Immunostimulatory RNA Oligonucleotides Induce an Effective Antitumoral NK Cell Response through the TLR7

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J Immunol 2009; 183:6078-6086; doi: 10.4049/jimmunol.0901594
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RNA oligonucleotides containing immune-activating sequences promote the development of cytotoxic T cell and B cell responses to Ag. In this study, we show for the first time that immunostimulatory RNA oligonucleotides induce a NK cell response that prevents growth of NK-sensitive tumors. Treatment of mice with immunostimulatory RNA oligonucleotides activates NK cells in a sequence-dependent manner, leading to enhanced IFN-γ production and increased cytotoxicity. Use of gene-deficient mice showed that NK activation is entirely TLR7-dependent. We further demonstrate that NK activation is indirectly induced through IL-12 and type I IFN production by dendritic cells. Reconstitution of TLR7-deficient mice with wild-type dendritic cells restores NK activation upon treatment with immunostimulatory RNA oligonucleotides. Thus, by activating both NK cells and CTLs, RNA oligonucleotides stimulate two major cellular effectors of antitumor immunity. This dual activation may enhance the efficacy of immunotherapeutic strategies against cancer by preventing the development of tumor immune escape variants. The Journal of Immunology, 2009, 183: 6078–6086.

Viruses are recognized by the innate immune system through endosomal and cytosolic receptors that selectively detect conserved pathogen structures (1). Molecular patterns specific to nucleic acids from viruses are recognized by the cytoplasmic helicases retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) and by endosomal receptors of the TLR family, the TLRs 3, 7, 8, and 9 (1–8). Binding of nucleic acid ligands to their respective receptor activates intracellular signaling cascades that rapidly induce innate and adaptive immune responses (1).

An important goal of therapeutic cancer vaccines is the induction of Ag-specific responses that mediate protective immunity against tumors. Because of their immunostimulatory properties, nucleic acid ligands for TLRs are powerful tools that can be used to boost tumor-specific immune responses. Indeed, inclusion in cancer vaccines of Cpg oligodeoxynucleotides, that bind TLR9, leads to the production of IFN-γ and potentiates immune responses to tumor Ags in patients (9). Within the immune system, human TLR9 is expressed mainly on B cells and a subset of dendritic cells (DC), the plasmacytoid DC. The receptors TLR7 and TLR8 that are activated by ssRNA of viral origin are expressed in addition by human myeloid DC and monocytes that are essential for Ag presentation and for the initiation of immune responses against tumor Ags (5, 6, 10, 11). We have recently described RNA sequences that stimulate immune responses through the TLRs 7 and 8 (12). We have further shown that ssRNA oligonucleotides that activate TLR7 can trigger the generation of CTL by inducing Th1-type immunity (13).

In addition to the Ag-specific immune responses effected by CTL, a major component of antitumor immunity is the innate NK cell response (14). NK cells are involved both in tumor immunosurveillance and in the rejection of established tumors and can prevent the dissemination of metastases (14, 15). The ideal NK cell targets are tumor cells that have lost expression of MHC class I or that overexpress ligands for the activating receptor NKG2D (16). Upon interaction with target cells, NK cells exert cytotoxic functions that are determined by a balance of multiple activating and inhibitory signals (16). Two important antitumoral effector mechanisms triggered by target cell interaction are the direct cytolytic activity of NK cells and the production of IFN-γ. Strategies to exploit NK cell-mediated immunity may thus hold strong potential for cancer therapy.

The therapeutic potential of TLR7 agonists is supported by encouraging results with a recently developed class of antitumor agents, the imidazoquinolines, that acts in part through the activation of TLR7 (17). The lead compound, imiquimod, is however only approved for the treatment of skin tumors by topical use (18). Although some imidazoquinoline compounds support antitumor NK responses in vivo, disseminated tumor models (19), in solid tumors injection of imiquimod is only effective locally but not at
distant sites (20). Due to the short half-life of these small molecules in vivo, frequent applications are necessary.

In this study, we show for the first time that immunostimulatory RNA oligonucleotides elicit an effective antitumor NK response in vivo through TLR7. We demonstrate that RNA oligonucleotide treatment selectively inhibits growth of MHC class I-negative tumors but not growth of MHC class I-expressing tumors. Stimulation of mice with immunostimulatory RNA oligonucleotides leads to an activated NK phenotype with enhanced IFN-γ production and increased cytotoxicity. These effector functions are triggered indirectly through activation of DC to produce IL-12 and type I IFN. We further show that oligonucleotide-activated DC are sufficient to promote NK activation in vivo. Thus, the therapeutic application of RNA oligonucleotides represents a promising strategy to stimulate NK cell responses to induce effective antitumor immunity.

