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

Running title: Immunostimulatory RNA oligonucleotides

Carole Bourquin¹, Laura Schmidt¹, Veit Hornung¹, Cornelia Wurzenberger¹, David Anz², Nadja Sandholzer¹, Susanne Schreiber¹, Andreas Voelkl¹, Gunther Hartmann², Stefan Endres¹.

¹Division of Clinical Pharmacology, Department of Internal Medicine, Ludwig-Maximilian University of Munich, 80336 Munich, Germany

²Division of Clinical Pharmacology, University Hospital, University of Bonn, 53105 Bonn, Germany

Author contributions: CB, VH, GH and SE designed research; LS, CW, NS, SS, and AV performed research, VH and GH contributed vital new reagents, LS, CW, DA and NS collected data, CB, LS, DA and SE analyzed data, CB and SE wrote the paper.

Declaration of Commercial Interest: Gunther Hartmann is a consultant for Coley Pharmaceuticals whose product was studied in the present work.

This study was supported by grants from the Deutsche Krebshilfe 10-2214-En3 to S.E., from the Else-Kröner Fresenius Stiftung and the Deutsche Forschungsgemeinschaft En 169/7-2 and Graduiertenkolleg 1202 to C.B. and S.E. and by a grant from the Bundesministerium für Bildung und Forschung (Biofuture 0311896) to G.H. This work is part of the thesis of L.S. and C.W. at the University of Munich.

Corresponding author: Carole Bourquin
Phone: +49 89 5160 2230
Fax: +49 89 5160 4406
Email: carole.bourquin@med.uni-muenchen.de
Abstract

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 \textit{in vitro} and \textit{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.
Introduction

Recognition of pathogens by the innate immune system is mediated through pattern-recognition receptors that recognize distinct microbial components. Nucleic acids from pathogens are recognized by several classes of receptors, including Toll-like receptors (TLR) and cytoplasmic receptors. Microbial DNA, in particular DNA rich in unmethylated CpG motifs, is detected by TLR9 in the endosome. Long double-stranded RNA (more than 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. The 5'-triphosphate end of RNA generated by viral polymerases directly binds to RIG-I. Single-stranded RNA (ssRNA) from ssRNA viruses has been shown to be detected through TLR7 and TLR8. 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 (DC). The stimulatory activity on DC was also observed with the corresponding single-stranded RNA oligoribonucleotides (ORN).

DC are professional antigen-presenting cells that express a variety of pattern-recognition receptors. Exposure of DC 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 DC. Mature DC can directly interact with immune effector cells such as CTL, a process that is essential for the induction of protective immunity against infectious diseases and tumors. In addition, mature, activated DC also represent a critical source of IL-12 and IFN-α, two key cytokines in the driving of both innate and Th1-dependent acquired immune responses.
The immune-activating effects of TLR ligands have prompted their use in vaccine formulations. It is well established that binding of synthetic CpG oligodeoxynucleotides (ODN) to TLR9 both enhances the generation of an innate immune response and promotes protective Th1-type immunity in animal models\(^\text{17}\). In humans, clinical studies have demonstrated a potential for CpG ODN as adjuvant in antiviral vaccination\(^\text{18,19}\). Furthermore, CpG ODN combined with a peptide antigen promote CD8+ T cell responses to tumor antigens in melanoma patients\(^\text{20}\). However, TLR9 displays a restricted expression pattern in humans, where this receptor is expressed on B cells and plasmacytoid DC 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\(^\text{21}\).

Here we characterized the \textit{in vitro} and \textit{in vivo} immunostimulating potential of synthetic single-stranded ORN that activate TLR7. We show for the first time that ORN 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.
Methods

Mice

Female Balb/c and C57BL/6 mice were purchased from Harlan-Winkelmann (Borchen, Germany). Mice were 5-12 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 oligoribonucleotides 9.2dr (5’-UGUCCUUCAUGUCCUUCAA-3’) and PolyA in both the unmodified phosphodiester and fully phosphorothioated (PTO) forms and the oligoribonucleotides TLR4.1s (5’-UACUUAGACUACUACCUCG-3’), 9.2as (5’-UUGAAGGA-CAGGUUAAGCU-3’) and MyD88s (5’-CAGACAAACUAUCGACUGAtt-3’) in fully phosphorothioated 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). Chicken egg ovalbumin (OVA) was purchased from Sigma-Aldrich.

