FLT3 Ligand Enhances the Cancer Therapeutic Potency of Naked RNA Vaccines

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Abstract

Intranalonal immunization with antigen-encoding naked RNA may offer a simple and safe approach to induce antitumor immunity. RNA taken up by nodal dendritic cells (DC) coactivates toll-like receptor (TLR) signaling that will prime and expand antigen-specific T cells. In this study, we show that RNA vaccination can be optimized by coadministration of the DC-activating Fms-like tyrosine kinase 3 (FLT3) ligand as an effective adjuvant. Systemic administration of FLT3 ligand prior to immunization enhanced priming and expansion of antigen-specific CD8+ T cells in lymphoid organs, T-cell homing into melanoma tumors, and therapeutic activity of the intranodonal RNA. Unexpectedly, plasmacytoid DCs (pDC) were found to be essential for the adjuvant effect of FLT3 ligand and they were systemically expanded together with conventional DCs after treatment. In response to FLT3 ligand, pDCs maintained an immature phenotype, internalized RNA, and presented the RNA-encoded antigen for efficient induction of antigen-specific CD8+ T-cell responses. Coadministration of FLT3 ligand with RNA vaccination achieved remarkable cure rates and survival of mice with advanced melanoma. Our findings show how to improve the simple and safe strategy offered by RNA vaccines for cancer immunotherapy. 

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Introduction

Direct application of naked antigen-encoding RNA is for more than one decade under active investigation as a vaccine approach against cancer (1). Compared with DNA, RNA immunization lacks the risk of integration into the genome and due to transient expression of the encoded antigens allows better control of immune responses. In contrast to viral recombinant vectors, RNA does not feature immunodominant viral antigens that interfere with the preferred antigen-specific immune response. Moreover, RNA-based compounds not only deliver the complete antigen but also have an intrinsic adjuvant activity. Recombinant RNA is easy to produce by in vitro transcription in large amounts and high purity. The capability of RNA vaccines to confer antitumor immunity has been shown in model systems (2–6) and promising results of early clinical testing have been reported recently (7–9).

A few years ago, we developed a strategy to generate pharmacologically optimized RNA with improved stability, translational performance, and presentation of the encoded antigen on MHC class I and II molecules (4, 10, 11). Injection of such optimized RNA into lymph nodes is superior to any other application route with regard to induction of potent antigen-specific T-cell immunity (5). The RNA propagates a proinflammatory microenvironment in the lymph node, resulting in de novo priming and efficient expansion of antigen-specific CD8+ and CD4+ T cells (5). By immunizing tumor-bearing mice with this RNA vaccine, we achieved potent antitumor immunity and remarkable survival benefit (5).

The objective of the current study was to further augment the potency of intranodally administered RNA by combination with a suitable adjuvant. Adjuvants are compounds that enhance the magnitude, breadth, quality, and longevity of specific immune responses to antigens and have minimal toxicity. For a long time, adjuvant research progressed at a slow pace and adjuvant design was largely empirical. In the meantime, as the development of immunotherapeutics is gaining speed, the need for rational selection strategies for adjuvant formulations based on sound immunologic principles is becoming evident.

We recently revealed that RNA administered into the lymph node is selectively internalized by resident immature dendritic cells (DC) and that the uptake is highly efficient and is driven by macrophagocytosis (12). Both the cell type and the uptake mechanism were shown to be functionally relevant for the observed efficiency of intranodal RNA vaccination. These notions were factored into the rationally guided search for suitable adjuvants. With DCs being essential for vaccine
effects of intranodal RNA, we came across Fms-like tyrosine kinase 3 (FLT3) ligand (13, 14), a naturally occurring glycoprotein stimulating early hematopoietic progenitors via the FLT3 receptor (15) and mobilizing them from the bone marrow into the peripheral blood and secondary lymphatic organs. FLT3 ligand is known to induce profound expansion of DCs in the peripheral lymphoid organs (16–18), and FLT3 ligand–induced DCs have an immature phenotype (19, 20). However, it is not clear whether these DCs are capable of presenting antigens encoded by RNA. In addition to expansion of DCs, various other effects of FLT3 ligand have been reported, including stimulation of natural killer (NK), B, and T cells (21–24). These effects translated in improved T-cell immunity and antitumor potency in mouse models when FLT3 ligand was used as an adjuvant in vaccination approaches (16–18, 25–27).

