Immunostimulatory RNA is a potent inducer of antigen-specific cytotoxic and humoral immune response in vivo

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Abstract

Single-stranded RNA stimulates immune cells and induces the secretion of pro-inflammatory cytokines and type I IFN. As adjuvant RNA can induce a Th2 type of humoral response, however, its potency in the induction of cytotoxic T cells in vivo has not been analyzed. Here we show that immunization with the antigen ovalbumin (OVA) and the adjuvant phosphodiester RNA complexed to the cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) induced a Toll-like receptor-7-dependent cytotoxic T cell and humoral response. Staining with SIINFEKL-Kb tetramers demonstrated the induction of antigen-specific T cells that were functional in in vivo cytotoxic T cell assays against SIINFEKL-loaded target cells. In infection experiments with OVA-secreting Listeria monocytogenes, the cytotoxic T cell response strongly reduced the bacterial load in liver and spleen. The RNA-driven humoral response was characterized by OVA-specific antibodies of the IgG1 isotype whereas CpG-DNA induced antigen-specific antibodies of the IgG2a (BALB/c) or IgG2c (C57BL/6) isotype. Furthermore, stimulation with RNA did not induce splenomegaly, a common feature of CpG-DNA-driven immune activation in mice. Taken together, our data confirm that RNA can be used as a safe adjuvant and induces a strong antibody response of the IgG1 isotype. Additionally, we demonstrate that RNA induces an antigen-specific immunity characterized by a potent cytotoxic T cell response to infection.

Introduction

The innate immune system recognizes pathogens by means of germ line-encoded pattern recognition receptors (PRRs). PRRs sense various conserved molecular structures of pathogens that are not found in vertebrates (1, 2). A subfamily of PRRs, the Toll-like receptors (TLRs), is important for initiation of an immune response. So far, 13 members (TLR1–13) have been reported that recognize structures from different pathogenic origin such as bacteria, viruses, fungi or protozoan parasites [reviewed in (3)]. One group of TLRs consisting of TLR1, 2, 4, 5, 6 and 11 is located on the cell surface and is responsible for detection of external pathogen structures like lipopeptides (TLR1, 2 and 6) (4, 5), LPS (TLR2 and 4) (6–10), flagellin (TLR5) (11) and profilin (TLR11) (12). The other group is formed by TLR3, 7, 8 and 9, which are localized inside the cell in the endoplasmic reticulum and endosomal–lysosomal compartment where they recognize bacterial or viral nucleic acid. Double-stranded RNA (dsRNA) acts as a viral-associated recognition motif for TLR3 (13), whereas DNA with non-methylated CpG motifs is a pattern for TLR9 (14–16). TLR7 in mice and TLR7 and TLR8 in humans have recently been shown to recognize single-stranded viral and synthetic RNA molecules that are rich in guanosine and/or uridine, provided the RNA is formulated within cationic lipids (17–19).

Stimulation of TLR results in the activation of signaling pathways via MyD88 or TRIF leading to up-regulation of co-stimulatory molecules on antigen-presenting cells (APCs) and production of pro-inflammatory and/or anti-viral cytokines.
RNA induces a potent cytotoxic and humoral immunity

In general, the TLR-mediated signaling via the adaptor molecule MyD88 which is shared by all TLRs except TLR3 induces an antigen-specific T<sub>1</sub> response. Accordingly, in MyD88-deficient mice, antigen-driven immune responses are of a T<sub>1</sub> type suggesting that TLR signals play an important role in directing the immune response towards T<sub>1</sub> (20, 21). Nevertheless, it has been reported that some TLR ligands such as the lipopeptide Pam3Cys induce a predominant T<sub>1</sub> immune response via TLR2 and lead to aggravation of asthma (22).

Due to the T<sub>1</sub>-promoting immune response, TLR agonists have been heavily explored as immunotherapeutics and vaccine adjuvant (23–28). CpG-DNA has proven to be one of the strongest T<sub>1</sub> immune-response-inducing adjuvant known in vivo (27). With regard to RNA, immune activation has been demonstrated by various groups (17–19,29, 30). It has recently been reported that naked synthetic phosphorothioate-modified RNA oligonucleotides and β-galactosidase (29) or micro-particles containing polyuridylic acid and ovalbumin (OVA) (30) induce immune stimulation and specific antibody production. The antibody isotype was primarily of the IgG1 isotype suggesting that RNA in vivo induces a T<sub>1</sub> biased immune response (29, 30). However, induction of a cytotoxic immune response and protection against infection has not been analyzed in detail.

