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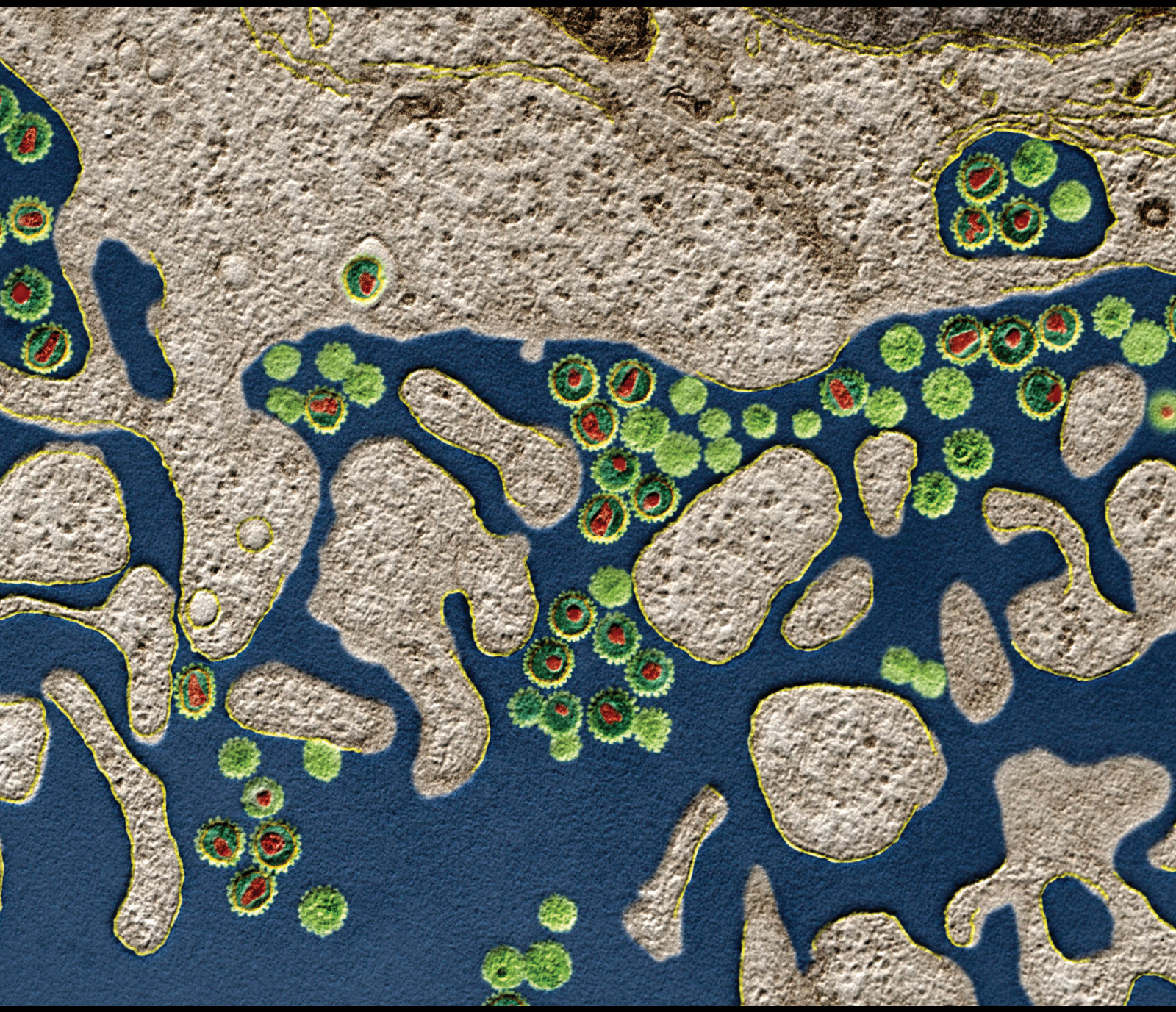
RNA Vaccination Therapy: Advances in an Emerging Field

Guest Editors: Sebastian Kreiter, Steve Pascolo, Smita K. Nair, Kris M. Thielemans, Mustafa Diken, and Andrew Geall



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Editorial

RNA Vaccination Therapy: Advances in an Emerging Field

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After more than two decades of research, the efforts to translate the concept of RNA based vaccination have reached a critical mass. Several preclinical and clinical projects located in the academic or industrial setting are underway and the coming years will allow us to get broad insight into clinical feasibility, safety, and first efficacy data. It can be anticipated that some RNA based vaccines will be approved within the near future.

The use of *in vitro* transcribed RNA is now viewed as an attractive approach for vaccination therapies, with several features contributing to its favorable characteristics. RNA allows expression of molecularly well-defined proteins and its half-life can be steered through modifications in the RNA backbone. Moreover, unlike DNA, RNA does not need to enter the nucleus during transfection and there is no risk of integration into the genome, assuring safety through transient activity. Rapid design and synthesis in response to demand, accompanied by inexpensive pharmaceutical production, are additional features facilitating its clinical translation.

The seminal work of Wolff et al. which showed that RNA injected directly into skeletal muscle can lead to protein expression opened the era of RNA based therapeutics [1]. This observation was followed by Martinon et al. and Conry et al. who performed the first vaccinations with viral- and cancer-antigen encoding RNA, respectively, and elicited antigen-specific immune responses [2, 3]. RNA based vaccination was also carried out by *ex vivo* transfection of mRNA into autologous dendritic cells (DCs) which was initially described by Boczkowski et al. [4]. Along with the introduction of highly efficient transfection methods for RNA [5], several preclinical and clinical studies showed the safety and efficacy of this RNA based vaccination strategy [6]. In a different setting, Hoerr et al. proved that direct injection of naked or protamine-protected RNA intradermally can lead to induction of T cell and antibody responses in preclinical models and then translated the approach into a clinical setting [7–10]. Personalized cancer vaccination with RNA and intravenous delivery of liposome-complexed RNA [11, 12] are other recent promising strategies that have reached the clinical stage. In addition to cancer, other disease settings such as infectious diseases as well as allergy were also shown to benefit from RNA based vaccination [13–15].

In this special issue, a number of papers will illustrate and summarize the advances in this emerging field. M. A. McNamara et al. will provide a comprehensive review on RNA based vaccines in cancer immunotherapy, which is further detailed for the use of mutanome engineered RNA by M. Vormehr et al. These will be complemented by a review from K. K. L. Phua describing targeted delivery systems for RNA based nanoparticle tumor vaccines. Other contributions will describe RNA based methods for *in vitro*

analytics such as cytotoxicity (T. A. Omokoko et al.) or effects of RNA on transcriptome of DCs (S. Hoyer et al.). Finally, E. Hattinger et al. will also demonstrate, with a different disease focus, the efficacy of prophylactic RNA vaccination against allergy.

In conclusion, this special issue covers many aspects of RNA based vaccines. As RNA based vaccination is not the only application of the RNA technology (RNA based protein replacement, immunomodulation, and cellular therapy are further promising fields of development), we hope to have sparked the readers interest in RNA based therapies in general.

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Research Article

Luciferase mRNA Transfection of Antigen Presenting Cells Permits Sensitive Nonradioactive Measurement of Cellular and Humoral Cytotoxicity

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Immunotherapy is rapidly evolving as an effective treatment option for many cancers. With the emerging fields of cancer vaccines and adoptive cell transfer therapies, there is an increasing demand for high-throughput *in vitro* cytotoxicity assays that efficiently analyze immune effector functions. The gold standard ^{51}Cr-release assay is very accurate but has the major disadvantage of being radioactive. We reveal the development of a versatile and nonradioactive firefly luciferase *in vitro* transcribed (IVT) RNA-based assay. Demonstrating high efficiency, consistency, and excellent target cell viability, our optimized luciferase IVT RNA is used to transfect dividing and nondividing primary antigen presenting cells. Together with the long-lasting expression and minimal background, the direct measurement of intracellular luciferase activity of living cells allows for the monitoring of killing kinetics and displays paramount sensitivity. The ability to cotransfect the IVT RNA of the luciferase reporter and the antigen of interest into the antigen presenting cells and its simple read-out procedure render the assay high-throughput in nature. Results generated were comparable to the ^{51}Cr release and further confirmed the assay's ability to measure antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity. The assay's combined simplicity, practicality, and efficiency tailor it for the analysis of antigen-specific cellular and humoral effector functions during the development of novel immunotherapies.

1. Introduction

Cancer immunotherapy is emerging as an important contributor to the armamentarium of future oncology treatments [1–4]. This was heralded by the advent of checkpoint inhibitors, which have made a paradigm shifting difference in the outcome of cancer treatment, resulting in sustained effects and long term survival [5, 6]. Checkpoint inhibitors only unleash the effector functions of preformed T cell specificities. This has motivated the reassessment of vaccination approaches as a complementary concept [7]. As a parallel development, due to maturation of technology and promising clinical data, the interest in redirecting adoptively transferred T cells by recombinant T cell receptors (TCRs) and chimeric antigen receptors (CARs) has moved into the spotlight [8, 9], as has the pursuit of cancer-cell surface directed antibodies recruiting and activating immune effectors such as FcR positive immune cells (ADCC) or the complement cascade (CDC).

