Modification of antigen encoding RNA increases stability, translational efficacy and T-cell stimulatory capacity of dendritic cells

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Abstract

Adoptive transfer of dendritic cells (DCs) transfected with \textit{in vitro} transcribed RNA encoding tumor-associated antigens has recently entered clinical testing as a promising approach for cancer immunotherapy. However, pharmacokinetic exploration of RNA as potential drug compound as a key aspect of clinical development is still pending. Investigating the impact of different structural modifications of RNA molecules on the kinetics of the encoded protein in DCs, we have identified components located 3’ of the coding region, which contribute to a higher transcript stability and translational efficiency. Using quantitative RT-PCR and eGFP variants to measure transcript amounts and protein yield, we show that (i) a poly(A) tail of 120 nucleotides length as compared to a shorter one, (ii) an unmasked poly(A) tail with a free 3’ end rather than one extended with unrelated nucleotides and (iii) two sequential β-globin 3’ untranslated regions cloned head-to-tail between the coding region and the poly(A) tail, independently enhance RNA stability and translational efficiency. Consecutively, the density of antigen-specific peptide/MHC complexes on the transfected cells as well as their potency to stimulate and expand antigen-specific CD4+ as well as CD8+ T-cells are increased as well. In summary, our data provides a strategy for optimization of RNA transfected DC vaccines and a basis for defining release criteria for such vaccine preparations.

Introduction

Antigen encoding RNA \textsuperscript{1,2} has the advantages of a genetic vaccine (e.g. delivery of all epitopes of the whole antigen, easy manufacturing and standardized purification) as well as the added value of a safe pharmaceutical characterized by transient expression and lack of integration into the genome of the treated host. The combination of this versatile antigen delivery molecule with DCs as most potent antigen presenting cells is regarded as an attractive approach to induce cellular and potentially therapeutic immune responses in individuals suffering from cancer. A number of reports demonstrated convincingly that the use of RNA results in efficient induction of antigen-specific immune responses \textit{in vitro} as well as in animal models \textsuperscript{1,3-7} and paved the way for trials in humans. Early clinical trials showed feasibility, lack of toxicity and promising efficacy based on immunological and clinical read-outs \textsuperscript{8-12} and encouraged further work in this field.
Having reached this early phase of clinical development, antigen-specific RNA has the status of a drug compound requiring detailed exploration. Basic pharmacological issues which need to be addressed in drug development include the pharmacokinetics of the compound of interest within the system of its physiological activity after administration. In case of the quoted clinical trials this system would be represented by either immature or mature DCs. A key objective of such investigations is a better understanding of the impact the structural features of the drug formulation have on its pharmacological properties. Neither of these questions has so far been addressed for antigen encoding RNA.

*In vitro* transcribed RNA generated from plasmid templates and used in such studies typically consists of a 5’ cap structure followed by the sequence encoding the antigen of interest and a poly(A) tail of 30 to 70 nucleotides length \(^4,5\). In some reports, the poly(A) tail is attached enzymatically by terminal polyadenylation instead of being encoded in the template \(^13\). The gene of interest is either used with its autochthonous non-coding regions \(^12\) or flanked by the untranslated regions (UTR) of the human or *Xenopus laevis* \(\beta\)-globin gene \(^14\). The effects of each of these structural features on the intracellular bioavailability of the RNA molecule have not been systematically investigated. In fact, surprisingly little is known about the time-dose curve and the translational efficiency of *in vitro* transcribed (IVT) antigen-encoding RNA after its electroporation into DCs. Likewise, a dose-response relationship between the levels of antigen encoding RNA within the cell and peptide/MHC molecules on the cell surface has not been established. Thus, the extent to which the density of peptide/MHC complexes on the cell surface can be influenced by augmenting RNA stability remains unclear.

Our objective was to characterize and improve the pharmacokinetic properties of IVT RNA as a tool for antigen delivery into DCs. To our knowledge this is the first study which determines structural modifications increasing the stability and translational efficiency of RNA molecules in DCs. We show that this optimization improves both surface presentation of epitopes and induction of T-cell responses. We therefore expect that implementation of modified RNAs into clinical vaccine constructs will contribute to an improved outcome.
Materials & Methods

Cells and cell lines

OT-I CD8+ T-cells transgenic for the T-cell receptor (TCR) and recognizing the Kb specific peptide SIINFEKL from chicken OVA (OVA257–264) were kindly provided by H. Schild (Institute for Immunology, University of Mainz). All animal experiments complied with the guidelines set by the Johannes-Gutenberg University of Mainz.

Human monocytes were enriched with anti-CD14 microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) from peripheral blood mononuclear cells (PBMC) of healthy blood bank donors. Immature DCs (iDC) were differentiated from these by culture in RPMI 1640 with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM Sodium pyruvate, non-essential amino acids and 10% heat inactivated human AB-Serum (all Invitrogen, Karlsruhe, Germany) supplemented with 1000 U/ml GM-CSF (Essex, Luzern, Switzerland) and 1000 U/ml IL4 (Strathmann Biotech, Hamburg, Germany). Maturation of human DCs (mDC) was induced by culturing for two days with 500 U/ml IL4, 800 U/ml GM-CSF, 10 ng/ml IL1β (Pharmingen, Hamburg, Germany) 10 ng/ml TNFα (Sigma, Taufkirchen, Germany), 1000 U/ml IL6 (Strathmann Biotech, Hamburg, Germany), 1 µg/ml PG-E2 (Sigma, Taufkirchen, Germany). Immature DCs were typically MHC IIpos, CD80low CD86low, CD83neg and CD14neg and after maturation MHC IIhigh, CD80high CD86high, CD83pos and CD14neg. Murine bone marrow derived DCs (BMDC) for in vitro or in vivo stimulation were generated as described by Lutz et al. 15 Briefly, bulk cells obtained from bone cavities of C57BL/J6 mice were cultured for 7 days in medium supplemented with 200 U/ml rmGM-CSF (Peprotech/Tebu, Frankfurt, Germany). At day 7 cells were CD11cpos, MHC-IIpos, CD80low, CD86low, Dec205neg. These cells were used for quantification of peptide/MHC complexes. For in vivo expansion of T-cells, BMDCs were matured after electroporation for 16h with poly(I:C) (50 µg/ml; Amersham Bioscience, Freiburg, Germany).