Materials and Methods

Mice and cell lines

Female C57BL/6 mice were purchased from Harlan-Winkelmann. TLR7-deficient mice (C57BL/6 background) were a gift from S. Akira (Osaka University, Osaka, Japan) and bred in the animal facilities of the Department of the Ludwig-Maximilian University of Munich (Munich, Germany). Female β2-microglobulin (β2m)-deficient mice and IL-12p40-deficient mice (both C57BL/6 background) were from Charles River Laboratories. IFNAR-deficient mice (129sv background) and wild-type (wt) 129sv mice as controls were provided by Dr. H. J. Anders (Ludwig-Maximilian University of Munich, Munich, Germany). Mice were 5–12 wk of age at the onset of the experiments. All animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany). The C57BL/6-derived T cell lymphoma cell line RMA and the TAP-deficient variant RMA-S were provided by Dr. J. Charo (Berlin, Germany).

Oligonucleotides

The 20-mer oligoribonucleotides 9,2dr (5’-UGUCCUUCAAUUGUCCUUC AA-3’) and poly(A) in both the unmodified phosphodiester (PD) and fully phosphorothioate (PTO) forms were purchased from CureVac. To protect against degradation, all oligoribonucleotides used were PTO unless indicated otherwise. The PTO-modified CpG oligoribonucleotide 1826 (5’-TCCATGACGTTCCTGACGTT-3’) was obtained from the Coley Pharmaceutical Group (Langenfeld, Germany). Oligoribonucleotides were complexed with N-[1-(2,3-dioleoylpropyl)-N,N,N-trimethylammonium methosulfate (DOTAP; Roche) before use by incubation for 20 min at a ratio of 1:2-1:5 (w/w).

For stimulation of human NK cells, the sequence 9.3as (5’-UGUUAA-3’) was incorporated twice and 2 μg/ml CpG complexed with DOTAP for 6 h at 1 × 10⁶/ml in 200 μl of complete medium. For NK-DC co-cultures, stimulated DC were washed twice and 2 × 10⁵ purified NK cells or 2 × 10⁶ total splenocytes in 200 μl of fresh medium/well were added. For cultures of NK cells with DC supernatant, 200 μl of BMDC supernatant was added to 2 × 10⁶ purified NK cells or total splenocytes. Cell activation was measured 18 h later. Neutralizing Abs against IL-2 (20 μg/ml, clone JES6-1-A12; R&D Systems), IL-6 (5 μg/ml, clone MP520F3; R&D Systems), IL-12-L23p40 (40 μg/ml, clone C17.8; BioLegend), and IL-15 (2 μg/ml, clone AIO3; MBL) were added to the supernatant, as indicated, 2 h before coincubation with NK cells.

Human NK cells (1 × 10⁶ in 200 μl of medium) were cocultured with accessory cells (monocytes) at the indicated ratios. Cells were stimulated for 12 or 36 h with 1 μg/ml 9.3as or 10 μM 3M-001, 3M-002, or 3M-003 before cell activation or IFN-γ production was measured.

In vivo NK activation and tumor therapy

For in vivo immunostimulation, 20–40 μg of oligonucleotides complexed with DOTAP were injected i.v. or 100 μg of uncomplexed CpG was injected s.c. into TLR7-deficient or wt C57BL/6 mice. Single-cell suspensions were prepared from spleen 22 h after injection unless indicated otherwise. For DC transfer, TLR7-deficient mice were injected twice i.v. with 2.5 × 10⁶ wt BMDC in 200 μl of Heps-buffered saline at a 2-h interval. Wild-type BMDC were either unstimulated or stimulated in vitro with 1 μg/ml 9,2dr for 6 h before transfer. Two hours after the cell transfer, mice were injected i.v. with RNA oligonucleotides. For tumor therapy, groups of five mice were injected on day 0 with 10⁷ TAP-deficient RMA-S or wt RMA tumor cells s.c. Mice were treated i.v. with RNA oligonucleotides on days 0, 3, and 6 (RMA-S) and additionally on days 9 and 12 (RMA). For depletion of NK cells, 0.5 μg of the IL-2R chain-specific mAb TMB1 was given i.p. 2 days before and 2 days after tumor challenge as described previously (21). For depletion of CD8 T cells, 0.1 μg of the mAb CD8α was given i.p. 2 and 1 days before and 7 days after tumor challenge. Efficacy of depletion was confirmed by flow cytometry. Tumor size was expressed as the product of the perpendicular diameters of individual tumors. For assessing NK activation after repeated injections of RNA oligonucleotides in tumor-bearing mice, 4 × 10⁶ B16 cells were injected i.v. and animals were treated with RNA oligonucleotides eight times at 3-day intervals.

