in this process by secreting type 1 IFN and stimulating NK cells to produce IFN-γ.[23]

Unlike immunostimulatory DNA sequences, single-stranded RNA molecules are highly susceptible to degradation by RNases. Encapsulation in liposomes, complexing to a cationic peptide, or chemical modifications are necessary to enhance RNA stability and provide immunostimulatory activity.[11-13,24] Cationic liposomes not only protect the ORNs from degradation, but also increase the uptake into cells and target oligonucleotides to the endosomal compartment containing TLR7.[25] In the present study, ORNs were complexed with cationic liposomes (DOTAP), as we have previously shown that in the absence of DOTAP, no immunologic activity is observed.[13] Furthermore, we assessed in this study the influence of the ORN backbone on the immunostimulatory activity by comparing 2 ORNs with the same nucleotide sequence 9.2dr containing either an unmodified phosphodiester backbone or a PTO-modified backbone. In vitro, cytokine induction was consistently higher using the PTO-modified ORN. In vivo, injection of unmodified 9.2dr only increased serum levels of IL-12p40. In

contrast, production of IFN-α and IL-6 as well as phenotypic activation of DCs and lymphocytes was detected only after injection of PTO-modified ORNs. The enhancement of the immunostimulatory effect may be due to the stabilizing function of the PTO modification. Although the PTO backbone itself has been described to have immunostimulatory properties,[24] we observed only minor immunostimulation with the PTO-modified control sequence PolyA that did not reach the significance level.

The generation of an antigen-specific CTL response is of paramount importance for the development of an effective protective antiviral or antitumor immune response. The formation of CTLs is potentiated by DC activation and production of Th1-associated cytokines that enhance antigen presentation and priming of T cells.[14] In particular, selective activation of IL-12–producing DCs may be beneficial for developing a vaccine directed at inducing Th1-dependent cellular immunity.[26,27] Here we show that coinjection of ORNs with the model protein OVA effectively promoted the generation of antigen-specific cytotoxic T cells. We thus demonstrate for the first time that an ORN not only promotes innate immunity, but also stimulates the adaptive arm of the immune response in a sequence-dependent manner. One of the few vaccine adjuvants in clinical trials that promote the generation of CTLs are CpG oligonucleotides, which bind to TLR9.[28,29] In our model, the percentage of CTLs induced by ORNs was similar to that induced by CpG ODNs.

Immunostimulatory ORNs triggered the generation of a specific antibody response to OVA. While antibodies of the IgG1 isotype were induced by immunization with OVA alone, OVA-specific antibodies of the IgG2a isotype were only induced by immunostimulatory ORNs in a sequence-dependent manner. The generation of IgG2a antibodies is indicative of a bias toward a Th1-like response.[30] In a similar way, CpG ODNs have been described to promote a Th1-type response associated with induction of IgG2a antibodies.[31]

Whereas the function of pattern recognition receptors is the stimulation of an immune response to protect the host against invading pathogens, inappropriate stimulation through these receptors can lead to autoimmunity. In systemic lupus erythematosus, the uncontrolled activity of self-reactive T and B cells leads to the sustained production of tissue-damaging autoantibodies against nuclear antigens. Interestingly, the RNA component within the prototype autoantigen U1 small nuclear ribonucleoprotein (U1snRNP) is in itself immunostimulatory, inducing IFN-α and proinflammatory cytokines in a TLR7-dependent manner.[32,33] Here we show that immunostimulatory RNA can enhance a Th1-type

immune response to antigen. In a similar manner, specific RNA sequences within U1snRNP may therefore not only produce an antigen-unspecific activation of the immune system but also potentiate the generation of T and B cells specific for autoantigens.

A promising strategy in the immunotherapy of tumors is the use of mRNA-encoding tumor antigens to induce T-and B-cell immunity to the encoded antigens. In vivo application of mRNA induced cytotoxic T-cell activity and specific antibodies in mice.[34] Furthermore, human DCs transfected ex vivo with mRNA induced an antigen-specific immune response both in vitro to a viral antigen and in vivo to a tumor-associated antigen in patients with prostate cancer.[34,35] In some studies, mRNA transfection also contributed to activate DCs and enhance maturation.[35,36] The sequence-dependent adjuvant effect of ORNs we describe here may play a role in enhancing the effect of mRNA-based vaccines. Furthermore, the addition of immunostimulatory RNA sequences to mRNA vaccines could be used to enhance the potency of the vaccines.

The immunostimulatory effects of TLR ligands on both the innate and the adaptive arms of the immune system have prompted the investigation of their therapeutic potential in vaccine formulations. In particular, CpG ODNs, like immunostimulatory RNA, promote innate immune responses characterized by DC activation associated with IL-12 and IFN-α production. Stimulation of innate immunity by CpG ODNs can have a marked antitumor effect.[37] Furthermore, the strong Th1-type immune responses and cell-mediated immunity promoted by CpG ODNs in animal tumor models have led to the initiation of clinical trials studying the effectiveness of CpG ODNs in immunotherapy of tumors.[20,37-39]

Although preliminary results from clinical trials with CpG ODNs are encouraging, the restricted expression pattern in humans of TLR9, the receptor for CpG ODNs, may represent an additional difficulty. We have now shown that a similar Th1-type immune response can be induced by ORNs and thus that ORNs can serve as vaccine adjuvant to induce a CTL immune response. The immunostimulating activity of ORNs and ssRNA is mediated through TLR7 and TLR8,[11-13] which, in contrast to TLR9, are expressed in humans on a broad range of immune cells, including professional antigen-presenting cells such as MDCs and monocytes.[40,41] Indeed, ligands for TLR7 and TLR8 stimulate human monocytes as well as PDCs and B cells.[42,43] Therefore, a ligand for TLR7 and TLR8 such as an immunostimulatory ORN may, in patients, show a therapeutic efficacy in vaccine formulations that is superior to TLR9 ligands.

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Authorship

Contribution: C.B., V.H., G.H., and S.E. designed research; L.S., C.W., N.S., S.S., and A.V. performed research; V.H. and G.H. contributed vital new reagents; L.S., C.W., D.A., and N.S. collected data; C.B., L.S., D.A., and S.E. analyzed data; and C.B. and S.E. wrote the paper.

Conflict-of-interest disclosure: G.H. is a consultant for Coley Pharmaceuticals, whose product was studied in the present work. The other authors declare no competing financial interests.

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