In this study, we detail our efforts to evaluate FLT3 ligand in combination with intranodally administered RNA and to dissect the mechanisms underlying the strong synergy of both compounds. Surprisingly, we revealed that plasmacytoid DCs (pDC) are central to the observed adjuvant effects of FLT3 ligand. Our findings not only open new paths for improved RNA vaccination strategies but also give unexpected insights into the functional potency of pDCs.

Materials and Methods

Animals

C57BL/6 mice were obtained from Jackson Laboratories. TCR transgenic OT-I mice that recognize the H2-Kb-restricted SIINFEKL epitope (OVA257-264) from chicken ovalbumin (OVA) gene (Mainz, Germany) was received in 2008 and subjected to reauthentification of cells has not been done since receipt. FLT3 ligand-conditioned bone marrow–derived DCs (FLT3L-BMDC) were generated by 8-day culture of bone marrow precursors with 200 ng/mL FLT3L-IgG4.

FLT3 ligand plasmid construction and protein purification

A cDNA representing the signal peptide and extracellular domain of human FLT3 ligand (aa 1–185) was cloned into an expression vector and carboxy-terminally fused to a 675-bp fragment coding for the hinge region and heavy chain constant regions 2 and 3 [(CH2 and CH3) of human IgG4], to obtain FLT3L-IgG4. As control, the 675-bp IgG4-Fc fragment (IgG4) was cloned separately in the expression vector. HEK293 cells were transfected with these constructs using polyethylimine (PEI; Sigma-Aldrich) for production of FLT3L-IgG4 or IgG4 proteins, which subsequently were purified from culture supernatants by affinity chromatography with Mab-Select SuRe columns (GE Healthcare) and stored at −80°C. FLT3L-IgG4 or IgG4 fragments were administered intraperitoneally (i.p.). In some experiments, human IgG4 kappa antibody (Sigma-Aldrich) was used as a control.

RNA vaccines, in vitro transcription, and intranodal immunization

All plasmids for in vitro transcription of naked antigen-encoding RNA encoding luciferase (Luc) are based on the pST1-2hBgUTR-A120 backbone (10), which feature a 3′ human β-globin untranslated region (hBgUTR) and a pol(A) tail of 120 nucleotides and allow generation of pharmacologically improved in vitro transcribed RNA. The MITD vectors pST1-sec-SIINFEKL-MITD-2hBgUTR-A120 (SIINFEKL; ref. 11) and pST1-sec-Influenza-HA-MITD-2hBgUTR-A120 (HA; ref. 11) allow tagging of the respective protein with a secretion signal (Sec) and the transmembrane and cytosolic domains of the MHC class I (MITD) ensuring efficient presentation on MHC class I and class II. The SIINFEKL construct contains aa 257 to 264 of chicken OVA and the HA construct a codon-optimized partial sequence of influenza HA (aa 60–285 fused to aa 517–527; influenza strain A/PR/8/34) designed to combine major immunodominant MHC epitopes.

In vitro transcription and purification of RNA were previously described (29). HA-RNA was labeled with Cy5-UTP (Cy5-RNA) according to the manufacturer’s instructions (Amer sham). Purified RNA was assessed by spectrophotometry, gel electrophoresis, and BioAnalyzer (Agilent).

As described previously (5), for intranodal immunization of mice with naked RNA, the inguinal lymph node of anaesthetized mice was surgically exposed, 20 μg RNA in 10 μL RNase-free PBS (Ambion) were injected intranodally, and the wound was closed.