Flow cytometry and ELISA

Single-cell suspensions from splenocytes or blood were treated with ammonium chloride buffer to lyse erythrocytes. RMA-S tumors were mechanically disrupted and incubated with collagenase (Sigma-Aldrich) at 37°C for 20 min, then passed through a 40-μm cell strainer (BD Biosciences) to obtain single-cell suspensions. For analysis of activation markers, cells were stained with fluorochrome-coupled mAbs and analyzed by flow cytometry. Anti-mouse CD3-allophycocyanin, NK1.1-PerCP, and IFN-γ-FITC, anti-mouse and anti-human CD69-PE, and anti-human leukocyte Ag DR-PerCP were from BD Biosciences.

To assess IFN-γ production by NK cells, splenocytes were isolated 4 h after RNA injection and incubated for 4 h with brefeldin A at a concentration of 1 μg/ml. Cells were stained with PerCPCoujugated anti-NK1.1 (BD Biosciences), fixed with 2% parafomaldehyde, and permeabilized with 0.5% BSA, 0.5% saponin, and 0.02% sodium azide in PBS. Fixed cells were stained with FITC-conjugated anti-IFN-γ Ab (BD Biosciences) for 25 min. The percentage of IFN-γ-positive NK cells was determined by flow cytometry. Levels of human or murine IFN-γ in the supernatant were quantified by ELISA (BD Biosciences) according to the manufacturer’s protocol.

Cytotoxicity assays

For in vivo determination of cytotoxicity, target splenocytes from wt and TLR7-deficient mice were labeled for 15 min at room temperature with 15 and 0.15 PM CFSE, respectively. Cells were washed twice with PBS and resuspended at 1 × 10⁶/ml. The two populations were mixed at a 1:1 ratio and 1 × 10⁵ cells were injected i.v. into wt C57BL/6 mice injected 4 h before.
the RNA lymphoma that is selectively targeted by NK cells, unlike the MHC-sufficient parent cell line (24). Mice were treated with the highly immunostimulatory oligonucleotide 9.2dr (12, 13) after inoculation of either NK-sensitive RMA-S cells or wt RMA cells. Three injections of the 9.2dr oligonucleotide complexed with DOTAP at 3-day intervals suppressed progression of RMA-S tumors compared with untreated mice (Fig. 1A). In contrast, after injection of a control poly(A) sequence of the same length, no effect on tumor growth was observed. Treatment with RNA oligonucleotides did not prevent tumor progression in the MHC-sufficient RMA tumors (Fig. 1B), suggesting that NK cells are the critical effectors of the antitumor effect of immunostimulatory RNA. Within the tumor, NK cells represent 11.6% of small lymphocytes (∼2.4%; n = 5). Results for one tumor are shown in Fig. 1C. Indeed, during treatment with 9.2dr, RMA-S tumors progressed more rapidly in NK-depleted mice than in CD8-depleted mice or undeployed group (D). SSC, Side scatter.

Statistics
Comparisons in tumor size among groups were made using the Mann-Whitney U test for various time points. For cytokine ELISAs and cytotoxicity assays, significance was assessed by Student’s t test. Statistical analysis was performed using SPSS software. Error bars indicate SEM.

Results
Immunostimulatory RNA sequences suppress growth of MHC-negative tumors
We have recently described immunostimulatory sequences within RNA oligonucleotides that activate a Th1-type immune response, an important effector of antitumoral immunity (12, 13). To assess whether immunostimulatory RNA oligonucleotides can suppress tumor growth through activation of NK cells, the second major cellular component of antitumoral immunity, we examined the efficacy of RNA oligonucleotides in the treatment of RMA-S tumors. The RMA-S cell line is a MHC class I-negative variant of the RMA lymphoma that is selectively targeted by NK cells, unlike the MHC-sufficient parent cell line (24).