Vectors for in vitro transcription (IVT)

A list of all vectors is provided in Fig. 1.

A series of vectors for the in vitro transcription of polyadenylated RNA molecules under the control of the SP6-promoter were derived from a modified pGEM3Z-Vektor (Promega, Madison, USA) into which we had cloned a 120 bp poly(A) tail (pGEM3Z-A(120). To
engineer the pGEM3Z-β-globinUTR-A(120) series, the 3’ untranslated region (UTR) of the β-globin molecule flanked by Xho1/Sal1 restriction enzyme sites was amplified from human bone marrow (3’ β-globinUTR-sense: 5’-tta gtc gac gca gca atg ata aat gtt ttt tat tag gca-3’, 3’ β-globinUTR-antisense: 5’-tta ctc gag agc tcg ctt tct tgt cca att tac tc-3’) and a single (pGEM3Z-1β-globinUTR-A(120)) or two serial fragments (pGEM3Z-2β-globinUTR-A(120)) were inserted in front of the poly(A) tail.

All vector backbones were equipped with the open reading frame of the reporter molecule eGFP amplified from the peGFP-C1 vector (Becton-Dickinson, Heidelberg, Germany) using specific primers (eGFP-sense: 5’-gga tcc acc atg gtg agc aag ggc gag gag-3’, eGFP-stop-antisense: 5’-gga tcc tta ctt gta cag ctc gtc cat gcc g-3’). Vectors carrying the d2 variant of eGFP, which owing to a PEST domain has a shorter half-life, were obtained by substituting the eGFP-fragment in the respective construct with a BamH1/Xho1-site-flanked d2eGFP-fragment amplified from pTRE-d2GFP (BD Bioscience, Heidelberg, Germany) with specific primers (d2GFP-sense: 5’-gag gga tcc acc atg gtg agc aag ggc gag ggg ctg-3’, d2GFP-stop-antisense: 5’-gag ctc gag gaa ttc cta cac att gat cct agc aga -3’).

The pST1-2β-globinUTR–A(120) construct obtained by introduction of a T7-promoter, two serial human 3’ β-globinUTR, poly(A) tails of different length and the neomycin-resistance gene into the pCMV-Script-Vector (Stratagene, LaJolla, USA) served as ancestor for a second series of vectors allowing in vitro transcription of polyadenylated RNAs under the control of the T7-promoter.

To introduce sites for linearization with Sap1 (pST1-2β-globinUTR-A(120)) and Bpi1 (pGEM3Z-A(120)), fragments encoding consensus sequences for these enzymes on the complementary strand were synthesized (GENEART, Regensburg, Germany) and inserted 3’ to the poly(A) tail. Bpi1 and Sap1 are type IIS restriction enzymes, which in contrast to the broadly used type II endonucleases cleave staggered to rather than within their specific recognition site. Also into pST1-A(120) and its variants eGFP and d2eGFP were inserted.

To obtain pST1-Sec-SIINFEKL vector a BamH1-site-flanked fragment representing base pairs 766-801 (aa 255-266) of the ovalbumin sequence encoding the immunodominant peptide epitope was synthesized by GENEART and cloned into the backbone. In addition a 78 bp signal peptide derived from a MHC class I molecule (Sec) was amplified from activated PBMC (primers: Sec-sense 5’-aag ctt agc ggc cgc acc atg cgg gtc aeg ggc ccc cga acc-3’, Sec-antisense 5’-ctg cag gga ggc ggc cca ggt ctc ggt cag-3’) and inserted in front of the SIINFEKL-sequence. The pST1-Sec-HCMV pp65 vector was generated accordingly by ligation of a PCR product obtained by amplification of the pp65 (UL83) ORF from a lysate of
HCMV-infected fibroblasts (BioWhitaker, Walkersville, USA) using specific oligonucleotides (pp65-sense 5’-gga tcc acc atg gag tgc cgc cgt tgt ccc gaa atg-3’, pp65-Stop-antisense 5’-gga tcc tca acc tgg gcg tcg agg cga tgc-3’).

A cDNA fragment amplified from human testis mRNA coding for NY-ESO-I using NY-ESO-sense 5’-gga tcc gcc acc atg cag gcc gaa ggc cgg ggc aca-3’ and NY-ESO-antisense 5’-gga tcc gcc cct ctg ccc tga ggg agg-3’ oligonucleotides was cloned into the pST1-vector and used for generation of control RNA.