We show here that injection of phosphodiester RNA and OVA induced OVA-specific cytotoxicity and antibody responses in a TLR7-dependent manner. In comparison with CpG-DNA, immunostimulatory RNA induced lower frequencies of cytotoxic T cells, but these were active in vivo and significantly reduced the bacterial load after Listeria monocytogenes infection. As shown previously, RNA did not induce spleenomegaly which is in contrast to the effect of CpG-DNA (29, 31). Further, we observed and confirmed that RNA induced antibodies of the IgG1 isotype indicating a T<sub>1</sub>, type of immune response. Overall, our data suggest that RNA is a weaker but safer adjuvant than CpG-DNA and induces an antigen-specific immunity characterized by a potent cytotoxic T cell response.

**Methods**

**Mice**

C57BL/6 and BALB/c mice were purchased from Harlan (Borchen, Germany). TLR7<sup>−−</sup> mice have been described (32). All animals were kept under specific pathogen-free conditions and only female mice were used for the experiments at 8–12 weeks of age.

**Reagents**

Chicken egg albumin (OVA) was obtained from Sigma–Aldrich (Taunfichken, Germany). The dominant CD8 T cell epitope of OVA, the peptide SIINFEKL (OVA peptide 257–264), was custom synthesized by Research Genetics (Huntsville, AL, USA). Phosphorothioate-modified CpG-DNA oligonucleotide 1668 with the sequence 5′-TTCACTGACGTTCCTGTATGC-3′ (Huntsville, AL, USA). Phosphorothioate-modified CpG-DNA oligonucleotide 1668 with the sequence 5′-TTCACTGACGTTCCTGTATGC-3′ (Huntsville, AL, USA). Phosphorothioate-modified CpG-DNA oligonucleotide 1668 with the sequence 5′-TTCACTGACGTTCCTGTATGC-3′ (Huntsville, AL, USA). Phosphorothioate-modified CpG-DNA oligonucleotide 1668 with the sequence 5′-TTCACTGACGTTCCTGTATGC-3′ (Huntsville, AL, USA).

**Injection of mice**

Mice were injected subcutaneously with 100 μl liposomal buffer alone (20 mM HEPES, 150 mM NaCl, pH 7.4), with 200 μg OVA and 30 μg DOTAP in buffer, with 200 μg OVA, 30 μg DOTAP and 100 μg RNA40 in buffer or with 200 μg OVA and 10 nM ODN 1668 in buffer. The injection scheme is presented in Fig. 1(A).

**Ex vivo tetramer staining of primed SIINFEKL-specific, CD8<sup>+</sup>-positive CD62L<sup>low</sup> T cells**

Seven days after the second injection, ~150 μl blood was analyzed for the presence of SIINFEKL tetramer binding by CD8-positive CD62L<sup>low</sup> T cells as described (33). In short, blood cells were depleted of erythrocytes, followed by a staining with 1 μg ml<sup>−1</sup> ethidium monoazide (EMA) (Molecular Probes, Karlsruhe, Germany) for 30 min at 4°C for a life/death discrimination. Subsequently, a triple staining with anti-CD8 allophycocyanin (clone CD8α, Caltag, Hamburg, Germany), anti-CD62L FITC (clone MEL-14, BD Biosciences, Heidelberg, Germany) and MHC SIINFEKL tetramer PE [H-2K<sup>b</sup>/SIINFEKL (OVA 257–264)/murine β<sub>2</sub>-microglobulin/ streptavidin–PE] was performed for 45 min at 4°C. Additionally, an Fc block (CD16/CD32, 2.4G2, BD Biosciences) was used to avoid unspecific antibody binding. At least 1 × 10<sup>6</sup> CD8<sup>+</sup>-positive events were analyzed on a FACSCalibur flow cytometer.