FIGURE 1. RNA oligonucleotides suppress growth of MHC-negative tumors. Mice were inoculated s.c. on day 0 with 10^6 RMA-S (A, C, and D) or RMA (B) tumor cells. Mice were treated at 3-day intervals from day 0 with 20 μg PTO 9.2dr or poly(A) i.v. complexed with DOTAP. A and B. Data show the mean tumor sizes ± SEM (n = 5) and are representative of two independent experiments. C: Percentage of NK cells (NK1.1^+CD3^−, gated on small lymphocytes) in RMA-S tumors was measured by flow cytometry. Data are shown for one representative tumor (n = 5). D. Mice injected with NK-depleting (depl.) or CD8-depleting Abs were treated with 9.2dr as in A. *, p < 0.05; **, p < 0.01; ns, not significant; comparison to poly(A)-treated group (A), untreated (B), or undepleted group (D). SSC, Side scatter.

Previously with 9.2dr, CFSE staining in splenocytes was analyzed by flow cytometry 4–10 h after target cell injection. Specific lysis was calculated as follows: specific lysis (percent) = 100 – [100 × (CFSE<sup>low</sup> cells in stimulated mice/CFSE<sup>low</sup> cells in stimulated mice)/(CFSE<sup>low</sup> cells in unstimulated mice/CFSE<sup>low</sup> cells in unstimulated mice)] (22).

For determination of cytotoxicity in vitro, splenic NK cells were either isolated from treated mice 4 h after oligonucleotide injection or were activated with 100 μl of supernatant from RNA-stimulated DC for 4 h. NK cells were plated at 5 × 10<sup>5</sup> cells/100 μl in complete RPMI 1640 without phenol red (PAA). YAC-1 target cells (1 × 10<sup>6</sup>) were labeled with CFSE without further stimulation (C). Specific lysis for individual mice (data points, n = 3) and mean (bar). B–D, Results are representative of two independent experiments. E, CD69 was measured as in A on circulating and intratumoral NK cells in mice bearing RMA-S tumors (n = 6) treated with 9.2dr as in Fig. 1. F, CD69 was measured on circulating NK cells 22 h after either one injection or the last of eight injections of 9.2dr (at 3-day intervals) in mice inoculated i.v. with B16 cells (n = 5). *, p < 0.05; **, p < 0.01; and ***, p < 0.001. No stim, No stimulation.
Immunostimulatory RNA sequences rapidly activate NK cells in vivo

To characterize the effect of RNA oligonucleotides on NK cells, mice were injected i.v. with RNA oligonucleotides complexed with DOTAP. In addition to oligonucleotides comprising a PTO backbone, oligonucleotides of the same sequence with an unmodified PD backbone were used. As shown in Fig. 2A, a single application of 9.2dr PTO resulted in up-regulation of the early activation molecule CD69 on 80% of splenic NK cells. CD69 expression was also up-regulated on a small proportion of splenocytes in mice injected with the unmodified 9.2dr PD oligonucleotide. Furthermore, 9.2dr PTO enhanced production of IFN-γ by NK cells as early as 4 h after injection (Fig. 2B). Notably, this increase in IFN-γ production was observed directly ex vivo without the need for restimulation in culture by target cells. Activation of NK cells was clearly sequence dependent, since NK cells were not activated by treatment with poly(A) oligonucleotides of the same length in either the PTO or PD form. Because of their stronger immune-activating capacity, PTO oligonucleotides were used for all additional experiments.

To assess the cytotoxicity of NK cells following RNA oligonucleotide application, an in vivo assay was performed (22). In this assay CFSE-labeled splenocytes from β2m-deficient mice were injected i.v. as target cells. Splenocytes from wt mice labeled with a higher CFSE concentration were injected simultaneously as reference. Blood was collected from recipients 10 h after target cell injection and PBMC were analyzed for CFSE staining by flow cytometry. Lysis of CFSElow β2m-deficient cells was selectively increased in 9.2dr PTO-treated mice with a specific lysis of >60% (Fig. 2C and D). Indeed, an increased specific lysis was detected as early as 4 h after target cell injection (data not shown).

We then examined CD69 expression on NK cells from tumor-bearing mice. As with healthy animals, CD69 was up-regulated in mice bearing RMA-S tumors upon treatment with 9.2dr PTO (Fig. 2E). Within the tumor, NK cells also showed increased CD69 expression following treatment with 9.2dr PTO (Fig. 2E).