One of the many technical challenges in immunotherapy development is the assessment of cytotoxicity induced by immune effectors, whether engineered or therapeutically

elicited, in biological assays. Such assays are required for different stages of immunotherapeutic product development, including but not limited to high-throughput discovery/selection of clinical lead candidates, mechanism-of-action or pharmacodynamics, biomarker studies accompanying clinical trial protocols, and potency assays for release of immunotherapeutic compounds.

Biological cytotoxicity assays for immunotherapeutic concepts may be more challenging as compared to those for chemical compounds due to various reasons. These include the use of difficult-to-label target cells, or, regarding reporter gene transfection-based assays, the use of difficult-to-transfect targets such as primary human professional antigen presenting cells (APCs). These have to be modified to efficiently express not only the reporter gene but also the antigen of interest when measuring the cytotoxicity of cytotoxic T lymphocytes (CTLs).

Many cytotoxicity assays assess the integrity of target cell membranes after coincubation with killing reagents, for example, CTLs or monoclonal antibodies (mAbs). The Chromium-51- (^{51}Cr-) release assay, first described in 1968 [10], is still the gold-standard but has the drawback of being radioactive and consequently hazardous. Newer nonradioactive assays using vital dyes [11], fluorescent dyes [12, 13], and combinations thereof [14] as well as bioluminescence-based assays [15, 16] have various disadvantages ranging from suboptimal labelling of targets to spontaneous release by leaky cells and inacceptable labor intensiveness [14, 17, 18].

A commonly used nonradioactive reporter gene is the luciferase enzyme [19–21]. When expressed in living cells, luciferase produces bioluminescence through a photogenic reaction in which it catalyzes the oxygenation of luciferin taken up from a substrate buffer that is added to the wells in the presence of intracellular oxygen and ATP.

Existing plasmid-based approaches using luciferase for the assessment of cytotoxicity such as the one described by Brown et al. [22] have the drawbacks of insufficient transfection efficiencies and significant decreases in vitality when using nondividing primary cells [23].

Therefore, the objective of the project presented here was to develop an efficient nonradioactive firefly luciferase-based cytotoxicity assay system compatible with dividing and primary nondividing APCs and suitable for high-throughput screening of cytotoxicity of immunotherapeutic formats. More specifically, the assay should robustly allow the assessment of antigen-specific CTL responses, antibody-dependent cell-mediated cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC).

To this end, instead of using a plasmid-based reporter gene delivery, a gene-encoding RNA was used. RNA is a versatile format to not only deliver the nonradioactive firefly luciferase reporter into the target cells, but also allow the antigen to be recognized by the respective immune effectors. Gene-encoding RNA for engineering of cells has the advantages of being easy to produce in large amounts by *in vitro* transcription (IVT) and easy to deliver by electroporation without compromising cell viability and, since it does not need to enter the nucleus, it is also an efficient system to transfect both dividing and nondividing cells. Furthermore, this approach circumvents transcriptional regulation issues faced when using DNA plasmids [23–26].

As previously reported, we have developed a plasmid construct (pST1-*Insert*-2BglobinUTR-A120-Sap1), which upon *in vitro* transcription gives rise to a 3′ modified RNA with optimized stability and translational efficiency [27]. This is achieved by fusing the cDNA of the gene of interest to the plasmid's cassette featuring two sequential human beta-globin 3′ untranslated regions (UTRs) and a 120-nucleotide long poly(A) tail with an unmasked 3′ end.

Taking advantage of our plasmid construct, this paper presents a sensitive, rapid, and simple-to-perform luciferase IVT RNA-based bioassay applicable for high-throughput screening of cytotoxicity mediated by antigen-specific CTLs or ADCC- or CDC-inducing mAbs.

2. Materials and Methods

2.1. Cells and Cell Lines. The human erythromyeloblastoid leukaemia cell line K562 stably transfected with human HLA-A*0201 (referred to as K562-A2) was cultured under standard conditions [28]. Endogenously human Claudin 18.2 (hCLDN18.2) expressing human gastric cancer cell lines NUGC-4 and KATO-III were maintained in RPMI 1640 (Life Technologies) supplemented with 10% foetal calf serum (Biofluid Inc., Gaithersburg, MD, USA) at 37°C, 5% CO_2, and RPMI 1640 supplemented with 20% foetal calf serum at 37°C, 7.5% CO_2, respectively. The CHO-K1 cell line stably expressing hCLDN18.2 was cultured in DMEM-F12 (Life Technologies), supplemented with 10% foetal calf serum, 1% Penicillin-Streptomycin (Life Technologies), and 1.5 mg/mL Geneticin (GE Healthcare Life Sciences) at 37°C, 7.5% CO_2. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation from buffy coats obtained from healthy blood bank donors. Monocytes were enriched from PBMCs with anti-CD14 microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Immature (iDCs) and mature dendritic cells (mDCs) were generated as previously described [27]. The monospecific CTL cell line IVSB specific for the HLA-A*0201-restricted tyrosinase-derived epitope $tyr_{368–376}$ [29, 30] was cultured as previously described [31].

2.2. In Vitro Expansion of Human T Cells. $CD8^+$ T cells were purified from PBMC of human cytomegalovirus $(CMV)^+$ donors by positive magnetic cell sorting (Miltenyi Biotec) and expanded by coculturing 2×10^6 effectors with 3×10^5 autologous DCs either electroporated with IVT RNA or pulsed with overlapping peptide pool for 1 week in complete medium supplemented with 5% AB serum, 10 U/mL IL-2, and 5 ng/mL IL-7.

For nonspecific expansion, 2×10^6 per well naïve $CD8^+$ T cells purified from CMV^- donors were stimulated in OKT3 mAb coated 24-well plates (Janssen-Cilag GmbH, Neuss). Coating was performed using 300 μL/well PBS-diluted mAb (10 μg/mL) for 2 h at 37°C. After 24 h of culture, 50 U/mL IL-2 was added to the stimulated CTLs. On day 3, the cells were resuspended in fresh medium supplemented with 50 U/mL

IL-2 and cultured for another 4 days in 24-well plates without OKT3.

2.3. Peptides and Peptide Pulsing of Stimulator Cells. Pools of N- and C-terminally free 15-mer peptides (all peptides purchased from JPT Peptide Technologies GmbH) with 11 amino acid overlaps corresponding to sequences of CMV-pp65 and HIV-gag (referred to as antigen pool), the latter used as negative control, were dissolved in DMSO to a final concentration of 0.5 mg/mL. The HLA-A*0201 restricted peptides derived from the CMV-pp65 ($pp65_{495–503}$, NLVPMVATV), tyrosinase ($tyr_{368–376}$, YMDGTMSQV), and SSX2 ($SSX2_{41–49}$, KASEKIFYV) antigens were reconstituted in PBS 10% DMSO. For pulsing, stimulator cells were incubated for 1 h at 37°C in culture medium using concentrations of 1–3 μg/mL, where not otherwise indicated.

2.4. Vectors for In Vitro Transcription. A plasmid for *in vitro* transcription of the synthetic firefly luciferase reporter gene (*luc2*) was constructed based on the previously described pST1-insert-2hBgUTR-A(120) vector, which allows the generation of RNA with optimized stability and translational efficacy [27]. The *luc2* gene was subcloned from the pGL4.14[luc2/Hygro] vector (Promega Corporation, Madison, WI, USA) and an internal EciI restriction site deleted by site-directed mutagenesis (Agilent) using the oligo luc2mut sense (5′-CTA CCA GGC ATC CGA CAG GGC TAC GGC CTG ACA GAA AC-3′) and the reverse complement (Eurofins Genomics).

The pST1-2hBgUTR-A(120)-IVT vectors containing enhanced green fluorescent protein (eGFP) and the full-length TCR alpha and beta chains of the $pp65_{495–503}$-specific and HLA-A*0201-restricted TCR-8-CMV#14 have been previously described [27, 31]. The pp65 antigen-encoding vector pST1-sec-pp65-MITD-2hBgUTR-A(120) features a signal sequence for routing to the endoplasmic reticulum and the MHC class I transmembrane and cytoplasmic domains to improve MHC class I and II presentation [32].

2.5. Generation of IVT RNA and Transfer into Cells. IVT RNA was generated as previously described [27] and added to cells suspended in X-VIVO 15 medium (Lonza) in a precooled 4 mm gap sterile electroporation cuvette (Bio-Rad). Electroporation was performed with a Gene-Pulser-II apparatus (Bio-Rad; human iDC: 276 V/150 μF; human mDC: 290 V/150 μF; $CD8^+$ T cells: 450 V/250 μF; K562-A2, CHO and NUGC-4: 200 V/300 μF; KATO III: 250 V/475 μF).

2.6. Flow Cytometric Analysis. Flow cytometric analysis was performed on a FACS-Calibur analytical flow cytometer using CellQuest-Pro software (BD Biosciences). DC maturation markers were detected by staining with PE-labelled anti-CD83 and APC-labelled anti-HLA-DR antibodies (BD Biosciences).