**Generation of IVT RNA**

To generate templates for *in vitro* transcription (IVT), plasmids were linearized downstream of the poly(A) tract and purified by phenol/chloroform extraction and sodium acetate precipitation. Linearized vector DNAs were quantified spectrophotometrically and subjected to *in vitro* transcription using commercial kits according to the manufacturer’s instructions. *In vitro* transcription of pST1-A120 based plasmids was carried out with the mMessage mMachine T7 ultra kit (Ambion, Austin, TX), which attaches the ARCA (7-methyl(3’-0-methyl)GpppG) cap analog m7G(5’)ppp(5’)G in an ultra high-yield transcription reaction. The pGEM3Z-A(120) based plasmids were capped with 7-methylGpppG using the mMessage mMachine SP6 Kit (Ambion Austin, TX) and. For some experiments vectors were linearized upstream of the plasmid encoded poly(A) tail and after *in vitro* transcription RNA was enzymatically polyadenylated using the poly(A) tail kit (Ambion, Austin, TX). Capped polyadenylated RNA was purified using MEGAclear™ Kit (Ambion, Austin, TX).

**Transfer of IVT RNA into cells**

Cells were suspended in 250 µl X-VIVO 15 medium (Cambrex, Verviers, Belgium) and transferred into a 4 mm gap sterile electroporation cuvette (BioRad, Hercules, USA). After addition of the appropriate amount of IVT RNA, cells were electroporated using a GenePulser-II apparatus (Biorad, Munich, Germany) applying the voltage and capacitance settings established for each cell type (K562: 200 V/300 µF, human iDC and mDC: 290 V/150 µF, murine BMDC: 276 V/150 µF, EL4: 300 V/150 µF). Cells were diluted in culture medium immediately after electroporation.
Quantification of eGFP transcript levels by real-time RT-PCR

Total cellular RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany), reverse transcribed with hexamer primers using Superscript II (Invitrogen, Carlsbad, California) and subjected to real-time quantitative analysis on ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems) with QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany). Reactions were performed for 40 cycles in triplicates with specific primers amplifying both eGFP and d2eGFP (eGFP-sense: 5’-cac atg aag cag cac gac ttc-3'; eGFP-antisense: 5’-cac ctt gat gc g gtt ctt ctg-3’, each 300 nM) with initial denaturation/activation for 15 minutes at 95 °C, 30 seconds at 94 °C, 30 seconds at 62 °C, and 30 seconds at 72 °C.

Expression of eGFP transcripts was quantified relative to 18sRNA as internal standard to normalize for variances in the quality of RNA and the amount of input cDNA.

Flow cytometric analysis

For immunophenotyping of immature and mature dendritic cells (DCs), antibodies were used with nonreactive isotype matched controls (BD Biosciences, Heidelberg, Germany).

For quantification of eGFP or d2eGFP protein, cells were washed in PBS and incubated with propidium iodide (10 µg/ml) prior to flow cytometric analysis. For eGFP analysis in DC cultures, gating was performed on cells exhibiting a large forward (FSC) and side scatter (SSC) for exclusion of contaminating lymphocytes. Propidium iodide negative cells were gated and the mean fluorescence intensity (MFI) expression was determined.

For quantification of the SIINFEKL peptide presented on the H-2 K\textsuperscript{b} molecule, mouse thymoma EL4 cells or mature bone marrow derived dendritic cells (BMDC) from mice were harvested, washed three times with cold buffer (PBS-1% FCS), blocked with PBS-10% FCS, stained for 30 min at 4 °C with 25.D1.16 antibody which specifically detects OVA\textsubscript{257-264} peptide SIINFEKL in conjunction with H-2 K\textsuperscript{b} molecules \textsuperscript{16}, and thereafter stained with goat anti-mouse-APC secondary antibody (JacksonImmuno Research, West Grove, USA). For generation of a standard curve for conversion of MFI values to equivalent molar concentrations of SIINFEKL peptide loaded on cells, the respective cells were pulsed for 2h and 37 °C with titrated amounts of SIINFEKL peptide (Biosyntan, Berlin, Germany; 2 pM to 30 µM, 3 fold dilutions).

Flow cytometric analysis was performed on a FACS-Calibur analytical flow cytometer (BD Biosciences, Heidelberg, Germany) using Cellquest software.
**In vivo expansion of murine T-cells**

For the assessment of antigen-specific *in vivo* expansion, splenocytes from TCR tg OT-I mice were prepared and adoptively transferred at day 0 into the tail vein of recipient C57BL/J6 mice. The cell number was adjusted to 1x10^5 TCR tg CD8^+ T-cells. For immunizations BMDC were electroporated with 50 pMol of RNA and then activated by poly(I:C) for 16 h. 1x10^6 BMDC were administered at day 1 by intraperitoneal (i.p.) injection. Retro-orbital blood samples were collected at day 4 and stained with anti-CD8 (Caltag Laboratories, Burlingame, USA) and SIINFEKL tetramer (H-2Kb/SIINFEKL 257-264; Beckman Coulter, Fullerton, USA).

**In vitro expansion of human T-cells**

CD4^+ as well as CD8^+ T cells were isolated from PBMC of HCMV-seropositive healthy donors by positive magnetic cell sorting with antibody-coated microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). 2x10^5 autologous DCs were either electroporated with IVT RNA or pulsed with peptide pools, washed and cocultured with 1x10^6 CD4^+ or CD8^+ effector T-cells in complete medium supplemented with 10% AB-Serum, 10 U/ml IL2 (R&D, Minneapolis, USA) and 5 ng/ml IL7 (R&D, Minneapolis, USA) in 24-well plates (BD Biosciences, Heidelberg, Germany). ELISpot-assays were performed 7 days after initiation of stimulation.