In vivo NK activation by RNA oligonucleotides is mediated through TLR7

RNA from viral origin is recognized by a variety of different receptors of the innate immune system, including TLR7 (1). To determine whether NK cell activation by RNA oligonucleotides is mediated through TLR7, TLR7-deficient and wt mice were injected with 9.2dr complexed with DOTAP. As shown in Fig. 3A, TLR7-deficient mice injected with 9.2dr showed no up-regulation.
of the early activation molecule CD69 on NK cells. In contrast, injection of the CpG oligodeoxynucleotide 1826, a ligand for TLR9, resulted in expression of CD69 on 80% of NK cells in TLR7-deficient mice, demonstrating that TLR7-deficient NK cells can respond to activation through another TLR. Also, the percentage of IFN-γ-producing NK cells was not significantly increased by 9.2dr in TLR7-deficient mice (Fig. 3B). Furthermore, NK cells isolated 4 h after RNA oligonucleotide injection showed TLR7-dependent cytotoxicity. NK cells from 9.2dr-treated wt mice effected a specific lysis of 60% against the NK-sensitive cell line YAC-1 (Fig. 3C) in a standard in vitro cytotoxicity assay (25). In contrast, NK cells from TLR7-deficient mice showed no cytotoxic activity upon 9.2dr stimulation. Thus, NK cell activation by RNA oligonucleotides is TLR7-dependent.

**DC are essential for NK cell activation**

We have previously shown that stimulation with RNA oligonucleotides leads to DC activation through TLR7 (12, 13). Because NK cells can be efficiently activated by DC (26, 27), we investigated whether DC play a role in NK-cell activation by RNA oligonucleotides. When total splenocytes or CD11c-depleted splenocytes with 10 μg/ml 9.2dr or poly(A) complexed with DOTAP. B, BMDC were stimulated with 9.2dr for 6 h or left unstimulated. Cells were washed to remove oligonucleotides and cocultured for 18 h with splenocytes or purified NK cells. CD69 expression on NK cells and IFN-γ secretion were measured. C and D, BMDC were stimulated as in B, washed, and cultured for an additional 18 h. BMDC culture supernatant was harvested and added to splenocytes or purified NK cells. C, Expression of CD69 on NK cells and IFN-γ production were assessed after 18 h. D, NK cytotoxicity was measured after 4 h against YAC-1 target cells at an E:T ratio of 12.5:1. Results are representative of three independent experiments. **, p < 0.01; ns, not significant; No stim, no stimulation.

**FIGURE 4.** RNA oligonucleotides stimulate NK cells in a DC-dependent manner. A, Expression of CD69 on NK cells was measured 6 h after stimulation of total splenocytes or CD11c-depleted splenocytes with 10 μg/ml 9.2dr or poly(A) complexed with DOTAP. B, BMDC were stimulated with 9.2dr for 6 h or left unstimulated. Cells were washed to remove oligonucleotides and cocultured for 18 h with splenocytes or purified NK cells. CD69 expression on NK cells and IFN-γ secretion were measured. C and D, BMDC were stimulated as in B, washed, and cultured for an additional 18 h. BMDC culture supernatant was harvested and added to splenocytes or purified NK cells. C, Expression of CD69 on NK cells and IFN-γ production were assessed after 18 h. D, NK cytotoxicity was measured after 4 h against YAC-1 target cells at an E:T ratio of 12.5:1. Results are representative of three independent experiments. **, p < 0.01; ns, not significant; No stim, no stimulation.
Using a panel of neutralizing Abs, we showed that IFN-γ production by NK cells was entirely IL-12 dependent (Fig. 5A). In contrast, neutralizing Abs for IL-2, IL-6, and IL-15 had no effect on IFN-γ production. To confirm the role of IL-12 in NK activation, splenocytes from IL-12-deficient mice were stimulated with 9.2dr for 18 h and expression of CD69 on NK cells was assessed. Middle panel, BMDC from wt or IL-12−/− mice were stimulated with 9.2dr for 6 h. Cells were washed to remove oligonucleotides and cocultured for 18 h with purified NK cells from TLR7−/− mice. IFN-γ production was quantified by ELISA. Right panel, wt splenic NK cells were activated for 4 h with supernatant from stimulated wt or IL-12−/− BMDC and cytotoxicity against YAC-1 cells was measured at an E:T ratio of 20:1. C, Left panel, Splenocytes from wt or IFNAR −/− mice were stimulated with 9.2dr for 18 h and expression of CD69 on NK cells was assessed. Middle panel, wt BMDC were stimulated with 9.2dr for 6 h. Cells were washed and cocultured for 18 h with splenocytes from wt or IL-12−/− mice. IFN-γ production was quantified by ELISA. Right panel, Cytotoxicity of wt or IFNAR −/− NK cells activated with supernatant from stimulated wt BMDC was measured as in B. *p < 0.05; **p < 0.01; ***p < 0.001, ns, not significant, nd, not determined; No stim, no stimulation.