2.7. Luciferase-Based CTL Cytotoxicity Assay. APCs were electroporated with 10–50 μg of *luc2* IVT RNA. For coelectroporation experiments, *luc2* IVT RNA and either pp65 or control RNA were electroporated into the target cells simultaneously. After electroporation, cells were resuspended in prewarmed culture medium and incubated overnight at 37°C and 5% CO_2. 20 h later, cells were diluted to a final concentration of 2×10^6 cells/mL in culture medium containing 1–3 μg/mL specific peptide (pool) or control peptide (pool) and were incubated for 1 h at 37°C and 5% CO_2. After pulsing with peptides, cells were washed and resuspended in complete culture medium and 1×10^4 cells per well were plated in triplicate in 50 μL into white 96-well flat-bottom plates (Thermo Scientific). $CD8^+$ effector cells were washed, counted, and cocultured in different E : T ratios in a final volume of 100 μL per well at 37°C and 5% CO_2 for 3 h. Minimal and maximal lysis control wells contained 1×10^4 target cells alone in a total volume of 100 μL and 90 μL, respectively. After the specified time 50 μL of a D-luciferin substrate solution containing 3.6 mg/mL D-luciferin (BD Biosciences Pharmingen), 150 mM HEPES (Life Technologies) and 1.2 mU/μL Adenosine 5′-Triphosphatase (Sigma-Aldrich) were added to each well to a final volume of 150 μL. Maximum lysis control wells were treated with 10 μL 2% Triton X-100/PBS prior to addition of substrate. 96-well plates were incubated for another hour at 37°C and 5% CO_2. After a total coincubation time of 4 h, the intracellular luciferase activity of living cells was measured using a Tecan Infinite M200 reader (Tecan Group AG, Crailsheim, Germany). Percent specific lysis was calculated as follows:

$$\left(1 - \frac{\mathrm{CPS}_{\mathrm{experimental}} - \mathrm{CPS}_{\mathrm{minimal}}}{\mathrm{CPS}_{\mathrm{maximal}} - \mathrm{CPS}_{\mathrm{minimal}}}\right) \times 100. \quad (1)$$

2.8. ^{51}Cr-Release Assay. Autologous DCs were loaded with 3 μg/mL pp65 peptide pool or control peptide and labelled with 100 μCi of ^{51}Cr (NEN Life Science) for 90 min at 37°C and 5% CO_2. ^{51}Cr labelled DCs were washed and resuspended in complete culture medium and 1×10^4 targets per 200 μL per well coincubated in triplicate with effector T cells at different E : T ratios for 4 h. A total of 60 μL of the supernatant was harvested, and released ^{51}Cr was measured with a scintillation counter. Spontaneous release was also determined. Percent specific lysis was calculated using the following equation:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100. \quad (2)$$

2.9. Luciferase-Based ADCC Assay. Target cells were electroporated using 7 μg of *luc2* IVT RNA. After electroporation, cells were resuspended in 2.4 mL prewarmed culture medium. 2×10^4 KATO-III cells or 2.5×10^4 NUGC-4 cells per 50 μL per well were plated independently in triplicate into white 96-well flat-bottom plates (NUNC) and were incubated for 4–6 h at 37°C, 7.5%, and 5% CO_2, respectively. Different IMAB 362 concentrations ranging from 0.06 ng/mL to 200 μg/mL and Ficoll-Paque-purified PBMCs from healthy donors were added to each well (E : T ratio of 40 : 1). KATO-III and NUGC-4 cell-containing plates were incubated for 24 h at 37°C, 7.5%, and 5% CO_2, respectively. After overnight

incubation, 10 μL 8% Triton X-100/PBS solution was added to the maximum lysis control wells and 10 μL PBS to the other wells. Finally, 50 μL D-luciferin substrate solution containing 3.2 mg/mL D-luciferin, 160 mM HEPES, and 0.64 mU/μL Adenosine 5′-Triphosphatase was added to each well to a final volume of 160 μL and plates were incubated for 90 min at room temperature (RT) in the dark. Bioluminescence was measured using a luminometer (Infinite M200, TECAN). Percentage of specific lysis was calculated using the formula described above for the luciferase-based CTL cytotoxicity assay.

2.10. Luciferase-Based CDC Assay. CHO-K1~hCLDN18.2 cells were electroporated using 7 μg of *luc2* IVT RNA and resuspended in 2.4 mL prewarmed culture medium. 5×10^4 cells per 50 μL per well were plated in triplicate into white 96-well flat-bottom plates (NUNC) and were incubated for 24 h at 37°C, 7.5% CO_2. 44% human serum from healthy donors was prepared in RPMI medium supplemented with 20 mM HEPES. IMAB 362 antibody was diluted in human serum to final assay concentrations ranging from 31.6 ng/mL to 10 μg/mL. 50 μL of different IMAB 362 antibody concentrations were added to the target cells to achieve an end concentration of 20% (v/v) serum. The 96-well plate was incubated for 80 min at 37°C, 7.5% CO_2. After incubation, 10 μL 8% Triton X-100/PBS solution was added to control for maximum lysis and 10 μL PBS to the remaining wells. D-Luciferin substrate solution was added to each well as described above for the ADCC assay. Plates were incubated for 45 min at RT in the dark and then measured in a luminometer (Infinite M200, TECAN). Specific lysis was calculated as described above for the ADCC assay.

3. Results and Discussion

3.1. Electroporation of Firefly Luciferase IVT RNA into Dividing and Nondividing APCs Is Nontoxic and Leads to Strong and Long-Lasting Gene Expression. One of the key elements for the performance of a cytotoxicity assay system is the labelling of the target cell population with a reporter system without affecting cell viability. This limitation is pronounced when using nondividing cells, such as primary human APCs, frequently required in the context of immunotherapy drug development. Plasmid-based reporter gene assays are of low efficiency and do not provide a good solution [22]. Instead of using a plasmid-based delivery approach, the use of a luciferase gene-encoding mRNA was investigated here.

The firefly luciferase (*luc2*) gene was cloned into the pST1-2hBgUTR-A(120)-EciI vector and *in vitro* transcribed from this construct with a stability optimized 3′ end (Figure 1(a)). 5.4 μg of this IVT RNA was electroporated into K562 leukemic cells stably transfected with the human HLA-A*0201 gene (hereinafter referred to as K562-A2) [28]. In addition, difficult-to-transfect nondividing primary human cells, namely, iDCs and mDCs, were also used as targets (Figure 1(b)). Luminescence after D-luciferin substrate addition was instantly detected and strongly increased between 2 and 8 h after electroporation. Signals reached maximum levels after 10 to 24 h in all cell types. K562-A2 cells showed the highest signal levels. Activity in primary cells was also very robust, and a 2–4-fold higher maximum luciferase activity was detected in mDCs compared to iDCs. High and durable expression levels were achieved with an approximately 80% signal intensity still being detectable 36 h after electroporation into K562-A2 cells and mDCs, and 24 h in the case of iDCs. Luciferase expression kinetics exhibited batch consistency within each cell type and were not affected by the use of higher *luc2* IVT RNA amounts (data not shown).

To assess the viability of the target cells, 10 μg RNA encoding luciferase and eGFP were electroporated into human iDCs and mDCs generated from the same donor. Both iDCs and mDCs displayed excellent viability, ranging from 85 to 95% in the 72 h after electroporation with reporter RNA as determined by flow cytometry (Figure 1(c)). 80–90% of all living DCs expressed eGFP stably over 72 h illustrating high transfection efficiency. This gives the IVT RNA approach an advantage over plasmid based assays, which show low efficiencies when used with nondividing primary APCs, probably a consequence of using more stringent electrical settings [23]. Both iDCs and mDCs retained their phenotypes after electroporation as demonstrated by the sustained levels of the maturation markers HLA-DR and CD83 in *luc2* transfected cells compared to controls for as long as 72 hours after electroporation. As expected, mDCs showed a higher expression of both markers (Figure 1(c)).

In summary, the data demonstrate high, stable, and long-lasting expression of *luc2* reporter IVT RNA in dividing as well as nondividing primary APCs, without compromising the viability or immunological phenotype of the target cells.

3.2. Optimization of the Assay Parameters Enhances and Prolongs Luciferase Signals Whilst Minimizing Background and Reveals a Strict Luminescence to Cell Number Correlation. As a next step, the implementation of the IVT RNA-based reporter-gene engineering of target cells into a robust cytotoxicity assay with a favorable signal-to-noise ratio and a high sensitivity was investigated.

For K562-A2 cells electroporated with 20 μg of *luc2* IVT RNA, a D-luciferin substrate concentration of 1.2 mg/mL achieved the highest signals (Figure 2(a)). These signals were prolonged and stable, allowing continuous detection of living cells after a single administration of substrate for at least 4 h (Figure 2(b)). Bioluminescence dropped to levels close to zero following Triton X-100 detergent-mediated cell lysis, demonstrating responsiveness of the technique to cytotoxic events (Figure 2(b)). The rapid reduction of background signals from dying cells is further accelerated by the addition of ATPase to the substrate buffer, which results in the immediate hydrolysis of ATP released from these cells and ceasing of luciferase activity following cell death (data not shown). The stability of the signal over 4 hours and the direct assessment of cell death allow both endpoint measurements and the determination of killing kinetics, which is superior to many other assays such as the ^{51}Cr and the Europium release assays, that only allow the former [17, 18, 33].