**5,6-Carboxyfluorescein diacetate succinimidyl ester-based in vivo cytotoxicity assay (in vivo kill)**

Syngeneic spleen cells (3 × 10<sup>5</sup> to 4 × 10<sup>6</sup>) per immunized mouse were washed with FCS-free RPMI 1640 medium (PAA, Coelbe, Austria), divided into two groups and stained with 0.5 or 5 mM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) at 37°C. The staining was stopped by adding complete medium. The cells were washed twice with complete medium, and the 5-mM CFSE group was incubated with 1 μM SIINFEKL peptide for 30 min at 37°C. Subsequently, both cell groups were washed three times with PBS, counted and mixed at a 1:1 ratio. The cell mixture was injected into the tail vein of the immunized mice, and after 20 h, blood and spleen were prepared, depleted of erythrocytes and analyzed for CFSE-stained cells by FACS. Percentage of in vivo kill is calculated by using the following formula: % in vivo kill = [(number of unloaded cells (0.5 mM CFSE) – number of SIINFEKL-loaded cells (5 mM CFSE))/number of unloaded cells] × 100.

**Intracellular cytokine staining**

For the intracellular cytokine staining, BD Cytofix/Cytoperm Plus kit with BD Golgi Plug (BD Pharmingen, Heidelberg, Germany) was used. Ten days after the second injection, spleens were removed and depleted of erythrocytes. From each spleen, 10<sup>7</sup> cells were stimulated for 5 h with 1 μM...
Simultaneously, Golgi Plug was added to block the secretion of newly synthesized cytokines. After the incubation, cells were first stained with 1 µg ml⁻¹ EMA (Molecular Probes) for 30 min at 4°C for a life/death discrimination. Then an extracellular staining with CD8–allophycocyanin/CD62L–PE (clone CD8α, Caltag/clone MEL-14, BD Biosciences) for 30 min at 4°C followed. Subsequently, cells were permeabilized and fixed according to the manufacturer’s instructions, followed by a staining with anti-IFN-γ–FITC antibody, anti-tumor necrosis factor-α (TNF-α)–FITC SIINFEKL. Simultaneously, Golgi Plug was added to block the secretion of newly synthesized cytokines. After the incubation, cells were first stained with 1 µg ml⁻¹ EMA (Molecular Probes) for 30 min at 4°C for a life/death discrimination. Then an extracellular staining with CD8–allophycocyanin/CD62L–PE (clone CD8α, Caltag/clone MEL-14, BD Biosciences) for 30 min at 4°C followed. Subsequently, cells were permeabilized and fixed according to the manufacturer’s instructions, followed by a staining with anti-IFN-γ–FITC antibody, anti-tumor necrosis factor-α (TNF-α)–FITC
antibody or an FITC-conjugated isotype control antibody (dilution 1:500 for 30 min at 4°C) (all from BD Biosciences).

Antibody ELISA
For antibody detection, serum was obtained from blood of immunized mice 1 week after the second injection. ELISA plates (Nunc, MaxiSorp) were coated overnight with 0.1 mg ml⁻¹ OVA. Sera were diluted 1:300 000 and for detection the following antibodies were used: biotin-Sp-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch, Hamburg, Germany), biotin anti-mouse IgG1, biotin anti-mouse IgG2a and biotin anti-mouse IgG2a b (IgG2a b equals IgG2c) (BD PharMingen). A streptavidin–HRP (Amersham Biosciences) was used according to the manufacturer’s instructions.

Listeria monocytogenes infection
Five weeks after the second injection, immunized mice were injected in the tail vein with 4 × 10⁶ recombinant L. monocytogenes secreting OVA (L. monocytogenes-OVA, kindly provided by H. Shen (University of Pennsylvania School of Medicine, Philadelphia, PA, USA) (34). Listeria monocytogenes were grown in brain–heart infusion (BHI) medium (Oxoid GmbH, Wesel, Germany) to the density of OD₆₀₀ = 0.1, which equals 10⁶ bacteria ml⁻¹ for this strain. The amount of injected bacteria was controlled by plating the bacteria on BHI plates and colony numbers were determined after overnight incubation at 37°C. Three days after the infection, spleen and liver were removed, homogenized, lysed in 0.1% Triton solution and plated on BHI plates in three different dilutions as follows: 1:5000, 1:50 000 and 1:500 000. The plates were incubated at 37°C overnight and colonies were counted.