Since type I IFN has also been implicated in the activation of NK cells (28, 29), we measured the activation of NK cells from type I IFN receptor-deficient mice upon RNA stimulation. Both CD69 up-regulation and cytotoxicity of NK cells were significantly reduced compared with wt NK cells (Fig. 5C). In contrast, IFN-γ production in type I IFN receptor-deficient NK cells was preserved. We conclude that the effector functions of NK cells following RNA oligonucleotide application are differently regulated. Although IFN-γ production is dependent on IL-12, cytotoxicity depends on type I IFN. Thus, DC-derived IL-12 and type I IFN are both critical cytokines for the activation of NK cells by RNA oligonucleotides.

**Immunostimulatory RNA activates human NK cells more rapidly than small molecule agonists**

In contrast to mice in which TLR7 is the sole receptor for ssRNA in the endosome, in humans TLR7 and TLR8 share the detection of ssRNA (5, 6). Small molecule TLR7 and TLR8 agonists, the imidazoquinolines, have been shown to activate NK cells (30). We compared the effect of immunostimulatory RNA with three small molecule agonists on human NK cells. Peripheral blood monocytes were used as accessory cells, because these cells express TLR8 and can differentiate to monocyte-derived DC. Immunostimulatory RNA up-regulated CD69 expression on up to 60% of NK cells in a monocyte-dependent manner (Fig. 6A). Stimulation with small molecule TLR7/8 agonists also increased CD69 expression, albeit at lower levels. An increase in CD69 expression was also seen when NK cells were cocultured with plasmacytoid DC for both immunostimulatory RNA and imidazoquinolines (data not shown). Furthermore, stimulation of the monocyte-NK coculture with immunostimulatory RNA induced IFN-γ production by NK cells as early as 12 h after stimulation (Fig. 6B). IFN-γ induction by imidazoquinolines occurred later and did not reach the levels obtained by immunostimulatory RNA (Fig. 6C). For both imidazoquinolines and immunostimulatory RNA, stimulation was clearly dependent on the presence of monocytes.

**DC mediate RNA activation of NK cells in vivo**

To determine whether DC also mediate NK cell activation by RNA oligonucleotides in vivo, TLR7-deficient mice were reconstituted with wt BMDC before treatment with 9.2dr. CD69 expression on NK cells was up-regulated in these mice upon 9.2dr treatment (Fig. 7, A and B). In contrast, we have shown that TLR7-deficient mice without transferred DC show no NK cell activation following 9.2dr injection (Fig. 3A). NK cell activation was also seen when DC were stimulated with 9.2dr before transfer, in addition to the in vivo 9.2dr treatment (Fig. 7). Transfer of DC alone without RNA stimulation did not induce NK activation. Thus, DC are sufficient to mediate activation of NK cells by RNA oligonucleotides in vivo.

**Discussion**

We have recently described immune-activating sequences within ssRNA oligonucleotides that can act through TLR7 (12). These oligonucleotides induce Th1-type cytokines in vivo and trigger the generation of CTL and Ab production when coinfected with Ag.
Indeed, in a murine lung metastasis model, IFN-β was essential for efficient protection against infections and tumors. Thus, both IFN-α and IFN-β demonstrated that IFN-β production by NK cells upon RNA oligonucleotide treatment suggests that IFN-γ may play an important role in antitumor efficacy. Indeed, in a murine lung metastasis model, IFN-γ was essential for the NK-dependent antitumor effect of 3M-011, a TLR7 agonist (19). We further show that RNA oligonucleotides potentiating NK cells are not directly activated to produce IFN-γ, but they respond to triggering by target cells (37). In contrast to this two-step process, we show here that a single injection of RNA oligonucleotides rapidly stimulates IFN-γ production by NK cells in vivo without the need for restimulation with target cells. Furthermore, an increase in cytotoxicity was seen in vivo as early as 4 h after injection of the target cells. Thus, RNA oligonucleotides rapidly activate NK cells in vivo while bypassing the need for additional restimulation. Activation was clearly sequence-specific, as a poly(A) sequence of the same length induced neither an activated phenotype nor IFN-γ production. We thus show for the first time that RNA oligonucleotides can activate NK cells in vivo in a sequence-dependent manner.