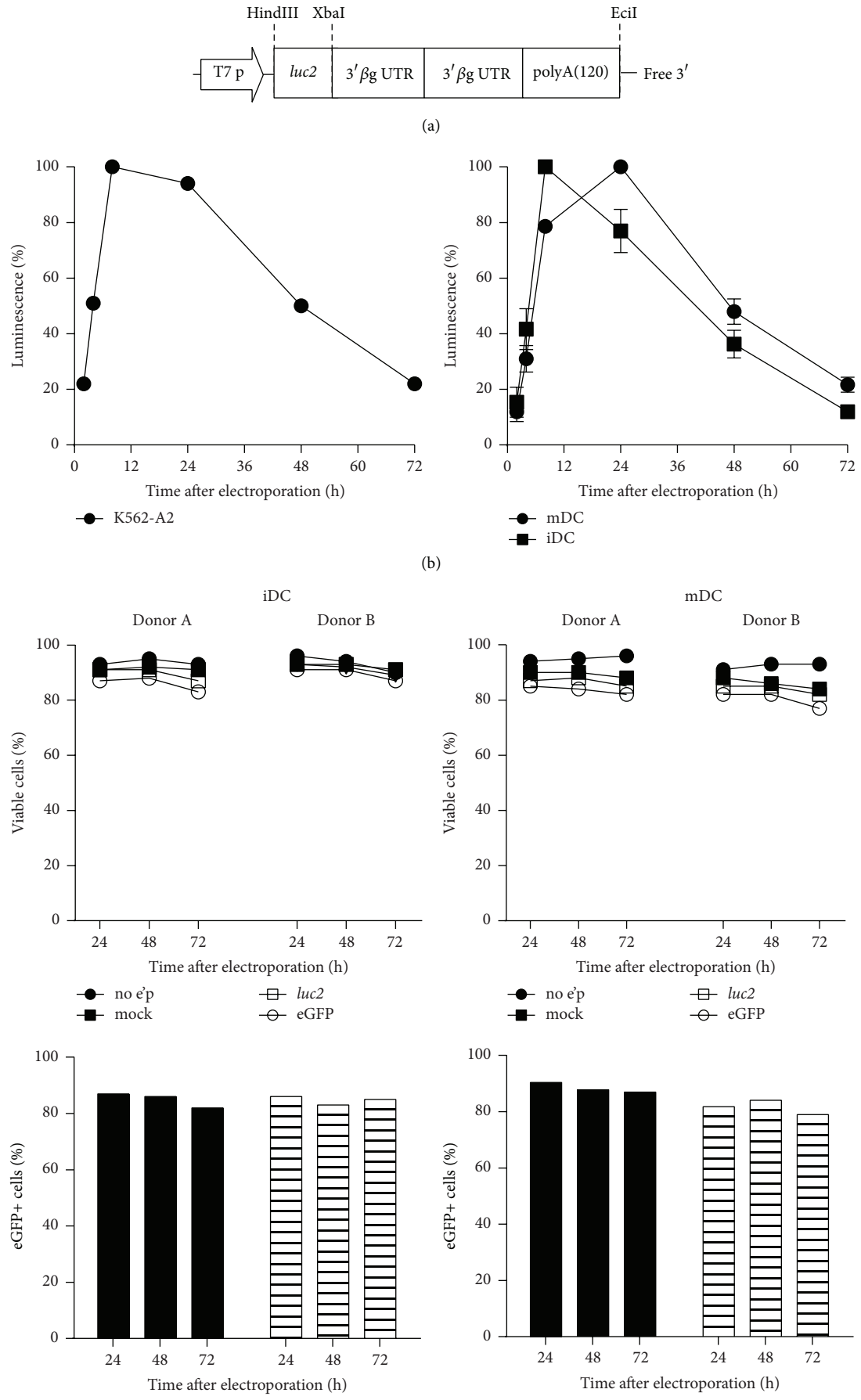
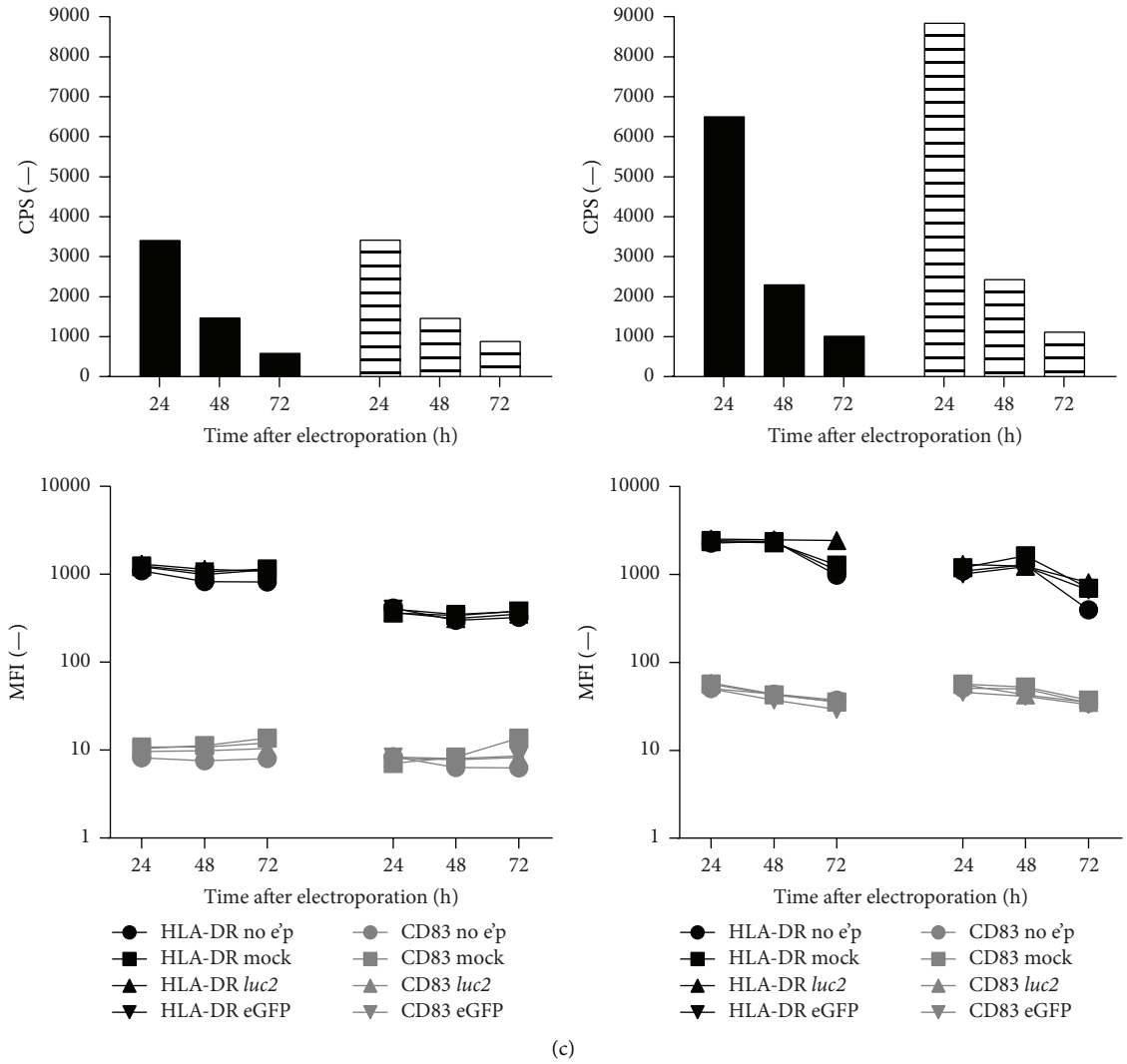


Figure 1: Continued.



(c)

Figure 1: Electroporation of firefly luciferase IVT RNA into DCs and K562-A2 cells is nontoxic and leads to strong and long-lasting gene expression without affecting target cell phenotype. (a) Optimised *luc2* reporter vector: composed of a gene-optimized synthetic firefly luciferase reporter gene cloned in front of two human β-globin 3′ untranslated regions (UTRs) fused head to tail and an unmasked free poly(A) tail of 120 bp. (b) Kinetics of *luc2* expression in K562-A2 cells ($n = 1$), human iDCs ($n = 3$), and mDCs ($n = 3$). Cells transfected with 8 pmol of *luc2*-encoding IVT RNA were harvested at different time points to measure luminescence from 1×10^4 cells (Bright-Glo Luciferase Assay Kit for 96-well plates (Promega)). Results are the mean ± SD luminescence. Percent luminescence is relative to the highest luminescence signal obtained in each experiment. (c) Viability, reporter gene expression of iDCs and mDCs after eGFP and *luc2* electroporation and phenotype after electroporation are depicted in descending order, respectively. iDCs (left panel) and mDCs (right panel) of 2 different donors were transfected with 10 μg eGFP- or *luc2*-encoding IVT RNA. Negative controls: cells electroporated without RNA (mock) and unelectroporated (no e'p) cells. Cells were harvested at different time points. Viability and HLA-DR, CD83, and eGFP expression levels were determined by flow cytometry. Luciferase activity of 1×10^4 viable cells was measured by luminescence in triplicate.

Electroporation of K562-A2 cells with increasing amounts of *luc2* IVT RNA displayed a dose-dependent increase in luminescence (Figure 2(c)).

The strict linear dependence between the detectable bioluminescence and transfected cell numbers further verified the sensitivity of the method (Figure 2(d)).

Next, these conditions were tested on nondividing primary cells, namely, human monocyte-derived iDCs and mDCs. Addition of D-luciferin to human iDCs and mDCs 24 h after their electroporation with *luc2* IVT RNA also demonstrated a linear correlation between cell number and bioluminescence (Figure 2(e)). Luciferase activity from as few as 1,000 cells was more than 24-fold higher than background levels, implying that luminescence from such few cells suffices for accurate reporter gene detection (Figure 2(e)).