Bone marrow cell culture and generation of DC

Bone marrow cells were harvested from murine femur and tibia and erythrocytes were lysed with ammonium chloride buffer (BD Biosciences, Heidelberg, Germany). 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 DC, 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. DC (CD11c+ cells) generally represented 70% of the preparation. DC were enriched by magnetic cell sorting after labeling with anti-CD11c microbeads (Miltenyi Biotec) (CD11c+ cells above 95%). Bone marrow cells were activated with 5 µg/ml ORN complexed with DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate; Roche, Germany) according to the manufacturer’s instructions or with 6 µg/ml CpG ODN 1826 or 1 µg/ml LPS. Bone marrow–derived DC were activated with 10 µg/ml ORN or 1 µg/ml CpG ODN complexed with DOTAP or 1 µg/ml LPS. For coculture experiments, BMDC were stimulated with ORN or CpG ODN complexed with DOTAP for 6 h, then washed and cocultured with splenocytes (2 x 10^5 BMDC with 2 x 10^5 splenocytes) in 96-well U-bottom plates for an additional 18 h. Supernatants were harvested for detection of cytokines by ELISA. For analysis of activation markers, cells were stained with fluorochrome-coupled 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) together with HRP-conjugated donkey antibody to rabbit IgG as the secondary reagent (Jackson Immunolaboratories). 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 were incubated with 20 µg of oligonucleotides and 20 µl HBS for 20 min and injected i.v. into the retroorbital plexus. Single cell suspensions were prepared from spleen and lymph nodes 18 h 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 retroorbital puncture at the indicated time points. Serum was prepared by centrifugation and stored at –20°C. For immunization, 20-50 µg OVA were injected s.c. 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, 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 h with 1 % BSA in PBS. After incubation of serum samples for 1 h, plates were washed with PBS/1 % Tween 20 and goat anti-mouse IgG, IgG1 or IgG2a conjugated to horseradish peroxidase (Southern Biotech Laboratories, Alabama, USA) was added at 1 µg/ml for 1 h. Plates were again washed and ELISA was developed by o-phenylenediamine (Sigma Aldrich). Reaction was stopped by 1 M H₂SO₄ 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<sup>b</sup>-OVA<sub>257-264</sub>-PE pentamers (Proimmune, Cambridge, UK) and anti-CD8. For the detection of intracellular IFN-γ, cells were restimulated for 1 h with 5 µg/ml OVA<sub>257-264</sub> peptide, brefeldin A was added at a concentration of 1 µg/ml and cells were incubated for a further 4 h. 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 min. The percentage of CD8+ T cells expressing IFN-\(\gamma\) was determined by flow cytometry.

**In vivo cytotoxicity assay**

Targets were prepared from C57BL/6 splenocytes. Suspension was divided into two populations, pulsed or unpulsed with 100 \(\mu\)g/ml OVA\(_{257-264}\) for 1 h at 37°C, washed extensively, and labeled with a high concentration (15 \(\mu\)M) or with a low concentration (1.5 \(\mu\)M) of CFSE (Molecular Probes) respectively. Peptide-pulsed CFSE\(^{\text{high}}\) cells and unpulsed CFSE\(^{\text{low}}\) cells were mixed and a total of 10\(^7\) CFSE-labeled cells were injected i.v. into immunized mice. After 16 h, splenocytes were isolated and analyzed by flow cytometry to detect the CFSE-labeled target cells. Specific lysis was calculated by the following formula:

\[
\text{specific lysis (\%)} = 100\% \times \left[1 - \frac{(\text{CFSE}^{\text{high}} \text{ cells/CFSE}^{\text{low}} \text{ cells})}{(\text{CFSE}^{\text{high}} \text{ cells in naive mice/CFSE}^{\text{low}} \text{ cells in naive mice})}\right]
\]

**Statistics**

All data are presented as mean +/- s.e.m. and were analyzed by unpaired, one-way analysis of variance (ANOVA) with the Newman-Keuls multiple-comparison test. Significance was set at a P value of less than 0.05. Statistical analysis were performed using SPSS software.
Results

Phosphorothioated RNA oligonucleotides activate DC 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 phosphorothioated (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 (Fig. 1A). IL-10 was not induced by ORN. Cytokine production was sequence-dependent, as 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 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 ORN on DC, CD11c+ DC from a 7-day bone marrow culture were stimulated with ORN. IL-12p40 was detected in the supernatant of 9.2dr-stimulated and 9.2dr PTO-stimulated DC, while only 9.2dr PTO induced IL-6 production (Fig. 1A). Neither IL-4 nor IL-10 production were detected after stimulation with ORN (Fig. 1A and data not shown). PolyA ORN did not induce cytokine production in DC.