**Enzyme-linked immunospot assay (ELISpot)**

96-well microtiter plates (Millipore, Bedford, USA) were coated overnight at 4 °C with 0,1 µg/ml anti-IFN-γ antibody 1-D1k (Mabtech, Stockholm, Sweden), washed four times with PBS and blocked with 2% human albumin. Autologous DC used as stimulator cells were plated in triplicates (2x10^4 target cells/well) after loading with 1,75 µg/ml pp65 or NY-ESO-I peptide pools. 15mer peptides with 11 amino acid overlaps covering the whole HCMV pp65 sequence and an analogous peptide pool derived from the NY-ESO-I antigen (both from Jerini, Berlin, Germany) as negative control were used. CD4^+ or CD8^+ responder cells from the T-cell expansion culture were added at a concentration of 1-3x10^4 cells/well. The plates were incubated overnight (37 °C, 5% CO_2), washed with PBS 0,05% Tween 20, and
incubated for 2h with the anti IFN-γ biotinylated mAB 7-B6-1 (Mabtech, Stockholm, Sweden) at a final concentration of 1 µg/ml at 37 °C. After washing in PBS containing 0,05% Tween 20, avidin-bound horseradish peroxidase H (Vectastain Elite Kit; Vector Laboratories, Burlingame, USA) was added and incubated for 1h at room temperature. Plates were developed after final wash with PBS containing 0,05% Tween 20 using 3-amino-9-ethyl carbazole (Sigma, Taufkirchen, Germany). Spots were counted using a computer-based evaluation system and the KS-ELISpot software version 4.4.35 (Carl Zeiss Vision, Eching, Germany).

Figure 1
Results

Density of peptide/MHC complexes on the cell surface correlates with the dose of antigen-encoding RNA electroporated into cells

To investigate the dose-response relationship between the levels of antigen-encoding RNA transferred into the cell and peptide/MHC molecules on the cell surface, we took advantage of an antibody allowing flow cytometric quantification of antigen-specific peptide/MHC complexes (H2-K\(^b\)-OVA\(_{257-264}\))\(^{17}\). Increasing amounts of RNA transcribed \textit{in vitro} from the linearized Sec-SIINFEKL-A67-Spe1 vector and encoding the OVA\(_{257-264}\) peptide SIINFEKL were electroporated into H2-K\(^b\) expressing EL4 tumor cells and analyzed for surface expression kinetics of specific peptide/MHC complexes. Even after electroporation of RNA concentrations as low as 10 pMol, surface expression of peptide/MHC molecules was easily detected at 12h after transfection, declined over time and was below detection level 24h after RNA transfer. (Fig. 2). Density and persistence of peptide/MHC complexes correlated with the amount of RNA transferred into the cells.

Figure 2

![Graph showing peptide concentration over time for different RNA concentrations.](image-url)
Enzymatic polyadenylation of RNA results in a mixture of transcripts varying in length

Poly(A) tailing of RNA can be either accomplished by enzymatic polyadenylation after linearization of the plasmid template 13 or a poly(A) stretch is cloned into the template vector. We observed that the length of an enzymatically synthesized poly(A) tail highly depends on the reaction conditions. Variations in the quantity of poly(A) polymerase (Fig. 3A) as well as in the incubation time (data not shown) lead to profound differences in poly(A) length. Since many parameters influence poly(A) tail length during the polyadenylation reaction, the number of attached ATP nucleotides cannot be precisely controlled even if the same reaction conditions are applied. Accordingly, enzymatically polyadenylated RNA samples generated in independent production runs are not directly comparable. In addition to this variability between different preparations, each individual preparation appears to contain a mixture of RNA species differing in the length of their poly(A) tail. This is documented by the fuzzy signal observed in Bioanalyzer™ electropherograms and gel images of enzymatically tailed RNA (Fig. 3B). In contrast, IVT RNA with a template encoded poly(A) tail appears as a steep peak and shows a sharp band in the gel image (Fig. 3C). In summary, template-encoded poly(A) tails are clearly superior since they reproducibly provide a well-defined product of sufficient purity.
Figure 3

A

B Enzymatic polyadenylation

C Template encoded poly(A) tail

12
An overhang at the 3’ end of the poly(A) tail hampers translational efficiency of IVT RNA

Vectors encoding the antigen of interest together with a poly(A) tail have to be linearized down-stream to the poly(A) stretch prior to in vitro transcription. This is typically achieved by using a type II restriction enzyme that cuts the vector backbone 3’ of the poly(A) tract. Thereby, part of the consensus sequence of the restriction enzyme remains as overhang extending the poly(A) tail at the 3’ end (Fig. 4a, left). To investigate the impact of this on protein yield, we generated IVT RNA with a free-ending poly(A) tail taking advantage of type IIS restriction enzymes such as Sap1 and Bpi1. Whereas type II restriction endonucleases digest within their recognition site, type IIS enzymes cut several base pairs aside of it (Fig 4A). We replaced the Spe1 restriction enzyme site in the original vector encoding eGFP with a poly(A)$_{67}$ tail by introducing a Sap1 site on the complementary strand. Thus, the poly(A) tails of the two eGFP encoding RNA species we obtained, had either an additional 3’ extension (eGFP-A(67)ACUAG) or not (eGFP-A(67)).

For assessment of RNA stability, we used the standard technique of monitoring decay kinetics of the translation product in transiently transfected cells plotting the amount of the specific protein as a function of time. Protein decay kinetics is regarded as state-of-the-art tool which is favored over RNA kinetics, since it allows integrated recording of all relevant translational characteristics in the functionality oriented way required for the objective of our investigations.