Flow cytometry and tetramer staining

All monoclonal antibodies (mAb) were from BD Pharmin gen, except anti-PDCA1 (Miltenyi Biotec). Hypotonically lysed blood and splenocyte samples were incubated at 4°C with mAbs. Lymph node cells were obtained by digestion with collagenase (1 mg/mL; Roche). Direct ex vivo quantification of SIINFEKL-specific CD8+ cells with H-2Kb/SIINFEKL tetramer (Beckman-Coulter) without in vitro stimulation (10) and intracellular cytokine staining (29) were previously described. Absolute quantification of NK cells in blood was carried out with Trucount tubes (BD Biosciences) by adding 50 μL heparinized blood to the Trucount tube, staining with antibodies against CD45, CD3, and NK1.1, and quantification of cells after lysis of erythrocytes with fluorescence-activated cell-sorting (FACS) lysing solution (BD Biosciences). Regulatory T cells (Treg) were analyzed with the FoxP3 Staining Kit (eBioscience). Conventional DCs (cDC; CD11c+PDCA1+) and pDCs (CD11c+PDCA1+) were sorted by FACSaria (BD Biosciences). Quantification of H-2 Kb/SIINFEKL complexes by
25-D1.16 antibody was carried out as previously described (10). Flow cytometric data were acquired on a FACSCalibur and FACSCanto analytic flow cytometers (BD Biosciences) and analyzed with FlowJo (Tree Star) software.

**In vivo bioluminescence imaging**

Uptake and translation of Luc-RNA were evaluated by in vivo bioluminescence imaging (BLI) using the IVIS Lumina imaging system (Caliper Life Sciences; ref. 4). Briefly, an aqueous solution of d-luciferin (150 mg/kg body weight; BD Biosciences) was injected intraperitoneally 24 hours after administration of Luc-RNA. After 15 minutes, emitted photons were quantified (integration time of 1 minute). In vivo bioluminescence in regions of interest (ROI) were quantified as total flux (photons per seconds) using IVIS Living Image 3.0 Software.

**Quantitative real time PCR**

Extraction of total cellular RNA, first-strand cDNA synthesis, real-time PCR (RT-PCR) using TaqMan Gene Expression Assays (Applied Biosystems), and normalization to the housekeeping gene Hprt1 were described previously (30).

**Tumor experiments**

Tumor vaccination protocols were previously described (5). Briefly, 2 × 10^6 B16-OVA melanoma cells were inoculated subcutaneously into the flanks of C57BL/6 mice. FLT3L-IgG4 (10 μg) or human IgG4 control antibody were administered intraperitoneally on day 3, day 7, day 14, and day 18 after tumor inoculation. Four intranodal immunizations of SINIFEKL-RNA (20 μg) in 3- to 4-day intervals were initiated 11 days after tumor cell inoculation (diameter of tumors 2 mm). The tumor sizes were measured every 3 days. Mice were sacrificed when tumor diameter reached 15 mm.

Tumor-infiltrating cells (TIC) were obtained by mechanical disruption of the tumor tissue and phenotyping myeloid-derived suppressor cells (MDSC; CD11b^+^, Gr1^+^), Treg cells (CD4^+^, CD25Foxp3^+^), NK cells (NK1.1^+^, CD3^+^, B cells (CD19^+^, CD13), SIINFEKL^+^ CD8^+^ T cells (CD3^+^, CD8^+^, tetramer^+^), CD8^+^ T cells (CD3^+^, CD8^+^, CD4^+^), CD4^+^ T cells (CD3^+^, CD4^+^CD8^+^), pDC (CD11c^+^, PDCA1^+^, NK1.1^+^), and cDC (CD11c^+^, PDCA1^−^, NK1.1^−^).

**In vivo depletion of pDCs**

C57BL/6 mice received 500 μg of anti-PDCA1 (Miltenyi Biotec) mAb or a monoclonal rat IgG1 mAb (Jackson Immunoresearch) as a control 2 times in 6-day intervals.

**Enzyme-linked immunospot assay**

Enzyme-linked immunospot (ELISPOT) assay was carried out as described before (5). Briefly, 1 × 10^4 CDcs or pDCs sorted from SIINFEKL-RNA or control RNA (HA-RNA) electroporated FLT3L-BMDCs were coinubcated with 1 × 10^6 magnetic-associated cell-sorted CD8^+^ OT-I T cells in a microtiter plate coated with anti-IFN-γ antibody (10 μg/mL, clone AN18; Mabtech). After 18 hours at 37° C, cytokine secretion was detected with an anti-IFN-γ antibody (clone R4-6A2; Mabtech).