The equipment and the read-out conditions of the assay greatly affect the specific signal, background reading, and the cross-talk between wells. In our hands, white polystyrene

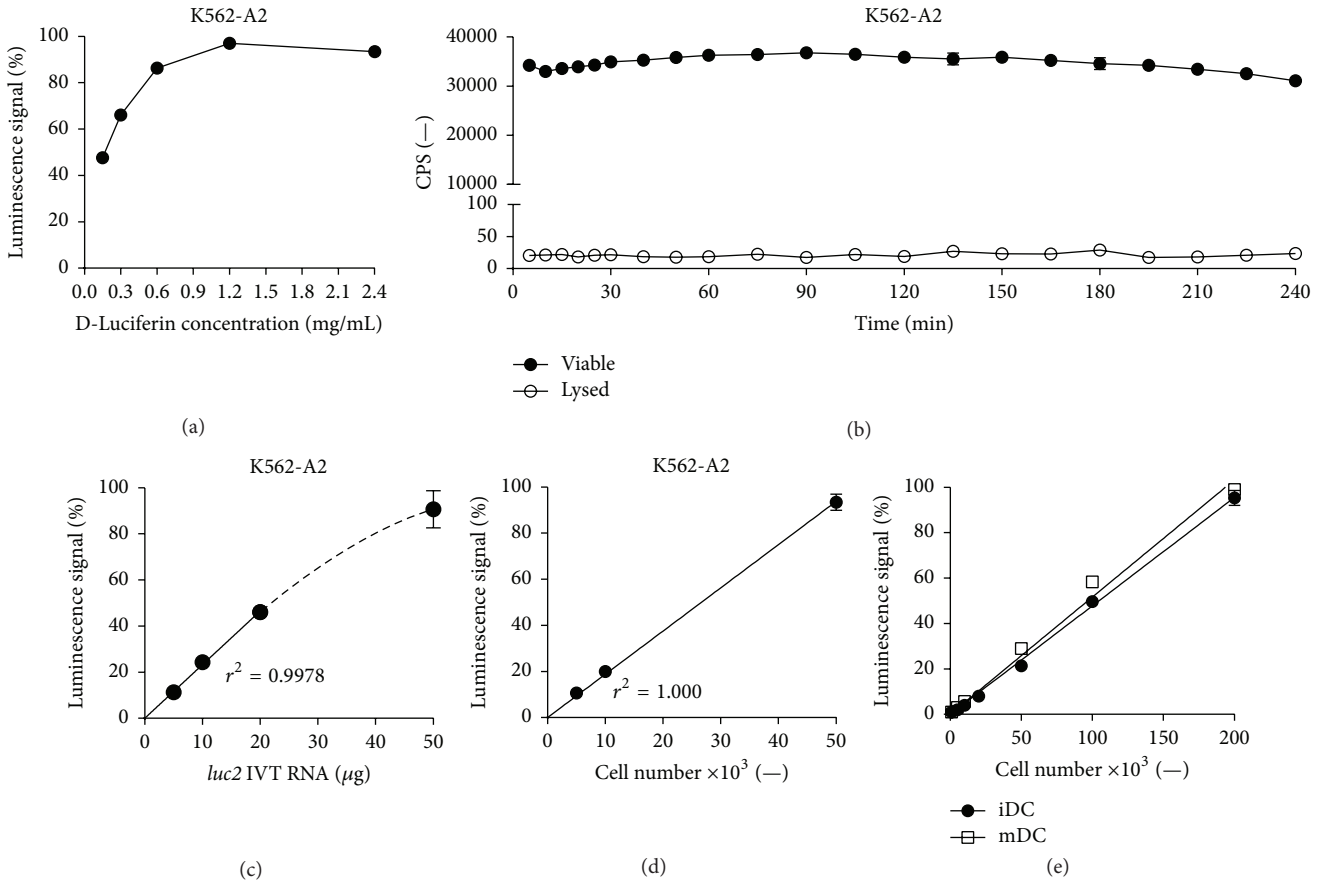


Figure 2: Optimization of the assay parameters enhances and prolongs luciferase signals whilst minimizing background reading and reveals a strict luminescence to cell number correlation. (a) Optimal D-luciferin substrate concentration. 1×10^6 K562-A2 cells were transfected with 20 μg *luc2* IVT RNA. After 24 h, luminescence of 5×10^4 cells per well was measured following addition of D-luciferin in different concentrations. (b) Stable bioluminescence upon D-luciferin substrate addition and immediate abolition of signals following total cell lysis. 2.5×10^6 K562-A2 cells were transfected with 50 μg *luc2* IVT RNA. 24 h after transfection, luminescence of 5×10^4 viable or 0.2% Triton X-100 treated cells was repeatedly measured after a single administration of 1.2 mg/mL D-luciferin substrate. Graph represents mean ± SD luminescence ($n = 3$). (c) Luminescence is dependent on *luc2* IVT RNA dose. 1×10^6 K562-A2 cells were transfected with different amounts of *luc2* IVT RNA. 24 h after transfection, luminescence of 5×10^4 cells per well was measured. (d) Luminescence is linearly dependent on the number of transfected K562-A2 cells. 1×10^6 K562-A2 cells were transfected with 50 μg *luc2* IVT RNA. 24 h after transfection, 1.2 mg/mL D-luciferin was added and luminescence of different amounts of cells was measured. (e) Luminescence is linearly dependent on the number of transfected primary cells. Human iDCs and mDCs were electroporated with 50 μg *luc2* IVT RNA. 24 h after transfection, 1.2 mg/mL D-luciferin was added and luminescence of different amount of cells was measured. Cell number and signal intensity correlation ($p < 0.0001$, $r^2 = 0.9984$ (iDC) and 0.9923 (mDC)). Graphs (a), (c), (d), and (e) represent mean ± SD luminescence ($n = 3$) relative to the highest luminescence signal obtained within each experiment.

flat-bottom plates that reflect light and maximize the output signal and the more cost-efficient Tecan Infinite M200 luminescence plate reader (Tecan, Crailsheim, Germany) resulted in an excellent signal-to-noise ratio, achieving specific signals with multiple logs above background (Table 1; Figure 2(b)). That, along with the advancements in plate readers, such as the automatic regulation of temperature and reagent addition, further promotes the automation of this assay for high-throughput screening. It should be noted that one can easily modify the assay according to one's needs, for example, target cell type and amount of cells usually available, by choosing a suitable plate reader and adjusting the amount of luciferase IVT RNA used for electroporation.

3.3. Luciferase IVT RNA Electroporation Permits Assessment of Antigen-Specific CTL Activity Comparable to the ^{51}Cr Release and Superior in the Ability to Monitor Killing Kinetics. Having optimized the key performance parameters of the assay system, the CMV-pp65 model antigen was used to measure primary antigen-specific CTL responses, as is frequently required in vaccine approaches. To generate the respective reagents, effector T cells from a CMV$^+$ donor were expanded by coculture with pp65 antigen pool loaded autologous iDCs. Simultaneously, mDCs of the donor were generated and electroporated with *luc2* IVT RNA. 20 h after transfection, the autologous target cells were loaded with either pp65 antigen pool or control antigen pool before being cocultured with