Human immature DCs were electroporated with 50 pMol of both eGFP IVT RNA species and the kinetics were determined by flow cytometric quantification of eGFP protein. Linearization with Sap1 improved expression significantly as compared to restriction with Spe1 as the RNA with free ending poly(A) tail resulted in 1.5 fold higher maximum protein levels and prolonged detectability of the protein. Interestingly, visual inspection of the curves showed that the message stability (e.g. the length of time over which protein continued to accumulate) of both eGFP-A(67) and eGFP-A(67)ACUAG RNAs appeared to be comparable (Fig. 4B). Also functional RNA half-life (e.g. time needed to complete a 50% decay in the capacity of an RNA to synthesize protein, which is a more accurate parameter than physical half-life since it also considers translational competence of an RNA species) is comparable for eGFP-A(67) and eGFP-A(67)ACUAG RNAs. Apparently, the difference in protein yield can be accounted for by the higher translational efficiency (e.g. the slope of the curve) of RNA with a free poly(A) tail as compared to one with a 3’ extension.
The length of the poly(A) tail has an impact on the translational efficiency

To assess the impact of the poly(A) tail on protein yield, we used a series of green fluorescence protein variant encoding IVT RNAs differing merely in their poly(A) tail lengths and electroporated equimolar amounts of them into different cell types. eGFP is a protein with a relatively long physical half-life of 17.3h. We were wondering whether the stabilizing effect of the modification we introduced still applies for proteins with much shorter half-life and therefore included a recently described destabilized eGFP variant d2eGFP in several of the experiments. This variant has a half-life of 2h due to a C-terminal PEST region which promotes protein degradation.
As exemplified for immature as well as mature DCs by real-time RT PCR analysis, intracellular IVT RNA levels of d2eGFP variants at different time points after transfection were highest for poly(A)$_{120}$ tailed RNA, suggesting that RNAs with shorter poly(A) tails undergo faster degradation (Fig. 5A). Flow cytometric time course analysis of d2eGFP protein in the same batch of cells showed that this contributes to an increased and prolonged protein expression (Fig. 5B). Interestingly, both level and stability of d2eGFP RNA as well as protein amounts were higher in immature as compared to mature DCs. Moreover, in immature DCs decay rate at the onset of degradation appeared to be protracted (Fig. 5B).

As expected, with eGFP IVT RNA variants protein yield was higher than for d2eGFP RNA species (Fig. 5C). Importantly, however, the poly(A)$_{120}$ tailed RNA as compared to the shorter ones resulted in higher protein levels. Again protein levels were higher and decay was protracted in immature DCs as compared to mature ones with maximum protein levels sustained in steady state until up to 120h after electroporation (Fig. 5C).

Poly(A) tails longer than 120 bases did not have a significant effect on expression of eGFP or d2eGFP (data not shown).

**Figure 5**
Two 3’ UTRs of the human β-globin gene cloned in tandem improve protein level

In several preclinical studies the antigen of interest is flanked by the 3’ UTR of the human or *Xenopus laevis* β-globin gene. The impact of this modification has never been systematically investigated. We compared poly(A)120 tailed eGFP RNA without any UTR with RNA incorporating the human β-globin 3’ UTR. As a cloning artifact we also generated a plasmid template, which incorporated two sequential β-globin 3’ UTRs fused head-to-tail.
Interestingly, increase in protein yields in particular in immature dendritic cells was not as pronounced as expected when comparing RNA with one β-globin 3’ UTR to the tailed control RNA lacking a 3’ UTR (Fig. 5D). However, two reiterated β-globin 3’ UTR between the coding sequence and the poly(A) tail resulted in significantly higher maximum protein levels and prolonged persistence of the protein. Also for this structural modification, the translational characteristics influenced in immature DCs (primarily message stability and functional half-life) differed from those in mature DCs (primarily translation efficiency). Notably, inclusion of the human β-globin 5’ UTR did not have an additional beneficial effect on protein yield (data not shown).

Figure 5

The combination of the optimal poly(A) length, a free ending poly(A) tail and an optimized 3’ UTR act synergistically on RNA stability and translational efficiency

In summary, our data showed that a poly(A) tail of 120 nucleotides as compared to a shorter one, a poly(A) tail with a free 3’ end rather than one with an extension and two tandemly reiterated β-globin 3’ UTRs each improve the translational characteristics of RNA and result in higher and sustained protein expression. We combined these features in one RNA molecule (eGFP-2β-globinUTR-A(120)) and compared it to a standard IVT RNA containing a masked poly(A) tail of 67bp and lacking regulatory UTR (eGFP-A(67)ACUAG). Quantification of the transcript in immature DCs by real-time RT-PCR 48h after RNA transfer disclosed that combining these modifications significantly improves RNA stability by approximately two
orders of magnitude (Fig. 6A). Fluorescence microscopy of transfected mature DCs after 24h confirmed this differential stability on protein level (Fig. 6B).

In flow cytometric time kinetics with the short-lived d2eGFP variant we observed that the combination of the respective structural features results not only in higher protein levels, but is also sustained over a longer time and that this also applies for molecules with short half-life (Fig. 6C). Again prolonged stability was particularly prominent in immature dendritic cells (Fig. 6C), in which we observed significant MFIs even ~80h after electroporation.