**Immunohistochemistry and immunofluorescence**

Cryosections (4–6 μm) from mouse lymph nodes were fixed with cold acetone and blocked with PBS-containing 5% mouse serum.

For indirect staining, rat-anti-PDCA1 (JF05-IC2-4.1; Miltenyi), rat-anti-F4/80 (BM8; eBioscience) or rat-anti-NK1.1 (PK136; BD Biosciences) as primary antibodies were combined with Cy3-anti-rat-IgG (N418; Molecular Probes). For direct staining, reticular fibroblasts and fibers were visualized with Alexa488-anti-ER-TR7 (BMA), sections were mounted in Vectashield mounting medium (VECTORLabs), analyzed with a Zeiss immunofluorescence microscope (Carl Zeiss Micro-Imaging GmbH), and digital images were prepared using Photoshop CS4 (Adobe).

**Statistics**

GraphPad Prism software using unpaired 2-tailed Student’s t test or ANOVA with Tukey's multiple comparison test was used. Differences in survival were analyzed by the log-rank test. Values of P < 0.05 were considered statistically significant.

**Results**

**FLT3 ligand treatment expands DCs and NK cells in the lymph node and spleen**

In an attempt to test compounds expanding the DC population in combination with intranodal RNA, we came across FLT3 ligand. As the half-life of FLT3 ligand in serum is very short (31), we engineered a recombinant fusion protein (Fig. 1A) linking the extracellular domain of human FLT3 ligand to the heavy chain constant regions 2 and 3 (CH2-CH3 domain) of human IgG4 to increase the serum half-life (data not shown). It was already shown that human FLT3 ligand can cross-react with mouse FLT3 (32), which enabled the use of human FLT3 ligand in the murine setting. Two intraperitoneal applications of this FLT3 ligand IgG4 fusion protein (referred to as FLT3L-IgG4) resulted in significant increase of cellularity in lymph nodes and spleen as compared with controls [PBS or the IgG4-Fc protein fragment lacking the FLT3 ligand moiety (referred to as IgG4; Fig. 1B: Supplementary Fig. S1Ai)]. As DCs resident in lymphoid organs are of major relevance for the mechanism of action of intranodally administered RNA, we analyzed these cells in more detail. Subpopulations of cDCs and pDCs were profoundly expanded in both compartments of FLT3 ligand–treated mice as compared with controls (Fig. 1C; Supplementary Fig. S1B). As revealed in lymph node sections, pDCs accumulated in the T-cell zones (Fig. 1D). Noteworthy, neither lymph node cDCs nor pDCs of FLT3 ligand–treated mice showed molecular signs of maturation (Fig. 1E).

As described recently (32, 33), CD3^−^NK1.1^−^ NK cells were significantly expanded in lymph nodes and spleen of mice treated with FLT3 ligand (Fig. 1D and F) and were most prominent around day 7 in blood (Supplementary Fig. S2). In line with reports on FLT3 ligand application in rats (34), we found no expansion of macrophages in mice (Fig. 1D).
FLT3 ligand and intranodal RNA vaccination synergize
in inducing a Th1 lymph node milieu and in activation
of DCs and NK cells

Next, we investigated the fate and effects of intranodally
injected RNA when combined with FLT3 ligand as adjuvant.
We found that Cy5-labeled HA-RNA injected into lymph nodes
of mice preconditioned with 2 treatment cycles of FLT3 ligand
was internalized by both pDCs and cDCs with high efficiency
(Fig. 2A). Moreover, as shown by in vivo BLI, translation of Luc-
RNA injected into lymph nodes of these mice was comparable
with that of control mice (Fig. 2B).

Most interestingly, when FLT3 ligand–treated mice re-
ceived RNA intranodally, maturation of pDCs was induced as
documented by increase of markers CD80, CD86, CD40, and
MHC II (Fig. 2C). DCs started secretion of high levels of inter-
leukin (IL)-12 (Fig. 2D, left). Most interestingly, pDCs were
preferentially expanded and activated by RNA so that their
absolute number per lymph node surpassed that of cDCs
(Fig. 2D, right). Expression profiling of pDCs and cDCs from
lymph nodes by quantitative RT-PCR revealed profound
A: Flow cytometry analysis of DC subsets.