Results
Phosphodiester RNA as adjuvant induces a potent cytotoxic immune response
To characterize the adjuvant function of RNA, we immunized mice with chicken OVA and RNA complexed to the cationic lipid DOTAP (DO). After a boost injection, we analyzed the induction of SIINFEKL-specific CD8⁺ T cells, their cytokine profile and assessed their cytotoxic activity in vivo by infection experiments (Fig. 1A). Injections with DOTAP/OVA containing no RNA (OVA + DO) or solely buffer (control) served as negative controls. Immunizations with CpG-DNA 1668 were performed in parallel to compare the adjuvant capacity of RNA.

One week after the second injection, ~4.7% of all CD8-positive cells in the blood of RNA-immunized mice were activated (CD62Llow) SIINFEKL-specific T cells (Fig. 1B). This number was significantly higher than the amount of SIINFEKL-specific cells in both negative control groups (P < 0.05). CpG-DNA is a strong inducer of a cytotoxic T cell response (27) and a high percentage of CD8-positive cells (24%) were SIINFEKL specific. The antigen-specific cytotoxic T cells in RNA-treated and CpG-DNA-treated mice produced IFN-γ and TNF-α after stimulation with SIINFEKL peptide indicating their functionality (Fig. 1C). The cytotoxic potential of the CD8⁺ T cells was tested using CFSE-stained SIINFEKL peptide-loaded syngeneic cells that were injected into immunized mice. On average, 20% of SIINFEKL-loaded cells were killed in RNA-treated mice 20 h after injection of the cells, whereas no cytotoxicity could be observed in both negative control groups (Fig. 1D) (P < 0.01). With CpG-DNA as adjuvant, almost all SIINFEKL peptide-loaded cells were eliminated.

Next, we analyzed the adjuvant ability of RNA in inducing immune protection against L. monocytogenes infection. Listeria monocytogenes is an intracellular pathogen whose elimination requires a Th1 immune reaction. CpG-DNA, as a very strong Th1 adjuvant, provides an optimal protection against L. monocytogenes (35–37). Five weeks after the second injection, immunized mice were infected with 4 × 10⁴ OVA-secreting bacteria, an amount corresponding to the LD₅₀. Three days after infection, spleen and liver of infected mice were analyzed for bacterial load. RNA-driven vaccination reduced the bacterial load in these organs by 90% whereas in both control groups no protective effect was observed (Fig. 1E). Similar results were obtained by injections with 1 × 10⁴ Listeria (data not shown). In comparison, CpG-treated animals cleared the Listeria infection efficiently.

Overall, immunizations utilizing RNA induce a strong cytotoxic response leading to the reduction of SIINFEKL-presenting cells in vivo and the reduction of bacterial burden in a L. monocytogenes infection model.

Phosphodiester RNA as adjuvant induces OVA-specific antibodies of Th2 type
To investigate the humoral immune response induced by the RNA-OVA mixture, we analyzed the production of OVA-specific antibodies in the sera of immunized mice 1 week after the second injection (Fig. 1A). C57BL/6 and BALB/c mice treated with RNA or CpG-DNA as adjuvant produced higher amounts of OVA-specific IgG antibody than control mice although injections of OVA/DOTAP induced some OVA-specific IgG (Fig. 2). As anticipated, CpG-DNA induced mainly antibodies of the IgG2c isotype in C57BL/6 mice [IgG2c substitutes the IgG2a isotype in C57BL/6 mice (38)] or IgG2a isotype in BALB/c mice which is the result of a Th1-driven immune response. It is of note that the BALB/c mouse strain tends to react towards infection with a Th2 response, a circumstance that can explain the relatively high amount of IgG1, the isotype typically associated with a Th2 immune response, in the sera of CpG-DNA-treated BALB/c mice. Interestingly, RNA predominantly induced IgG1 antibodies in both mouse strains.