Noteworthy, flow cytometric data presented in Fig 6C features three independent experiments conducted in parallel, showing nearly no standard deviation and proving high reproducibility of this procedure.
Figure 6

A

![Bar chart for relative transcript level comparison](image)

- eGFP-A67ACUAG
- eGFP-2βgUTR-A(120)

B

![Images of mDC with eGFP-A(67)ACUAG and eGFP-2βgUTR-A(120)](image)

C

![Graphs showing time course of MFI](image)

- d2a0FP-2βgUTR-A(120)
- d2a0FP-A(67)ACUAG
- RNA free control
Stabilized IVT RNA increases the number of antigen-specific peptide/MHC complexes on the cell surface and improves expansion of antigen-specific T-cells in vivo

Next we investigated, whether improved RNA stability and consecutively higher protein levels in antigen presenting cells translate into more efficient T-cell stimulation. We first quantified the effect on cell surface expression of peptide/MHC complexes. The SIINFEKL peptide was cloned into the vector representing all optimizations (pST1-Sec-SIINFEKL-2β-globinUTR-A(120)-Sap1) and into a vector with standard features (pST1-Sec-SIINFEKL-A(67)-Spe1). IVT RNAs derived from both vectors were electroporated into EL4 cells and BMDCs. We detected significantly higher numbers of OVA peptide/Kb complexes on the cell surface sustained over a longer time course upon electroporation of the genetically improved RNA Sec-SIINFEKL-2β-globinUTR-A(120) (Fig. 7A).

To assess the impact on stimulatory capacity, we resorted to the OT-I TCR, that has been used extensively on the C57BL/J6 (B6) background to detect MHC class I presentation of the SIINFEKL peptide. At day zero animals were adoptively transferred with OT-I CD8$^+$ T-cells. The following day BMDCs of C57BL/J6 mice electroporated with both SIINFEKL encoding RNA construct variants and matured for 16h were administered i.p. into mice. At day four, OT-I CD8$^+$ T-cells were measured in peripheral blood with tetramer technology. We found in vivo expansion of antigen-specific TCR transgenic CD8$^+$ T-cells to be significantly superior using Sec-SIINFEKL-2β-globinUTR-A(120) RNA for antigen delivery as compared to Sec-SIINFEKL-A(67)ACUAG RNA (Fig. 7B).

To assess whether stabilized IVT RNA constructs for antigen delivery improve antigen-specific stimulation of human T-cells as well, we resorted to HCMV pp65, the immunodominant antigen of human cytomegalovirus that is frequently used for the validation of autologous stimulation of polyepitopic T-cell responses. CD4$^+$ and CD8$^+$ T-cells purified from HCMV seropositive healthy donors were cocultured with autologous DCs electroporated with the respective IVT RNA variants encoding pp65. Expansion of T-cells measured on day 7 in an IFN-γ ELISpot using autologous DCs pulsed with a pool of overlapping peptides covering either the entire pp65 protein sequence or a control protein showed superiority of Sec-pp65-2β-globinUTR-A(120), with effects being most prominent for expansion of CD4$^+$ T-cells.
Figure 7

A

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<td>Sec-SIINFEKL-A(67)ACUAG</td>
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B

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C

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- Sec-pp65-2μgUTR-A(120)
- Sec-pp65-A(67)ACUAG
- pp65 peptide pool

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Discussion

The use of DCs transfected with antigen encoding IVT RNA in clinical applications provides an opportunity to address several of the shortcomings of current vaccination strategies at once. The success and the ultimate clinical utility of this approach will depend on the optimization of parameters contributing to the induction and efficient expansion of T-cell responses. Our aim was to refine translational characteristics of transiently expressed RNA in DCs and thus improve pharmacokinetics and consecutively T-cell stimulatory capacity of these engineered antigen presenting cells.

The mechanisms underlying the steady-state levels of naturally expressed mRNAs in the cytoplasm have received considerable attention over the last years, since the regulation of mRNA turnover plays a major role in the overall control of gene expression. mRNA stability and translation are controlled by a complex network of RNA/protein interactions and depend on the primary and secondary structures of mRNAs, their rates of translation and the initiation of one of the various degradation mechanisms. Sequence elements within the 3’ and 5’ non-coding regions of the RNA molecule have been characterized as important factors in regulating the stability of a transcript in ways, that are not completely understood. However, the vast majority of data stems from studies in yeast cell lysates or prokaryotic cells, whereas data in eukaryotic cell lines is less conclusive and seems to depend on the cell type used. For human DCs, this is the only study investigating systematically the influence of several 3’ elements on translational characteristics of transiently expressed RNA.

The poly(A) tail regulates the fate of mRNA by synergy with the 5’ cap, with the internal ribosomal entry site and with various RNA stability determinants. Antigen-encoding RNA constructs used currently for preclinical studies have poly(A) tail lengths of 30 to 70 nucleotides, whereas those administered in clinical trials have lengths of 64 nucleotides. According to our data, the pharmacokinetic properties of RNA in DCs can be further improved by a poly(A) tail of 120bp length. This impact of poly(A) tail lengths on the pharmacological properties of IVT RNA implicates the need for pure preparations with defined and uniform tail lengths of IVT RNA. As we show, this can be achieved by encoding the poly(A) tract in the template vector rather than attaching it by enzymatic polyadenylation. For linearization of such vectors down-stream of the poly(A) stretch prior to in vitro transcription, we propose type IIS endonucleases instead of the usually used type II restriction enzymes. This offers the advantage of cutting within the poly(A) stretch preventing
an overhang extending the poly(A) tail, which may compromise efficient protein synthesis of the encoded antigen. Vectors used currently for preclinical and clinical studies resort either to UTRs of β-globin or to autochthonous UTRs of the antigen they want to deliver, which are not further characterized in terms of their impact on RNA stability. According to our data, two serial 3’ UTRs of the β-globin gene upstream to the poly(A) tail as compared to one improve the translational characteristics of RNA. As previous reports show, the length of the 3’ UTR as such has an effect on the stability and translational efficiency of poly(A)-negative, but not of poly(A)-positive RNA for which a 27 bp 3’ UTR appears to be optimal. Therefore, we do not expect this effect to be mediated simply by the increase of the 3’ non-coding region. Systematic screening approaches for 3’ UTRs conferring even better translational characteristics may be worthwhile for further optimizing RNA vaccines.