B: luciferase signal in different conditions.

C: Expression levels of CD86, MHC II, CD80, and CD40.

D: IL-12 and IL-12 DCLN expression in different conditions.

E: Fold change in chemokine expression.

F: NK cell count and percentage.

G: CD3 and NK1.1 expression.
FLT3 Ligand Enhances Therapeutic Potency of RNA Vaccine

alteration of the lymph node microenvironment of mice receiving the combination of FLT3 ligand and RNA as compared with mice receiving FLT3 ligand alone (Fig. 2E). NK cells and Th1 lymphocytes attracting chemokines CXCL10 (IP10) and CXCL9 (35, 36) were induced up to 2 logs. IL-6, which supports expansion and survival of primed T cells (37) and CD40 receptor, a key mediator of DC activation by CD4+ T cells, were upregulated, the latter particularly in pDCs. DC recruiting chemokines CCL3 (MIP-1α) and CCL4 (38) were modestly elevated, whereas IFN-α expression did not change.

Intranasal administration of naked RNA to FLT3 ligand–treated mice also had a major impact on NK cells. The frequency and absolute number of lymph node NK cells (Fig. 2F) as well as their intracellular IFN-γ levels (Supplemental Fig. S3A) increased significantly and they exhibited an activated CD69+ phenotype (Fig. 2G). Moreover, absolute number and frequency of lymph node NK cells secreting high amounts of IFN-γ, when treated with PMA in vitro, were clearly higher in RNA treated group (Supplemental Fig. S3B), showing enhanced susceptibility of NK cells for inflammatory stimuli.

These findings underline that the combination of FLT3 ligand and intranasal RNA administration induces an inflammatory Th1 milieu, favorable for the efficient priming and expansion of antigen-specific T cells, and leads to functional activation of both antigen presenters as well as NK cells, thus integrating all factors required for an efficient immune response.

pDCs are essential for augmentation of RNA-induced antigen-specific T-cell response by FLT3 ligand preconditioning

We then characterized T-cell responses induced by combining FLT3 ligand with intranasal RNA vaccination. Mice received FLT3 ligand at day 0 and day 3 followed by intranasal SIINFEKL-RNA immunization at day 7 and day 10. Frequencies of SIINFEKL-specific CD8+ T cells measured 5 days after the last immunization were profoundly increased in blood, spleen, and lymph nodes of FLT3 ligand preconditioned mice (Fig. 3A), and these cells secreted IFN-γ upon antigen encounter (Supplemental Fig. S4). To determine the relevance of pDCs for the observed adjuvant effect, pDC-depleting antibody anti-PDCA1 was administered to mice preconditioned with FLT3 ligand prior to immunization with SIINFEKL-RNA, resulting in reduction of the pDC subpopulation in the lymph node to half of normal levels and nearly complete abrogation in the spleen (Fig. 3B), whereas cDC and NK cell numbers were not altered (data not shown). Frequencies of SIINFEKL-specific CD8+ T cells achieved by the combination of FLT3 ligand and intranasal SIINFEKL-RNA administration were significantly lower in pDC-depleted mice than in mice with normal pDC frequencies (Fig. 3C). pDC-depleted mice treated with FLT3 ligand and SIINFEKL-RNA had T-cell frequencies comparable with those of mice that were immunized with SIINFEKL-RNA alone, suggesting that the increment in T-cell response achieved by FLT3 ligand preconditioning is significantly mediated by pDCs.

To elucidate the mechanism by which pDCs mediate the FLT3 ligand adjuvant effect, we first tested their capability to act as antigen presenters. BMDCs were differentiated in vitro in FLT3 ligand–supplemented medium that resulted in a mixed population of cDCs and pDCs (FLT3-L-BMDCs). FLT3-L-BMDCs were transfected with SIINFEKL- or control-RNA, and SIINFEKL peptide/MHC molecule complexes were quantified with the 25-D1.16 antibody (Fig. 3D). Most interestingly, we found efficient processing and presentation of the peptide by pDCs (factor 3.0 vs. 3.7 as compared with cDCs). When these peptide-presenting cDCs and pDCs were sorted and tested in ELISPOT for recognition by TCR transgenic OT-I CD8+ T cells (Fig. 3E), pDCs strongly induced IFN-γ secretion, indicating their capability to act as efficient antigen-presenting cells.