Because the oligonucleotide backbone itself, independently of the nucleotide sequence, may play a role in TLR7 and TLR9 recognition and activation (40, 41), we compared in vivo NK activation by oligonucleotides with the same sequence containing either an unmodified PD backbone or a PTO-modified backbone. We observed low activation with the PD backbone, while activation was strongly enhanced by the use of the PTO backbone, although recognition and activation remained sequence-dependent. Similarly, we previously observed stronger T and B cell activation with PTO RNA oligonucleotides (13). This probably relates to the increased stability provided by the PTO backbone, since RNA is highly susceptible to nuclelease degradation (42).

RNA can be sensed by a variety of receptors of the innate immune system, including the TLRs 3 and 7, the cytoplasmic helices RIG-I and MDA-5, the serine-threonine kinase PKR, and the NALP-3 inflammasome (3–8, 43–46). Importantly, we showed that NK activation by the 9.2dr RNA oligonucleotide is entirely TLR7-dependent, since the increase in NK effector function following RNA treatment is absent in TLR7-deficient mice. This demonstrates that cytosolic receptors do not participate in the in vivo recognition of these RNA oligonucleotides. Thus, we showed for the first time that RNA oligonucleotides activate NK cells in

In this study, we show for the first time that RNA oligonucleotides can inhibit tumor growth in a sequence-dependent manner. Treatment of mice with TLR7-activating oligonucleotides selectively suppressed growth of NK-sensitive tumors, demonstrating that RNA oligonucleotides activate NK immunity (31, 32). Two main mechanisms are responsible for NK-mediated antitumor immunity, IFN-γ production and direct cytotoxicity (16). IFN-γ is a crucial mediator of antitumor immunity in experimental models and elevated levels of IFN-γ are associated with disease outcome in several clinical studies (14, 33). The increase in IFN-γ production by NK cells upon RNA oligonucleotide treatment suggests that IFN-γ may play an important role in antitumor efficacy. Indeed, in a murine lung metastasis model, IFN-γ was essential for the NK-dependent antitumor effect of 3M-011, a TLR7 agonist (19). We further show that RNA oligonucleotides potentiating NK cytotoxic activity in vivo. The close contact of activated NK cells with target cells is a prerequisite for cytotoxic activity (34) and indeed we clearly show the presence of activated NK cells within the s.c. tumors. Thus, both IFN-γ production and direct cytotoxicity may synergize to provide NK-mediated antitumor immunity upon RNA oligonucleotide treatment.

In the absence of infection, NK cells present a naive phenotype and do not efficiently up-regulate effector functions upon contact with target cells expressing NK-activating receptors (35, 36). To achieve efficient protection against infections and tumors, NK cells therefore require an initial priming that can be effected by bacterial, viral, or even parasitic infectious agents (37–39). Primed NK cells are not directly activated to produce IFN-γ, but they respond to triggering by target cells (37). In contrast to this two-step process, we show here that a single injection of RNA oligonucleotides rapidly stimulates IFN-γ production by NK cells in vivo without the need for restimulation with target cells. Furthermore, an increase in cytotoxicity was seen in vivo as early as 4 h after injection of the target cells. Thus, RNA oligonucleotides rapidly activate NK cells in vivo while bypassing the need for additional restimulation. Activation was clearly sequence-specific, as a poly(A) sequence of the same length induced neither an activated phenotype nor IFN-γ production or cytotoxicity. We thus show for the first time that RNA oligonucleotides can activate NK cells in vivo in a sequence-dependent manner.

**FIGURE 6.** Immunostimulatory RNA activates human NK cells more rapidly than small molecule agonists. In brief, 10⁴ NK cells were cocultured with monocytes at the indicated ratio. Cells were stimulated with 1 μg/ml of the immunostimulatory RNA oligonucleotide 9.3as or with 3M-001 (TLR7 agonist), 3M-002 (TLR8 agonist), or 3M-003 (TLR7 and 8 agonist) (10 μM). A, After 36 h, expression of CD69 was analyzed on NK cells (gated as MHC class II-negative cells). B and C, Supernatant was removed after 12 h (B) and 36 h (C) of coculture and IFN-γ production was assessed. Data show the mean of three donors ± SEM. n.d., Not determined.
vivo through TLR7. In vitro studies show that also human NK cells within PBMC can be stimulated by a TLR7-activating ssRNA sequence derived from HIV-1 (47). This suggests that a therapeutic strategy using RNA oligonucleotides to target NK cells may also be effective in humans.