Combining these structural components, we achieved not only a more than 5 fold increase of maximum protein concentration but also persistence of protein expression over a longer time. In principle, the improvement of RNA kinetics by these modifications is not restricted to DCs but is similar in K562 and may also be of interest for other cellular settings such as T-cells, B-cells or muscle cells, which are attractive host cells for expression of IVT RNA encoded proteins. Interestingly, we observed that stability and decay kinetics of the RNAs differ in immature and mature human DCs, most likely due to differences in the complex network of RNA/protein interactions in both cell types. Notably, revisiting our database of genome-wide expression profiles of immature and mature human DCs obtained with microarrays we found that factors involved in RNA stability are induced in the course of maturation (data not shown).

The mode of action of such a vaccine and its capacity to induce efficient T-cell responses depends on prolonged surface presentation of sufficient amounts of MHC-bound peptide epitopes derived from the RNA-encoded antigen. Previous studies reported that the density of MHC epitopes presented on the cell surface correlates with the amount of intracellular protein detected at a defined time point. However, it has also been reported that the production of MHC-associated epitopes and priming of cytotoxic T-lymphocytes is achieved even if the full-length protein encoded by the transferred RNA can not be detected in DCs at all. These observations are not contradictory but may be explained by the fact that in addition to steady-state protein levels, defective ribosomal products (DriPs) such as unfinished or misfolded proteins are very quickly processed into peptides and contribute significantly to MHC associated epitopes. The latter will obviously increase with the rate of de novo translation, which again is a function of stability and translational efficiency of the RNA
molecule. In line with this, with a SIINFEKL encoding RNA combining all structural improvements we described, more than 56 pMol of SIINFEKL/MHC complexes (having been reported as the amount required for induction of robust IFN-γ secretion of specific T-cells in this particular system \(^{17}\) are expressed on the antigen presenting cell up to 36h after electroporation. This is particularly interesting in light of migration kinetics of DCs from skin to regional lymph nodes. Mouse DCs start to enter the regional lymph node 18h after inoculation in the skin with reaching a maximum within 72h \(^{50,51}\). Similar time kinetics has been reported for intradermally injected DC in melanoma patients \(^{52,53}\). Thus, prolonging the duration of antigen presentation may have a significant effect on the stimulation of immune effectors in the lymph node.

Our findings also apply for expansion of human antigen-specific T-cells as exemplified by stimulation of sorted T-cell populations from CMV-seropositive donors with pp65 antigen. Antigen titration shows a higher impact of stabilized IVT RNA constructs on CD4\(^{+}\) as compared to CD8\(^{+}\) immune responses. The reason for this is most likely the preexistence of high-affinity CD8\(^{+}\) T-cells against immunodominant epitopes in the respective donor repertoires.

In summary, we confirm our starting assumption that optimization of structural features of antigen-encoding RNA increases density and stability of peptide/MHC complexes on the cell surface and translates into improved stimulation of CD4\(^{+}\) as well as CD8\(^{+}\) T-cells in murine and human systems. Moreover, our report underlines that one of the many advantages of formulating the antigen as IVT RNA is the added value provided by recombinant DNA technology of easily engineering elements into vaccine constructs that enhances their potency. We expect that strategies to manipulate RNA stability in genetically-engineered cells for control of heterologous protein expression will emerge into a promising future direction in pharmaceutical applications.

**Acknowledgements**

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Figure Legends

Figure 1. Vectors used for *in vitro* transcription and mRNAs derived from them. DNA templates coding for the marker proteins eGFP or d2eGFP but varying in (A) linearization site, (B) poly(A) tail length, and (C) the 3’ untranslated region (UTR) and (D) vectors encoding an ovalbumin-derived T-cell epitope and pp65 protein for investigation of antigen presentation and immune responses were transcribed *in vitro* in the presence of a cap analog to generate the mRNA species shown on the right.

Figure 2. Dose-response relationship between the levels of peptide-encoding RNA within the cell and peptide/MHC molecules on the cell surface. EL4 cells were electroporated (settings 300V/150µF) with different amounts of Sec-SIINFEKL-A(67)ACUAG RNA. Transfected cells harvested at different time points were stained with 25D1.16 antibody to determine surface SIINFEKL/Kb-complexes. Concentrations of SIINFEKL peptide were calculated from the mean fluorescence values of viable cells using a peptide titration as standard curve.

Figure 3. Comparison of enzymatically polyadenylated RNA with tailed IVT RNA transcribed from a template encoding the poly(A) tract. (A) Template IVT RNA prior to polyadenylation (lane 1) compared to IVT RNA products obtained in independent polyadenylation reactions with increasing concentrations of poly(A) polymerase (lane 2-5), resulting in increasing sizes of poly(A) tail lengths. (B) IVT RNA with an enzymatically attached poly(A) tail was purified using MEGAclear™ Kit (Ambion, Austin, TX) and analyzed on an Agilent 2100 Bioanalyzer™. The electropherograms (left) and gel images (right) disclose a mixture of RNA species differing in length. (C) Electropherograms (left) and gel images (right) of purified IVT RNA obtained from a plasmid template encoding the poly(A) tail show a well-defined homogenous RNA species.