Surface expression of the activation markers MHC II, CD80, CD86 and CD40 was measured on CD11c+ DC after stimulation of bone marrow cells with ORN or CpG ODN (Fig. 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 DC on immune effector cells, purified bone marrow-derived DC were activated with the PTO ORN 9.2dr or PolyA, washed and cocultured with splenocytes. The early activation marker CD69 was upregulated on CD3+ and CD8+ T cells as well as on B cells after coculture with 9.2dr PTO-activated dendritic cells (Fig. 2A). In contrast, DC incubated with the PolyA PTO ORN did not activate splenocytes. Furthermore, splenocytes cocultured with 9.2dr PTO-activated DC produced the effector cytokine IFN-γ. Similar levels of IFN-γ were induced in splenocytes by CpG ODN 1826-activated DC. In contrast, no IFN-γ was detected in the supernatant of cocultures with PolyA PTO-cultured DC (Fig. 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 ORN complexed with DOTAP. As shown in Figure 3 (A and B), 9.2dr PTO application resulted in a strong upregulation 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 upregulated 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 upregulated on both myeloid and plasmacytoid DC subpopulations in the spleen of mice treated with 9.2dr PTO, indicating in vivo activation of DC. Neither the unmodified 9.2dr ORN nor the PolyA ORN significantly increased CD69 expression on DC. As positive control,
CD69 expression was measured in mice injected with CpG ODN (CD8+ T cells: 77 %, CD4+ T cells: 40 %, B cells: 51 %, MDC: 19 %, PDC: 54 %).

In the serum of ORN-injected mice, IL-12p40 was increased 2 h after injection with 9.2dr and 9.2dr PTO and the levels remained high at 6 h after injection (Fig. 4). At 22 h 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 h in mice injected with 9.2dr PTO. IL-6 was no longer detectable in serum 6 h p.i. IFN-α serum levels were increased after 2 h and 6 h 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 ORN on the development of an immune response to antigen, mice were immunized with ovalbumin (OVA) together with PTO ORN 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 (Fig. 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 ORN 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. While a small increase in pentamer+ OVA-specific 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 (Fig. 6A and C). Splenocytes from immunized mice were restimulated with the MHC-I-restricted peptide OVA257-264 and assessed for IFN-γ production (Fig. 6B and 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 OVA257-264 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 (Fig. 7).
Discussion

ssRNA and ssRNA viruses such as vesicular stomatitis virus and influenza virus are recognized by immune cells through TLR7 and TLR811,12,22, but specific sequence motifs responsible for viral RNA recognition have not been described. We have recently characterized an immunostimulatory RNA sequence consisting of nine bases (GUCCUUCAA), which needs however to be part of a longer oligonucleotide to become active13. 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 ORN 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 DC to produce the proinflammatory cytokines IL-12, IFN-α and IL-6. While IFN-α was mainly produced by ORN-activated plasmacytoid DC (data not shown), myeloid DC produced high amounts of IL-12p40 and IL-6. The immunostimulatory effect of ORN was also seen in vivo, where injection of 9.2dr resulted in activation of both myeloid and plasmacytoid DC as well as in a rapid increase in serum concentrations of IL-12, IFN-α and IL-6. Clearly, the stimulation was sequence-dependent, as a PolyA ORN of the same length showed only minor immunological activity. The cytokines induced, in particular IL-12 and IFN-α, are critical in the driving of both innate and Th1-type immune responses14,16. They stimulate lymphocyte differentiation and activation via the induction of Th1 cytokines such as IFN-γ. Indeed, we observed that after stimulation with ORN, DC induced phenotypic activation of T and B lymphocytes in vitro and
were potent inducers of IFN-γ production in splenocytes. \textit{In vivo}, injection of ORN resulted in increased expression of the activation marker CD69 on B cells, 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. DC 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\textsuperscript{23}.