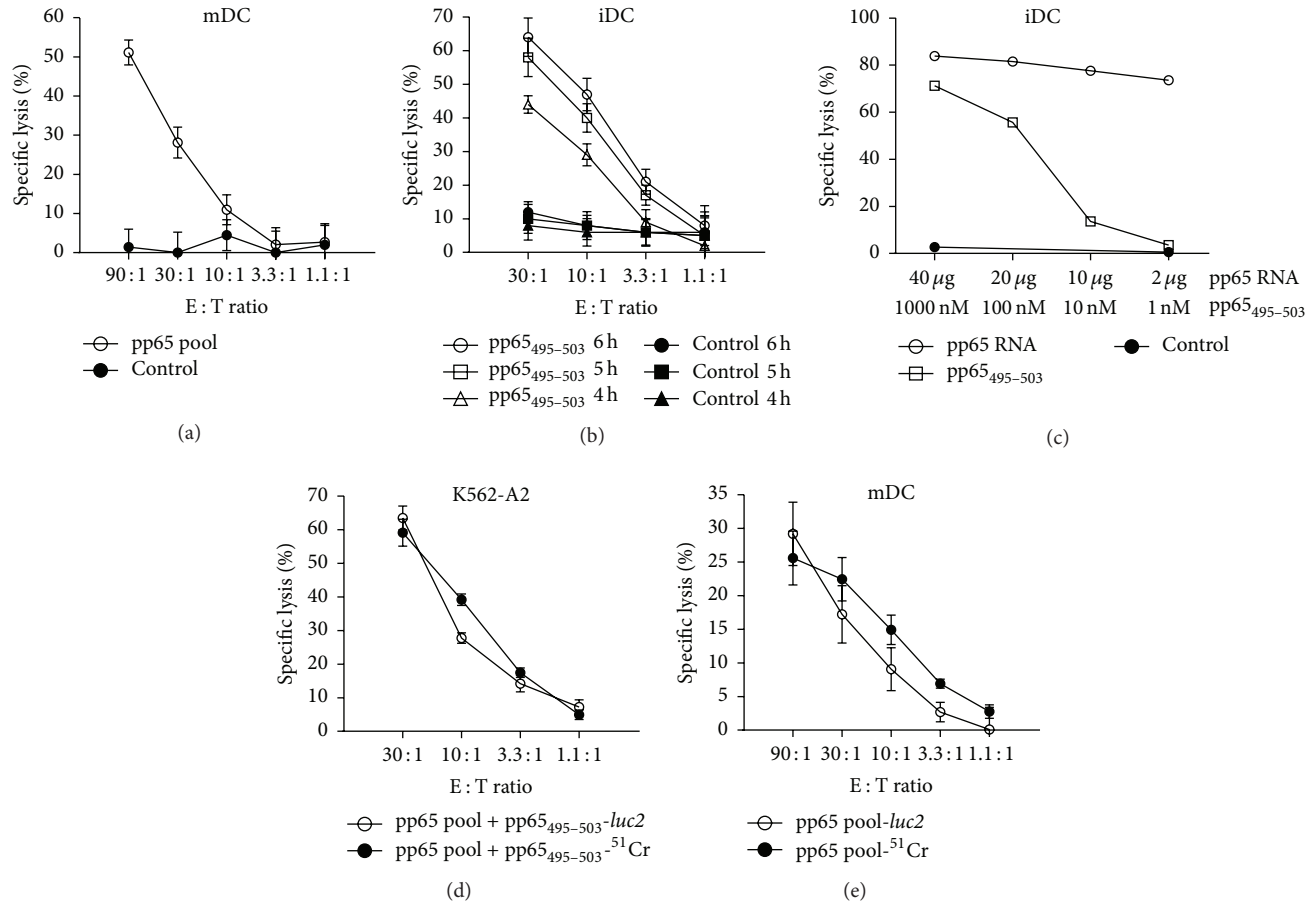


Figure 3: Luciferase IVT RNA electroporation permits assessment of antigen-specific CTL activity comparable to the ^{51}Cr release and superior in the ability to monitor killing kinetics. (a) Cytolytic activity of primary CMV-pp65-specific T cells. CMV$^+$ donor-derived CD8$^+$ T cells were expanded for one week and used to assess the killing of autologous mDCs transfected with 50 μg *luc2* RNA and loaded with overlapping peptide pools representing either CMV-pp65 or HIV-gag as control. Specific lysis was determined after 4 h incubation of peptide-loaded target cells with CD8$^+$ effector cells using different E : T ratios. (b) Kinetics of killing mediated by TCR-transfected CD8$^+$ T cells. OKT3-stimulated CD8$^+$ T cells of a CMV$^-$ HLA-A*0201$^+$ donor were transfected with 20 μg TCR-8-CMV#14 alpha and beta chain IVT RNAs. Autologous iDCs transfected with 20 μg *luc2* RNA were loaded with either peptide pp65$_{495–503}$ or tyr$_{368–376}$ as control. iDCs and CD8$^+$ T cells were cocultured at different E : T ratios and specific killing was assessed at different time points. (c) Dose-dependent killing of target cells using different antigen formats. OKT3-stimulated CD8$^+$ T cells from a CMV$^-$ HLA-A*0201$^+$ donor were electroporated with 20 μg TCR-8-CMV-#14 IVT RNA. Autologous iDCs were cotransfected with 20 μg *luc2* IVT RNA and decreasing amounts of a CMV-pp65 antigen-encoding IVT RNA or were *luc2* transfected and subsequently pulsed with titrated amounts of peptide pp65$_{495–503}$. iDC transfected with *luc2* IVT RNA and pulsed with 1000 nM SSX2$_{41–49}$ peptide served as a control. Effector and target cells were incubated at an E : T ratio of 19 : 1. ((d) and (e)) Comparability of the *luc2* IVT RNA assay with the ^{51}Cr assay. Cytotoxicity of CMV-pp65-specific CD8$^+$ T cells of a HLA-A*0201$^+$ CMV$^+$ donor against (d) K562-A2 cells or (e) autologous mDCs was assessed after one week antigen-specific expansion using the *luc2* IVT RNA assay in comparison to the ^{51}Cr assay. 20 h after *luc2* RNA electroporation, target cells were loaded with pp65$_{495–503}$ peptide either alone or together with 100 μCi of ^{51}Cr. 1×10^4 peptide-loaded targets were incubated at different E : T ratios with CD8$^+$ effector cells for 4 h. Cytotoxicity was determined via measurement of luminescence after addition of D-luciferin substrate or via measurement of released ^{51}Cr after harvesting of supernatant. All graphs represent the mean ± SD lysis (n = 3).

the CD8$^+$ effectors at different E : T ratios for 4 h. The calculated specific lysis of pp65 pulsed target cells increased with increasing E : T ratios, while target cells pulsed with control peptides were not lysed, illustrating the assays ability to detect and quantify antigen-specific CTL immune responses (Figure 3(a)).

In order to assess the assay's capacity of directly determining the kinetics of CTL-mediated killing, OKT3-stimulated CD8$^+$ T cells from a CMV$^-$ HLA-A*0201$^+$ donor were electroporated with IVT RNA encoding a previously isolated T cell receptor (TCR-8-CMV-#14) directed against the immunodominant CMV-pp65-derived HLA-A*0201-restricted peptide pp65$_{495–503}$ [31]. Autologous iDCs were transfected with *luc2* IVT RNA and 20 h later loaded with either the specific or a control peptide. Effector and target cells were incubated at different E : T ratios. D-Luciferin was

Table 1: White opaque flat-bottom plates together with the Infinite M200 (Tecan) plate reader result in a robust signal-to-noise ratio.

Parameter	Experiment	Outcome
Plate opacity	White opaque versus transparent	Specific luciferase signals obtained from white opaque plates were 2-3-fold higher
Plate design	Flat-bottom versus V-shaped bottom	Only negligible well-to-well cross-talk was observed when flat-bottom plates were used
Plate reader	Wallac VICTOR2 (Perkin Elmer) versus Infinite M200 (Tecan) versus GENios Pro (Tecan)	Signals from the Infinite M200 device were 4-fold and signals from the GENios Pro 20-fold higher than those detected with the Wallac VICTOR2

added once after 3 h. Following that, multiple luminescence readouts were taken at different time points and descriptive killing kinetics could be recorded (Figure 3(b)). For each E : T ratio, the specific lysis increased over time, with the 30 : 1 ratio showing the highest specific lysis at all time points, while control peptide loaded iDCs were not lysed.

Other popular flow cytometry based cytotoxicity assays monitor, for example, caspase activation or granzyme B substrate cleavage in target cells [34]. These alternatives have the ability to quantify target cell death at the single-cell level. However, one needs to carefully determine the best time for such endpoint measurements, as markers of apoptosis such as caspase activity are only transiently present. This may be challenging especially with regard to T cell populations with unknown or low frequency antigen-specific effectors. Due to the long-lasting signals, the luciferase assay, on the other hand, provides the opportunity to take multiple measurements over a longer time period.

A further advantage of using gene-encoding RNA is that, together with the luciferase reporter gene IVT RNA, any other antigen (or vaccine) IVT RNA of interest can be cotransfected. OKT3-stimulated $CD8^+$ T cells from a CMV^- HLA-A^*0201^+ donor were electroporated with the same TCR-8-CMV-#14 IVT RNA. Autologous iDCs were cotransfected with *luc2* IVT RNA and decreasing amounts of a pp65 antigen-encoding IVT RNA or were *luc2* transfected and subsequently pulsed with titrated amounts of the $pp65_{495–503}$ peptide. In addition to illustrating the efficient cotransfection of luciferase and varying amounts of antigen IVT RNA, the results show the sensitive recognition of antigen via the TCR with 74% specific lysis being detected using 2 μg of pp65 RNA for transfection (Figure 3(c)). In the context of this cotransfection ability, it should be noted that the use of a full-length antigen-encoding IVT RNA would allow the detection of CTL responses specific for naturally processed epitopes that are presented on the surface of the APCs.

The ^{51}Cr-release assay is widely used and is considered as the gold standard approach to assess T cell and natural killer cell-mediated cytotoxicity [32, 35–37]. The efficiency of the luciferase IVT RNA assay was thus further confirmed by a direct comparison with the ^{51}Cr-release assay using either K562-A2 cells or primary DCs as target cells. For the former, which were stably transfected with HLA-A^*0201 (Figure 3(d)), effector T cells of a CMV^+ HLA-A^*0201^+ donor were expanded using peptide loaded autologous iDCs. K562-A2 cells were electroporated with *luc2* IVT RNA. 20 h later, half of the cells were loaded with pp65 antigen pool and $pp65_{495–503}$ peptide alone and the other half were simultaneously labelled with ^{51}Cr. For the primary DCs (Figure 3(e)), effector T cells from a CMV^+ donor were expanded using peptide loaded autologous iDCs. In parallel, autologous mDCs were electroporated with *luc2* IVT RNA. 20 h later, half of the cells were loaded with pp65 antigen pool or control antigen pool alone and the other half were concurrently labelled with ^{51}Cr. In both settings, peptide-loaded targets and $CD8^+$ effector cells were then incubated at different E : T ratios for 4 h before luminescence and released chromium were measured. The IVT RNA-based assay yielded specific lysis levels that were as sensitive as, and almost identical to, the ^{51}Cr assay, with 60% and ~20% specific lysis of the K562-A2 cells (Figure 3(d)) and autologous mDCs (Figure 3(e)), respectively, at an E : T ratio of 30 : 1.