Figure 4. Role of a free-ending poly(A) tail on translation efficiency. (A) Rationale for using a type IIS restriction enzyme (e.g. Sap1) instead of a type II restriction enzyme (e.g. Spe1) for linearization of the plasmid template prior to *in vitro* transcription. Type IIS restriction enzymes cut adjacent to rather than within their recognition site and prevent an overhang of nucleotides derived from the vector backbone and remaining as 3’ attachment at the poly(A) tail. (B) Impact of an unmasked free poly(A) tail on translational efficiency in dendritic cells. Immature dendritic cells were electroporated with eGFP-A(67) RNA, which
contains an unmasked poly(A) tail or with eGFP-A(67)ACUAG, in which 4 additional nucleotides are attached 3’ to the poly(A) tail. Electroporation without RNA or with RNase digested RNA served as controls. Cells were harvested at different time points (3h, 6h, 24h, 48h, 72h, 120h, 168h, 192h) and the eGFP fluorescence of viable cells was measured by flowcytometry.

**Figure 5. Impact of regulatory components located 3’ of the coding region on transcript stability and protein yield in dendritic cells and cell lines.**

(A) Influence of poly(A) tail length on transcript stability in iDC and mDC. Cells were electroporated with equal amounts of d2eGFP encoding IVT RNA species, which differ in the length of their poly(A) tails. Cells were harvested after 6h, 24h, 48h, 96h and eGFP transcript levels were quantified by real time RT-PCR. Cells electroporated without RNA served as controls. For each time point the transcript levels were shown relative to expression levels obtained for d2eGFP-2β-globinUTR-A(120) in iDCs. (B) Flowcytometric analysis of protein levels in the same cells used in experiment 5A, which were transfected with the short-lived d2eGFP variant as reporter molecule. (C) Cells were transfected with eGFP encoding IVT RNA variants differing in the lengths of their poly(A) tails. RNase digested RNA and untailed RNA served as controls. Cells were harvested after 6h, 24h, 48h, 72h, 96h, 144h, 192h and eGFP fluorescence of viable cells was measured by flowcytometry. (D) Influence of the 3’ untranslated region (UTR) on translational efficiency. Immature and mature dendritic cells were electroporated with eGFP RNA variants differing in their 3’ UTR. Cells were harvested after 6h, 24h, 48h, 72h, 96h, 144h, 168h or 218h and the eGFP fluorescence of viable cells was measured by flowcytometry.

**Figure 6. Improvement of RNA stability by combination of the optimized structural features.** (A) Immature dendritic cells were transfected with different eGFP variants featuring combinations of the improved structural characteristics. Cells were harvested 48h after transfection and the eGFP transcript level was assessed by real time RT-PCR. Cells electroporated with buffer or with RNase digested RNA were used as controls and reference. Transcript levels were shown relative to expression levels obtained for eGFP-2β-globinUTR-A(120). (B) Fluorescence microscopy of mature dendritic cells 24h after transfection with standard eGFP-A(67)ACUAG and optimized eGFP-2β-globinUTR-A(120) IVT RNA. To allow comparison, images were obtained using equal acquisition parameters. (C) Immature and mature dendritic cells were transfected with different IVT RNA constructs encoding for
the short lived d2eGFP variant. Mean fluorescence intensities of viable cells were determined at different time points after transfection in three independent experiments. Data for both cell types is shown as the mean values of 3 experiments ± SEM.

Figure 7. Impact of stabilized IVT RNA constructs on T-cell stimulation in vivo and in vitro. (A) Increase of antigen-specific peptide/MHC complexes by using stabilized IVT RNA constructs. Cells were electroporated with Sec-SIINFEKL-A(67)ACUAG RNA or Sec-SIINFEKL-2β-globinUTR-A(120) RNA (EL4 cells: 10 pMol, 50 pMol; C57Bl/J6 immature BMDC in triplicates: 150 pMol). Electroporation with buffer only was used as control. Cells were stained for SIINFEKL/Kb-complexes with 25D1.16 antibody. Concentrations of SIINFEKL peptide were calculated from the mean fluorescence values of viable cells using a peptide titration as standard curve. Data for BMDC is shown as the mean values of 3 experiments ± SEM. (B) Improved in vivo T-cell expansion by using stabilized IVT RNA constructs. 1x10^5 TCR transgenic CD8+ OT-I cells were adoptively transferred to C57Bl/J6 mice. BMDC of C57Bl/J6 mice were transfected with 50 pMol RNA (Sec-SIINFEKL-A(67)ACUAG, Sec-SIINFEKL-2β-globinUTR-A(120) or control RNA), matured for 16h with poly(I:C) (50 µg/ml) and injected i.p. one day after T-cell transfer (n=3). Peripheral blood was taken at day 4 and stained for SIINFEKL tetramer positive CD8+ T-cells. Dot plots show CD8+ T-cells and the numbers given represent the percentage of tetramer positive CD8+ T-cells. (C) Improved in vitro expansion of human T-cells with stabilized IVT RNA constructs. CD8+ and CD4+ lymphocytes from HCMV-seropositive healthy donors were cocultivated with autologous DC transfected with Sec-pp65-A(67)ACUAG RNA or Sec-pp65-2β-globinUTR-A(120) RNA, pp65 peptide pool (1,75 µg/ml) as positive control or control RNA (data not shown). After expansion for 7 days each effector cell population (4x10^4/well) was tested in IFN-γ ELISpot on autologous DC (3x10^3/well) either loaded with pp65 peptide pool or a irrelevant peptide pool (1,75 µg/ml). The graphs represent the mean spot number of triplicates ± SEM.
References


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Modification of antigen encoding RNA increases stability, translational efficacy and T-cell stimulatory capacity of dendritic cells

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