Unlike immunostimulatory DNA sequences, single-stranded RNA molecules are highly susceptible to degradation by RNases. Encapsulation in liposomes, complexation to a cationic peptide or chemical modifications are necessary to enhance RNA stability and provide immunostimulatory activity\textsuperscript{11-13,24}. Cationic liposomes not only protect the ORN from degradation, but also increase the uptake into cells and target oligonucleotides to the endosomal compartment containing TLR7\textsuperscript{25}. In the present study, ORN were complexed with cationic liposomes (DOTAP), as we have previously shown that in the absence of DOTAP, no immunological activity is observed\textsuperscript{13}. Furthermore, we assessed in this study the influence of the ORN backbone on the immunostimulatory activity by comparing two ORN with the same nucleotide sequence 9.2dr containing either an unmodified phosphodiester backbone or a PTO-modified backbone. \textit{In vitro}, cytokine induction was consistently higher using the PTO-modified ORN. \textit{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 DC and lymphocytes was detected only after injection of PTO-modified ORN. The enhancement of the immunostimulatory effect may be due to the stabilizing function of the PTO modification. While the PTO backbone itself has been described to have immunostimulatory properties\textsuperscript{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 CTL 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 DC may be beneficial for developing a vaccine directed at inducing Th1-dependent cellular immunity\(^{26,27}\). Here we show that coinjection of ORN 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 CTL are CpG oligonucleotides, which bind to TLR\(^9\). In our model, the percentage of CTL induced by ORN was similar to that induced by CpG ODN.

Immunostimulatory ORN 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 IgG\(2a\) isotype were only induced by immunostimulatory ORN in a sequence-dependent manner. The generation of IgG\(2a\) antibodies is indicative of a bias towards a Th1-like response\(^{30}\). In a similar way, CpG ODN have been described to promote a Th1-type response associated with induction of IgG\(2a\) antibodies\(^{31}\).

While 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\textsuperscript{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 a 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\textsuperscript{34}. Furthermore, human
DC 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\textsuperscript{34,35}. In some studies, mRNA transfection also contributed to activate DC and enhance
maturation\textsuperscript{35,36}. The sequence-dependent adjuvant effect of ORN 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 ODN, 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 ODN can have a marked antitumor effect\textsuperscript{37}. Furthermore, the strong Th1-type immune responses and cell-mediated immunity promoted
by CpG ODN in animal tumor models have led to the initiation of clinical trials studying the effectiveness of CpG ODN in immunotherapy of tumors\textsuperscript{20,37-39}. While preliminary results from clinical trials with CpG ODN are encouraging, the restricted expression pattern in humans of TLR9, the receptor for CpG ODN, may represent an additional difficulty. We have now shown that a similar Th1-type immune response can be induced by ORN and thus that ORN can serve as vaccine adjuvant to induce a CTL immune response. The immunostimulating activity of ORN and ssRNA is mediated through TLR7 and 8\textsuperscript{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 myeloid DC and monocytes\textsuperscript{40,41}. Indeed, ligands for TLR 7 and 8 stimulate human monocytes as well as plasmacytoid DC and B cells\textsuperscript{42,43}. Therefore, a ligand for TLR7 and 8 such as immunostimulatory ORN may, in patients, show a therapeutic efficacy in vaccine formulations that is superior to TLR9 ligands.
Acknowledgments

We thank Susanne Wenk, Rosemarie Kiefl and Julia Vorac for technical assistance. This work is part of the thesis of L.S. and C.W. at the University of Munich.
References


Figure legends

Figure 1. Both unmodified and PTO RNA oligonucleotides stimulate bone marrow cells and DC in a sequence-dependent manner

Murine bone marrow cells or bone marrow-derived DC (BMDC) were activated for 24 h with ORN 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 +/- s.e.m. and are representative of at least two independent experiments. *, P<0.001; ns, not significant. * without brackets: comparison to unstimulated sample. (B) Surface expression of the activation markers CD80, CD86, CD40 and MHCII on DC was measured by FACS after activation of bone marrow cells with ORN or CpG ODN for 24 h. Data are expressed as % of CD11c+ cells expressing the respective activation marker and are representative of two independent experiments.