To investigate whether IFN-α secretion, one of the most prominent constitutive functions of pDCs, plays a role in the adjuvant effect of FLT3 ligand, we treated IFNAR−/− mice with FLT3 ligand or PBS as control followed by vaccination with SIINFEKL-RNA. The increased frequency of SIINFEKL-specific CD8+ T cells in FLT3 ligand–treated mice compared with control mice showed that IFN-α secretion is not key for the FLT3 ligand adjuvant effect (Fig. 3F).

FLT3 ligand improves antitumoral therapeutic immunity mediated by intranasal RNA vaccination

Next, we wanted to know whether the profound therapeuic benefit of intranasal RNA immunization in animal...
models can be topped by the addition of FLT3 ligand. For an advanced B16-OVA melanoma model, tumor cells were inoculated (day 0) and mice were conditioned with 4 administrations of FLT3 ligand. Tumors were grown to macroscopic visibility, and 4 cycles of SIINFEKL-RNA vaccination were initiated 11 days after tumor inoculation (Fig. 4A). As single agents, intranodal RNA immunization cured only 30% of mice and FLT3 ligand alone had a very moderate effect on survival. By combining the RNA vaccine and FLT3 ligand, however, 70% of the treated mice were cured from their cancer and survived or became tumor-free. When we characterized the TICs in the treatment groups, we found significant differences. Although FLT3 ligand as single agent did not alter the composition of immune cell infiltrations in the tumor nodule, intranodal administration of SIINFEKL-RNA alone expanded nearly all tested subpopulations, in particular of pDCs, cDCs, NK cells, T cells, MDSCs, and Tregs (Fig. 4B). The combination of both agents had a minor incremental effect on cellularity as compared with RNA alone. Interestingly, the percentage of antigen-specific CD8+ T cells was profoundly increased (Fig. 4B). As a consequence, in animals treated with the combination, Tregs were outnumbered by cytotoxic antigen-specific CD8+ effector T cells (Fig. 4C).

In summary, these data imply that FLT3 ligand preconditioning further improves the survival benefit achieved by intranodal vaccination with antigen-encoding RNA and that this is associated with an increase of antigen-specific cytotoxic T cells infiltrating the tumor lesion.

Discussion

The use of antigen-encoding RNA for cancer vaccination confers several advantages. These can be fully exploited when RNA is administered intranodally and unfolds its dual potential as a template for efficient translation as well as immunostimulatory ligand for Toll-like receptors (TLR).