Overall, vaccination with RNA and CpG-DNA as adjuvant induces comparable amounts of OVA-specific IgG antibodies, the various composition of antibody isotypes, however, suggests that RNA in contrast to CpG-DNA does not induce a Th1-based humoral immune response.

The adjuvant function of phosphodiester RNA is TLR7 dependent
Single-stranded RNA (ssRNA) stimulates the immune system through TLR7 in mice and through TLR 7 and 8 in humans (17–19). To investigate, if the adjuvant activity of ssRNA is
TLR7 dependent in vivo, we immunized TLR7-deficient C57BL/6 mice with RNA-OVA and control mixtures. CpG-DNA as adjuvant induced a high number of SIINFEKL-specific CD62L<sup>hi</sup>CD8<sup>+</sup> T cells and an efficient cytotoxicity against SIINFEKL-loaded syngeneic cells in TLR7<sup>-/-</sup> mice (Fig. 3A). In contrast, ssRNA did not induce SIINFEKL-specific cytotoxic cells and subsequently did not lead to SIINFEKL-specific cytotoxicity in these mice. Similar result could be observed for the humoral response against OVA. Whereas CpG-DNA induced the production of OVA-specific antibodies in TLR7-deficient mice, RNA failed to induce OVA-specific antibodies (Fig. 3B). Thus, the presence of TLR7 seems to be necessary for ssRNA-driven adjuvant function.

**Immunostimulatory phosphodiester RNA does not induce splenomegaly**

Although CpG-DNA is a strong T<sub>H</sub>1-promoting adjuvant, side effects such as splenomegaly and alteration of mouse lymphoid morphology have been reported and may jeopardize its application (31, 39). We analyzed the spleen weight of CpG-DNA- and RNA-treated mice 1 week after the second injection and observed that CpG-DNA-treated mice had a 50% larger spleen compared with mice from negative control groups (Fig. 4). Interestingly, mice injected twice with RNA as adjuvant showed no noticeable changes in the spleen weight, demonstrating that phosphodiester RNA does not induce a strong systemic immune response.

**Discussion**

The innate immune system senses nucleic acid via TLRs and leads to the stimulation of APCs. TLR3, TLR7, TLR8 and TLR9 are located within the endosomal compartment and recognize ssRNA (TLR7/8), dsRNA/mRNA (TLR3) and ssCpG-DNA (TLR9) (3). Overall, nucleic acids such as CpG-DNA, ssRNA and mRNA are characterized by a strong immunostimulatory capacity and therefore are good candidates for a new class of adjuvant (18, 29, 30, 40–42). Accordingly, CpG-DNA induces the production of T<sub>H</sub>1-biased cytokines like IL-12 and IFN-α <sup>in vitro</sup> (27) and has been

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**Fig. 2.** RNA induces OVA-specific antibodies of T<sub>H</sub>2 type. One week after the second injection, the presence and isotypes of OVA-specific antibodies were analyzed in the sera of immunized C57BL/6 (A) (n = 12) and BALB/c (B) (n = 6) mice. One representative experiment of at least two independent experiments is shown. Statistical analysis was performed using a Mann–Whitney test.

**Fig. 3.** The adjuvant function of RNA is TLR7 dependent. TLR7-deficient and wild-type mice were immunized and 1 week after the second injection percentage of SIINFEKEL-specific CD8<sup>+</sup> T cells and their cytotoxic potential in vivo was analyzed (A) (n = 3 for OVA + DO and OVA + CpG-DNA and n = 6 for OVA + DO + RNA). Similarly, the presence and isotypes of OVA-specific antibodies were analyzed in the sera of immunized mice (B). One representative experiment of at least two independent experiments is shown.
T cells were active in the in vivo induction of OVA-specific cytotoxic T cells and antibodies. This entirely mediated by TLR7 since TLR7-deficient mice show no response. L. monocytogenes cells and lead to protection against a Th1 Ig isotype profile as reported for CpG-DNA. Accordingly, it has been reported that low concentrations of the synthetic TLR2 ligand Pam3Cys induce a predominant T<sub>h</sub>2-biased immune response with high levels of IL-13 and IgG1 and aggravation of asthma (22). As well Porphyromonas gingivalis LPS, which signals through TLR2 (6), induces a T<sub>h</sub>2 response characterized by significant levels of IL-13, IL-5 and IL-10, but lower levels of IFN-γ (26). Therefore, evidence accumulates that TLRs influence T<sub>h</sub>2-driven immune responses that may be driven by low-level signaling via MyD88-dependent or -independent mechanism.