Small molecule agonists of the imidazoquinoline family have been shown to activate NK cells through TLR7 and TLR8 (30). Previous in vitro studies on the mechanisms of NK cell activation by imidazoquinolines have however been inconsistent. Direct activation of human NK cells by imidazoquinolines without the need for accessory cells has been shown in vitro but is dependent on the presence of the cytokines IL-12, IL-15, or IFN-α that are generally produced by accessory cells (48, 49). Contrasting reports claim that the induction of IFN-γ production in vitro by imidazoquinolines is dependent on activation of accessory cells such as monocytes (30, 50, 51). Furthermore, imidazoquinolines also activate the NALP3 inflammasome, which leads to the production of bioactive IL-1β and IL-18 (52). Indeed, IL-18 was shown to contribute to NK cell activation by imidazoquinoline compounds (51).

In this study, we clearly show that reconstitution of TLR7-deficient mice with wt DC restores activation of NK cells by immunostimulatory RNA. Thus, we provide in vivo data demonstrating that the main mechanism for in vivo activation of NK cells by immunostimulatory RNA is entirely TLR7-dependent and is mediated by accessory cells. Indeed, the induction of NK responses to pathogens in vivo requires DC (37–39).

Although DC can activate NK cells by direct contact (29, 53), cell-cell contact is not necessary for activation of NK cells by RNA oligonucleotides. Instead, activation is mediated by DC-secreted factors, as the stimulatory effect on NK cells can be achieved by transferring supernatant of activated DC. Several cytokines, the most important being IL-2, IL-10, IL-12, IL-15, IL-18, and IFN-α, have been implicated in the regulation of NK activation (26, 54–57). Type I IFN in particular plays an important role for the in vivo priming of NK cells in different infectious models (37–39). Depending on the pathogen, type I IFN affects NK cell priming by acting either on myeloid DC that are induced to produce IL-15 (37) or directly on NK cells (39). The cellular source of type I IFN necessary to activate NK cells upon TLR stimulation remains however unclear (37). Interestingly, depletion of plasmacytoid DC, the main producers of the type I IFN-α, does not impair activation of NK cells in vivo upon infection (38). We have previously shown that RNA oligonucleotides induce both IL-12 and IFN-α in vivo (13). We show here that the effector functions following RNA oligonucleotide treatment are differently regulated: whereas IFN-γ production is dependent on IL-12, cytotoxicity depends entirely on type I IFN.

The therapeutic potential of cancer treatment strategies targeting NK cells has gained momentum in recent years. NK cells are one of the cellular mediators of Ab-dependent cell-mediated cytotoxicity, an important effector mechanism for the therapeutic use of mAbs targeting tumor Ags. Other treatments targeting NK cells include NK cell adjuvants such as cytokines, soluble TRAIL, c-kit tyrosine kinase inhibitors, or even the transfer of preactivated NK cells (14). These therapeutic strategies could be profitably combined with RNA oligonucleotides to enhance NK cell activation in vivo.

In summary, RNA oligonucleotides possess an important therapeutic potential for the treatment of NK-sensitive tumors by triggering the activation of NK cells via the induction of cytokine production by DC. In addition to their direct antitumoral efficacy, NK cells favor the generation of CTL through IFN-γ production (21, 58). This mechanism may contribute to the ability of RNA oligonucleotides to induce an Ag-specific CTL response (13). Because both MHC-negative and MHC-positive tumor cells can thus be eliminated, the combined activation of CTL and NK cells by RNA-based therapies may help prevent tumor immune escape (59). Furthermore, RNA oligonucleotides can be designed to induce other antitumoral properties in the same molecule: RNA oligonucleotides containing a 5′-tripophosphate lead to antitumoral efficacy through activation of the RIG-I receptor (60). In addition, introduction of a specific inhibitory sequence (small interfering RNA) permits the knockdown of specific genes such as tumor-promoting genes (60). Thus, it will be possible to combine TLR7 activation with other potent antitumoral mechanisms in one RNA molecule, providing a promising multifunctional therapeutic approach.

Disclosures

The authors have no financial conflict of interest.

References