This study was undertaken to evaluate FLT3 ligand as a systemic adjuvant in conjunction with intranodal RNA vaccination. This report shows for the first time that FLT3 ligand potentiates immunogenicity of intranodal RNA vaccination. We reveal that several mechanisms contribute to this synergy. First, FLT3 ligand expands DC and NK populations in different compartments including the lymph node and thus mobilizes beforehand a higher number of antigen presenters and effectors to the site the antigen will be delivered to. Our data also convey a better understanding of adjuvant features of FLT3 ligand. It is known that daily FLT3 ligand injections alter cellular composition in blood and secondary lymphatic organs of mice including induction of a marked DC hyperplasia (16–18). In contrast to the reported regimens based usually on administering 10 to 20 μg FLT3 ligand for 7 to 9 days (18, 43), we achieved even superior antitumoral effects in vivo with only 2 injections of 10 μg (day 0 and day 3) most likely owing to the high cooperative complementarity of naked RNA and FLT3 ligand. Most interestingly, among the different vaccine formats with which FLT3 ligand have been combined in the past, DNA vaccines have yielded the best results (27, 44, 45). Neither uptake mechanisms of DNA
Figure 3. FLT3 ligand acts as adjuvant for intranodal RNA vaccination via its effect on pDCs. A, C57BL/6 mice (n = 4–5) received either Flt3L-IgG4 (10 μg; day 0 and day 3) or PBS (control) intraperitoneally, followed by 2 intranodal immunizations (day 7 and day 10) with SIINFEKL-RNA (20 μg) in several independent experiments. SIINFEKL-specific CD8+ T cells were quantified in different compartments by tetramer staining carried out ex vivo (left). Bar chart shows data (mean ± SEM) from 2 independent experiments. Representative dot plots show the percentage of SIINFEKL-specific CD8+ T cells in all CD8+ cells 5 days after the last immunization (right). B and C, mice were treated as described above. After Flt3L-IgG4 preconditioning, mice received anti-PDCA1 antibody (500 μg i.p.) or a control antibody (rat-IgG1) 1 day prior to each immunization at day 6 and day 9. B, dot plots provide percentages of pDCs within all CD11c+ DCs in lymph nodes and spleen. C, percentages (mean ± SEM) of SIINFEKL-specific T cells within the CD8+ population in different treatment groups were determined by tetramer staining. D, BMDCs generated with Flt3L-IgG4 (Flt3L-BMDCs) were electroporated with either SIINFEKL-RNA (30 μg) or HA-RNA (30 μg) as control together with eGFP-RNA (10 μg). Surface densities of SIINFEKL/H2-Kb complexes on eGFP+ cDCs and pDCs were assessed after 18 hours by flow cytometry with the 25-D1.16 antibody. Numbers in parentheses represent mean fluorescence intensity of 25-D1.16. Data are representative for 2 independent experiments. E, Flt3L-BMDCs electroporated with either SIINFEKL-RNA (30 μg) or HA-RNA (30 μg) as control were sorted into cDCs and pDCs (1 × 10^4), which were then coincubated with CD8+ OT-I T cells (1 × 10^5) and SIINFEKL-specific CD8 T-cell response (mean ± SEM) was analyzed in an IFN-γ ELISPOT assay 18 hours later. F, IFNAR−/− mice (n = 4) received either Flt3L-IgG4 (10 μg; day 0 and day 3) or PBS (control) intraperitoneally, followed by 2 intranodal immunizations (day 7 and day 10) with SIINFEKL-RNA (20 μg). Percentages (mean ± SEM) of SIINFEKL-specific CD8+ T cells were quantified in blood by tetramer staining 5 days after the last immunization. *, P < 0.05; ***, P < 0.001 [Student’s t test (A, E, and F); ANOVA with Tukey’s multiple comparison test (C)].
Intranodal vaccination with naked antigen-encoding RNA elicits pol- 

tational efficiency of RNA vaccines in immature dendritic cells and 

vaccines nor the reason for synergizing with FLT3 ligand is 

known, but it is well conceivable that our findings for RNA 

may apply for nucleic acids in general.

Safety and tolerability of FLT3 ligand have been shown in 

healthy human donors (46, 47) and cancer patients (48–51).

Two clinical trials in cancer patients combined peptide 

vaccination with FLT3 ligand as adjuvant but failed to 

augment antigen-specific T-cell immunity, which was 

attributed to the nonactivated state of FLT3 ligand–mobilized 

DCs (50, 52). By addition of the TLR7 ligand imiquimod to 

a peptide vaccine plus FLT3 ligand, the lack of peptide-specific 

T-cell responses could be overcome in a melanoma trial (53).

Similar data have been obtained when DNA vaccines were 

combined with FLT3 ligand (44). Future studies have to 

show whether the property of RNA to trigger TLR signaling 

pathways in conjunction with the adjuvant effect of FLT3 

ligand may further improve vaccine potency in the clinical 

setting as well.

In summary, these unexpected findings not only are of high 

relevance for design of future RNA-based cancer vaccine 

protocols but also contribute to a better conceptual under-

standing of adjuvant strategies.

Disclosure of Potential Conflicts of Interest

U. Sahin (founder and chief executive officer) and C. Huber (founder) are 

associated with BioNTech AG (Mainz, Germany), a company that 
develops RNA-based cancer vaccines in the indicated functions. U. Sahin, S. 
Kreiter, O. Türeci, and A. Selmi are inventors on a patent application, in which 
parts of this article are covered. The other authors disclosed no potential 
conflicts of interest.

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