3.4. Luciferase IVT RNA Electroporation Permits a Highly Sensitive Assessment of Antigen-Specific CTL Activity. Having proven the robustness of the system, the capability of the assay to detect low-frequency antigen-specific T cells was examined. The monospecific CTL cell line IVSB recognizing the HLA-A^*0201-restricted tyrosinase-derived epitope $tyr_{368–376}$ was used [29, 30]. Decreasing amounts of IVSB T cells were spiked into peripheral blood lymphocytes (PBLs). The specific lysis of autologous iDCs pulsed with the $tyr_{368–376}$ peptide was assessed. Since specific lysis is calculated using internal maximum and minimum references (see Section 2), iDCs plus PBLs without IVSB T cells were used as the minimum lysis reference in this experiment. Luciferase signals were analyzed after 5, 6, and 9 h of coincubation (Figure 4). After 5 h, the cytotoxic activity of 0.37% antigen-specific T cells corresponding to 740 IVSB cells in a total of 200,000 PBLs was easily detected based on the specific lysis of $tyr_{368–376}$ peptide pulsed target cells (Figure 4). When the incubation time was prolonged to 9 h, the detection

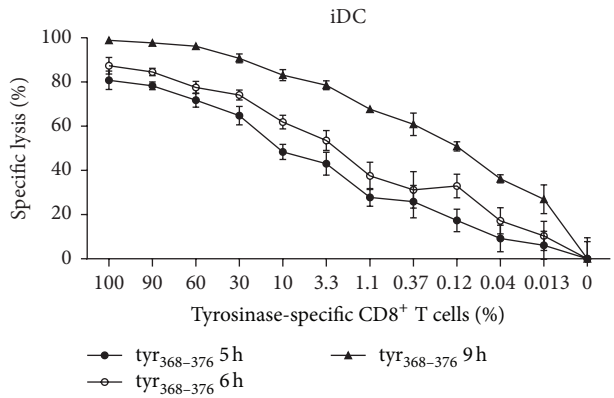


Figure 4: Luciferase IVT RNA electroporation permits a highly sensitive assessment of antigen-specific CTL activity. Titrated amounts of IVSB cells were spiked into PBLs and the specific lysis of autologous iDCs pulsed with the $tyr_{368-376}$ peptide was assessed using an E : T ratio of 20 : 1. Luciferase signals were analysed after increasing coincubation times. Results are the mean ± SD (n = 3).

limit was improved to as few as 26 antigen-specific T cells, corresponding to a frequency of 0.013% of PBLs.

The results confirm the suitability of the developed assay to sensitively detect cytotoxicity induced by very rare antigen-specific T cells, as is the case with *ex vivo* tumor-antigen specific effector cells in the blood of cancer patients.

In summary, the data indicates that the luciferase IVT RNA assay performs at least as well as the ^{51}Cr assay and is superior in its sensitivity, nonradioactivity, easy read-out procedure, and the monitoring of killing kinetics.

3.5. The Luciferase IVT RNA-Based Assay Efficiently Assesses mAb-Induced ADCC and CDC of Tumor Cell Lines. In addition to T cell-mediated cytotoxicity, other effector functions have also been shown to participate in antitumor responses [38]. The assay was therefore adopted to assess ADCC and CDC, which are mediated by the recruitment and activation of either FcR positive effector cells or complement factors by the Fc domains of cell-bound mAbs [39]. To this end, IMAB 362, a therapeutic mAb in advanced clinical development directed against the pan-cancer cell surface antigen Claudin 18.2 (CLDN18.2), which exerts tumor cell death via ADCC and CDC, was used [40–43]. KATO-III and NUGC-4 tumor cells endogenously expressing CLDN18.2 or CHO-K1 cells stably expressing the antigen were electroporated with *luc2* IVT RNA. To measure ADCC, IMAB 362 was added to the KATO-III (Figure 5(a)) and NUGC-4 (Figure 5(b)) target cells 4–6 h after *luc2* electroporation. The cells were then incubated with human PBMCs at a 40 : 1 E : T ratio for 24 h; then D-luciferin substrate was added for luminescence measurement. For the assessment of CDC, 24 h after *luc2* electroporation, the CHO-K1 cells were incubated with IMAB 362, diluted in human serum, for 80 minutes as a source of complement factors. Thereafter, D-luciferin was added for the signal read-out (Figure 5(c)).

Specific lysis via ADCC and CDC was found to be dependent on the IMAB 362 concentration. Dose-response curves were sigmoid with a good dynamic range. For IMAB 362-induced ADCC-mediated specific lysis of KATO-III cells, as few as 1.19 ng/mL antibody was sufficient to induce 25% killing (Figure 5(a)). For NUGC-4 cells, 24 ng/mL antibody induced 14 to 76% ADCC-mediated lysis among the different donors (Figure 5(b)). The maximum specific cell lysis was approximately 80% in both cell lines and was reached at concentrations of 9.88 μg/mL IMAB 362. Robust CDC was measured at a concentration of 1000 ng/mL and reached a maximum of up to 99% lysis of CHO-K1 cells at an IMAB 362 concentration of 3.16 μg/mL (Figure 5(c)).

The data demonstrates that the luciferase IVT RNA cytotoxicity assay may be used for both ADCC and CDC assessment. This may be very useful for high-throughput testing in the discovery and selection process of therapeutic mAb candidates as well as the assessment of immune cell and humoral responses in clinical vaccine development [44, 45].

4. Conclusions

This paper reports the establishment of a highly suitable nonradioactive IVT RNA firefly luciferase-based cytotoxicity assay. By directly measuring intracellular luciferase activity, the assay efficiently assesses effector cell cytotoxicity mediated by antigen-specific CTLs when using cell lines and primary nondividing APCs as targets. The results generated were comparable to the gold-standard ^{51}Cr-release assay. Taking advantage of an optimized IVT RNA reporter, the approach is extremely sensitive and rapid and has a simple read-out procedure, rendering it applicable for high-throughput screening. In further support of this, the assay allows for the cotransfection of luciferase and the antigen-encoding RNA into the APCs followed by the subsequent monitoring of killing kinetics. The assay was adopted for the evaluation of ADCC and CDC by a cancer cell surface antigen directed mAb. Together, the properties of the developed assay render it an attractive approach for measuring cytotoxicity *in vitro*,

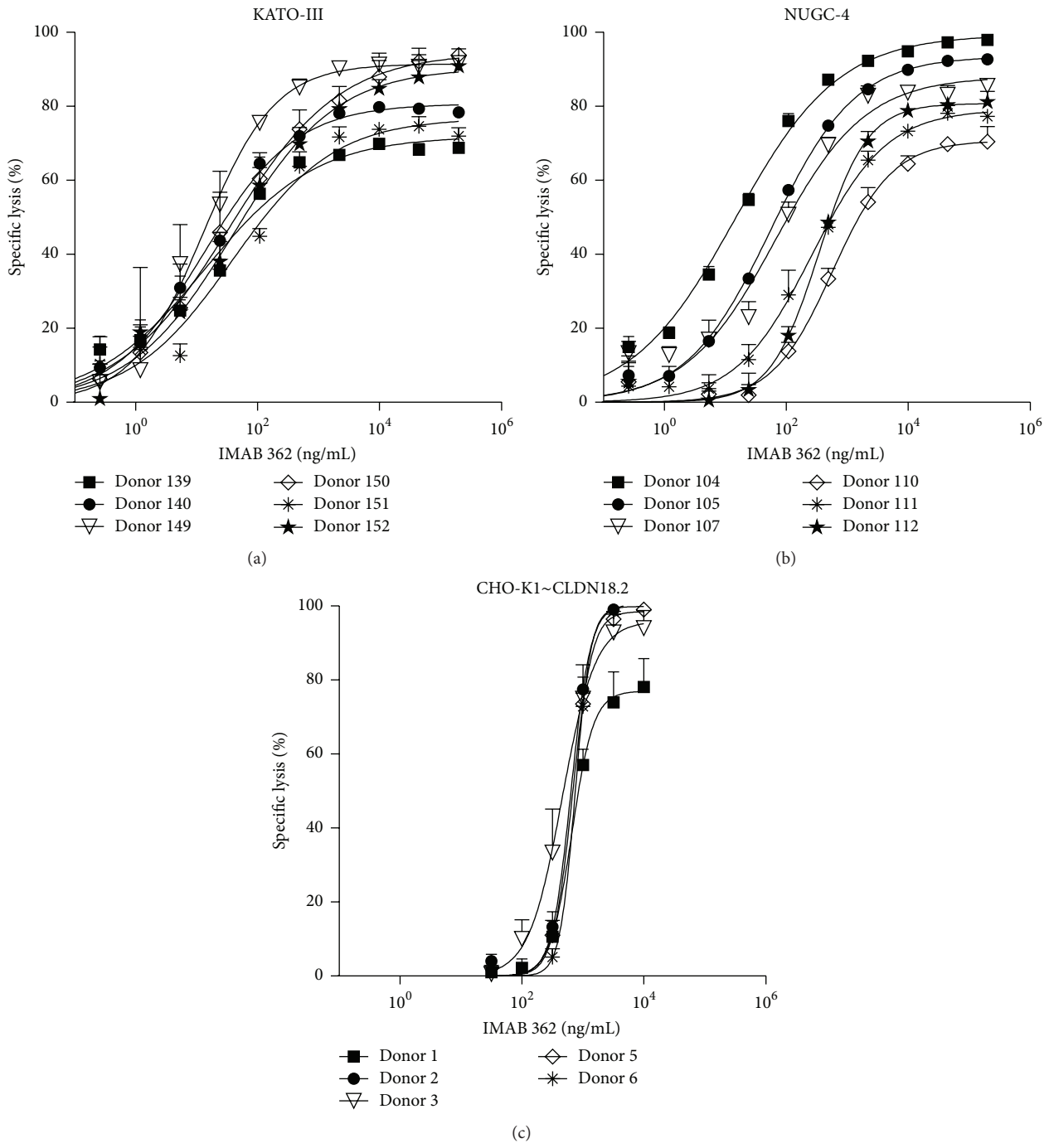


Figure 5: The luciferase IVT RNA-based assay efficiently assesses mAb-induced ADCC and CDC of tumour cell lines. ADCC assay using (a) KATO-III and (b) NUGC-4 cells. KATO-III and NUGC-4 cells endogenously expressing hCLDN18.2 were transfected with 7 μg *luc2* IVT RNA and seeded into 96-well plates independently. 4 h later, IMAB 362 at different concentrations and human PBMCs (E : T ratio = 40 : 1) from 6 different donors were added to the target cells and incubated for 24 h. ADCC was determined 40 and 45 min after addition of D-luciferin substrate to the KATO-III and NUGC-4 cells, respectively. (c) CDC assay. CHO-K1 cells stably expressing hCLDN18.2 were transfected with 7 μg *luc2* IVT RNA and seeded into 96-well plates. 24 h later, cells were incubated for 80 min with IMAB 362 diluted in human serum (final concentration of 20%) from 6 different healthy donors. CDC was determined 45 min after addition of D-luciferin substrate. Results are the mean ± SD (n = 3).