Figure 2. ORN-stimulated DC activate splenocytes to produce IFN-γ

BMDC 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 h, then washed and cocultured with naive splenocytes. A) After an additional 18 h 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 three 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 h coculture was measured in the supernatant by ELISA. Data show the mean of triplicate samples +/- s.e.m. and are representative of three independent experiments. *, P<0.001; ns, not significant. * without brackets: comparison to unstimulated sample.
Figure 3. PTO-modified immunostimulatory ORN activate lymphocytes and DC in vivo
Mice were injected i.v. with 20 µg ORN 9.2dr, 9.2dr PTO, PolyA or PolyA PTO and splenocytes were isolated 18 h after injection. CD69 expression was examined on CD4+ and CD8+ T cells and on B cells (B220+, CD11c−) (A and B) and on myeloid DC (CD11c+, CD11b+, B220−; MDC) and plasmacytoid DC (CD11c+, CD11b−, B220+; PDC) (C). (A) Representative data from one of five experiments are gated on CD8+, CD4+ T cells or B cells, respectively. Numbers indicate the percent of gated lymphocytes that are CD69+. (B and C) Data show the mean of individual mice in one experiment (PolyA: n=3, all other groups: n=4) +/- s.e.m. Results are representative of five independent experiments. *, P<0.001; ns, not significant. * without brackets: comparison to unstimulated group. The significance level was the same for all cell populations analyzed.

Figure 4. Immunostimulatory ORN induce production of Th1-type cytokines in vivo
Mice were injected as in Fig. 3 with ORN 9.2dr, 9.2dr PTO, PolyA or PolyA PTO. Blood samples were taken 2, 6 and 22 h 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) +/- s.e.m. Results are representative of two independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant. * without brackets: comparison with unstimulated group.

Figure 5. Immunostimulatory ORN 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 s.c. 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) +/- s.e.m. Results are representative of two independent experiments.
Figure 6. Immunostimulatory ORN trigger the production of antigen-specific, IFN-γ-producing CD8 cells

Mice were immunized as in Fig. 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 and C) The generation of OVA-specific CTL was assessed by flow cytometry using H-2kβ-OVA_{257-264} peptide pentamers and an anti-CD8 mAb. (B and D) Splenocytes were restimulated with the OVA_{257-264} peptide for 4 h and cytoplasmic expression of IFN-γ in CD8^{+} T cells was examined by flow cytometry. (A and B) Representative data from one of three experiments are gated on CD8^{+} cells. Numbers indicate the percent of CD8^{+} cells that are OVA-pentamer^{+} or IFN-γ^{+}. (C and D) Data show the mean values of individual mice (n=4) +/- s.e.m. Results are representative of three independent experiments. *, P<0.05; **, P<0.02; ***, P<0.001; ns, not significant. * without brackets: comparison to OVA group.

Figure 7. Immunostimulatory ORN induce antigen-specific cytotoxicity in vivo

Mice were immunized as in Fig. 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 two 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 +/- s.e.m. Results are representative of two independent experiments. *, P<0.01; **, P<0.001; ns, not significant. * without brackets: comparison to OVA group.
Fig. 1
Fig 2

A

<table>
<thead>
<tr>
<th></th>
<th>No stim</th>
<th>9.2dr PTO</th>
<th>PolyA PTO</th>
<th>CpG</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8</td>
<td>1.7</td>
<td>52.3</td>
<td>3.3</td>
<td>70.7</td>
</tr>
<tr>
<td>CD3</td>
<td>1.9</td>
<td>29.6</td>
<td>2.4</td>
<td>50.8</td>
</tr>
<tr>
<td>B220</td>
<td>1.3</td>
<td>54.4</td>
<td>2.9</td>
<td>68.4</td>
</tr>
</tbody>
</table>

B

![Graph showing IFN-γ levels with stimulants](image)

- □ 10 µg/ml (no splenocytes)
- □ 0.1 µg/ml
- □ 1 µg/ml
- ■ 10 µg/ml
Fig 3
Fig 4
Fig 6
Immunostimulatory RNA oligonucleotides trigger an antigen-specific cytotoxic T cell and IgG2a response

Carole Bourquin, Laura Schmidt, Veit Hornung, Cornelia Wurzenberger, David Anz, Nadja Sandholzer, Susanne Schreiber, Andreas Voelkl, Gunther Hartmann and Stefan Endres