Repeated injection with CpG-DNA can lead to adverse effects through over-stimulation of the immune system (31, 39). In our experiment, we observed a spleen enlargement in CpG-DNA-treated mice but not in animals treated with RNA as adjuvant which confirms the finding by Scheel et al. (29). Therefore, RNA seems to have lesser side effects than CpG-DNA in mice although its adjuvant capacity is weaker compared with CpG-DNA. Of note, this conclusion does not necessarily apply to humans since the expression pattern of TLR7, TLR8 and TLR9 varies in both species. In humans, TLR7 and TLR8 are expressed on various APCs, whereas TLR9 is basically only expressed on B cells and plasmacytoid dendritic cells (47, 48). In contrast, TLR8 is presumably non-functional in mice and TLR9 has a much broader expression pattern compared with humans (15, 49). Therefore, it is possible that ssRNA is a stronger adjuvant in humans than in mice and may even be superior to CpG-DNA in humans.

One disadvantage of RNA used as adjuvant may be the necessity of complexation to cationic lipids for efficient immune stimulation. Although Scheel et al. (29) report that naked phosphorothioate-modified RNA oligonucleotides induces immune stimulation in vivo, Westwood et al. (30) demonstrate that encapsulation of RNA and antigen is critical for immune stimulation. Of note, the RNA utilized in our study and by Westwood et al. (30) was phosphodiester RNA which presumably needs protection due to the short half-life. Naked phosphorothioate-modified RNA may circumvent the need for complexation and future developments could focus on RNA modifications that further enhance half-life and RNA uptake allowing the use of lower RNA concentrations for immunostimulation.

Overall, the antigen-specific immunity induced by RNA in vivo via TLR7 seems to be characterized by a potent cytotoxic T cell response to infection and a strong antibody response reflecting a T<sub>h</sub>2 immune response. The T<sub>h</sub>2 phenotype in regard to antibody isotype is surprising since TLR7-driven signaling strictly relies on MyD88 which is essential for a T<sub>h</sub>1-driven immune response (21). It is possible that signal strength may influence the outcome of the type of immune response. Since we used phosphodiester RNA molecules that have a relatively short half-life in vivo, it can be envisaged that the ligand is only available for a short time limiting the duration of cellular activation. Accordingly, one disadvantage of RNA used as adjuvant may be the necessity of complexation to cationic lipids for efficient immune stimulation. Although Scheel et al. (29) report that naked phosphorothioate-modified RNA oligonucleotides induces immune stimulation in vivo, Westwood et al. (30) demonstrate that encapsulation of RNA and antigen is critical for immune stimulation. Of note, the RNA utilized in our study and by Westwood et al. (30) was phosphodiester RNA which presumably needs protection due to the short half-life. Naked phosphorothioate-modified RNA may circumvent the need for complexation and future developments could focus on RNA modifications that further enhance half-life and RNA uptake allowing the use of lower RNA concentrations for immunostimulation.

Overall, we have confirmed that RNA is a potent adjuvant in vivo. Our study extends recent work on RNA as adjuvant (29, 30) by demonstrating that RNA induces cytotoxic T cells in a TLR7-dependent manner. The cytotoxic T cell response is potent and reduces bacterial load in an infection model with the intracellular pathogen L. monocytogenes. Future
investigation will focus on using ssRNA for tumor therapy and adjuvant application in humans.

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Abbreviations

APC antigen-presenting cell
BHI brain–heart infusion
CFSE 5,6-carboxyfluorescein diacetate succinimidyl ester
dsRNA double-stranded RNA
EMA ethidium monoazide
OVA ovalbumin
PRL pattern recognition receptor
ssRNA single-stranded RNA
TNF-α tumor necrosis factor-α

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