tailored for the use in the rapidly advancing tumor vaccine development, tumor-specific TCR, and mAb discovery fields.

Conflict of Interests

Ugur Sahin is associated with BioNTech RNA Pharmaceuticals GmbH (Mainz, Germany), a company that develops RNA-based cancer vaccines. Özlem Türeci and Ugur Sahin are inventors on patent applications featuring proprietary IVT RNA templates used in the process.

Authors' Contribution

Tana A. Omokoko and Uli Luxemburger contributed equally to the first authorship. Özlem Türeci and Ugur Sahin contributed equally to the last authorship.

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Review Article

Mutanome Engineered RNA Immunotherapy: Towards Patient-Centered Tumor Vaccination

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Advances in nucleic acid sequencing technologies have revolutionized the field of genomics, allowing the efficient targeting of mutated neoantigens for personalized cancer vaccination. Due to their absence during negative selection of T cells and their lack of expression in healthy tissue, tumor mutations are considered as optimal targets for cancer immunotherapy. Preclinical and early clinical data suggest that synthetic mRNA can serve as potent drug format allowing the cost efficient production of highly efficient vaccines in a timely manner. In this review, we describe a process, which integrates next generation sequencing based cancer mutanome mapping, *in silico* target selection and prioritization approaches, and mRNA vaccine manufacturing and delivery into a process we refer to as MERIT (mutanome engineered RNA immunotherapy).

1. Introduction

Somatic mutations are on the one hand a cause of cancer and drive the unlimited proliferation and malignant behavior of tumor cells. But on the other hand, the tens to hundreds of somatic nonsynonymous mutations [1] (the mutanome) displayed by a tumor are a rich source for highly specific targets for the recognition by cytotoxic and helper T cells with antitumor activity.

T cells are educated in the thymus, through a process called negative selection, to prevent the recognition of autoantigens. T cells readily recognize foreign antigens but in general are unable to recognize self-antigens, including most shared tumor antigens, with a high avidity. Mutated antigens on the other hand are not present in the thymus. Thus, the neoepitope-specific T cell repertoire is not affected by negative selection. Furthermore, as mutated antigens are only expressed in cancer cells, neoantigen-specific T cells would not cause on-target effects on healthy tissue. This renders mutated antigens ideal targets for therapeutic vaccination.

The importance of neoantigens in the rejection of transplantable murine tumors had already been recognized in the 1970s by Boon and colleagues [2, 3]. Only recently, however, the concurrence of technological and scientific breakthroughs has opened the way for exploitation of mutations for the development of truly personalized, mutation specific T cell vaccines. While deciphering the first human genome took about 13 years with a cost of about $2.7 billion [4], advances in next generation sequencing (NGS) make it possible today to sequence a genome, exome, or transcriptome within hours for approximately $1,000 [5]. This paved the way for a deeper understanding of neoantigen-specific T cells in cancer. Consequently, in 2012, we suggested that the mutanome could be exploited for tumor vaccination [6, 7]. Our team provided the preclinical proof of concept that NGS based mutation identification, followed by bioinformatic target selection and prioritization, could be utilized to produce a therapeutic vaccine that is effective in mice [6]. By now, several other groups demonstrated therapeutic

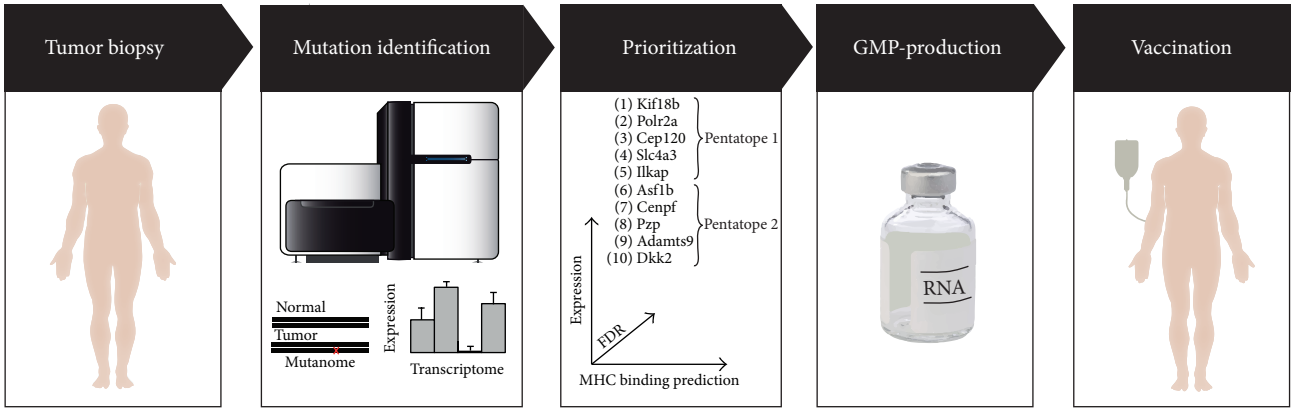

Figure 1: Concept of mutanome engineered RNA immunotherapy (MERIT). Next generation sequencing of nucleic acid from a tumor biopsy and healthy tissue is used to identify expressed, nonsynonymous, somatic mutations. Vaccine targets are selected based on several parameters such as expression, their MHC binding prediction, and restriction as well as a false discovery rate (FDR) [16]. Mutations encoded on pentatope RNAs are produced under GMP conditions and used for therapeutic vaccination.

efficacy of personalized vaccines with similar approaches [8–11]. Yadav and colleagues used mass spectrometry to select potential neoepitopes expressed on MHC class I molecules [9]. As pointed out by the authors, the complexity of mass spectrometry hampers its utility in a clinical setting.

Recent studies have further indicated the importance of neoantigen-specific T cells in the response against human tumors. Brown and coworkers showed that predicted neoepitopes, as well as CD8 and HLA-A expression, correlates with increased survival across different cancer types [12]. Furthermore, Snyder et al. [13, 14] and Tran et al. [15] recently demonstrated that mutation specific T cells play a pivotal role in the therapeutic efficacy of immune checkpoint blockade.

2. Concept

Putting the concept of personalized cancer vaccination into practice involves a step-wise process (Figure 1).

The tumor biopsy as source for the individual patient's DNA and RNA is retrieved. By comparison of exome sequencing data of healthy tissue and tumor DNA somatic nonsynonymous mutations are identified. Transcriptome sequencing of tumor RNA then provides information on the expression levels of identified mutations. Those neoantigens which are likely to induce a T cell response are to be selected. A vaccine encoding the targets of interest is manufactured, which finally is delivered to professional antigen-presenting cells such as dendritic cells (DCs) in combination with an adequate adjuvant. Each of these steps is critical for obtaining efficient and sustained immune responses and will be discussed in more detail in Sections 2.1 to 2.4.

2.1. Mutation Identification. NGS analysis of DNA and RNA for mutation detection requires a representative tumor and a healthy tissue sample. A blood sample is an easy to obtain source for healthy tissue and a few mL are sufficient for NGS analysis. Patients' tumors are rarely banked as fresh frozen samples. Therefore, we set up an optimized protocol for the efficient and reproducible isolation and NGS analysis of small amounts of nucleic acid from formaldehyde-fixed, paraffin-embedded (FFPE) samples as used in routine pathology. A few sections from FFPE blocks are sufficient and in our hands additional biopsies beyond those taken as part of routine diagnostic are not required. Generally speaking, mutation evaluation is error-prone and data depends on the algorithms used [16–18]. Moreover, correct identification of mutations within tumor samples may be compromised, by, for example, tumor heterogeneity and contaminations with healthy tissue or necrotic cells. For this reason we established a statistical value to gauge the false discovery rate (FDR) and accurately discriminate true mutations from erroneous calls [16].

2.2. Target Selection. Tumors of patients display hundreds of mutations. Selecting the right ones as neoantigens for vaccination is challenging and critical, as not every mutation is immunogenic. We perform *in vitro* immunogenicity testing of candidate mutations with blood samples of patients. These bioassays identify prevalent immune responses of the patient and thereby validate immunogenicity of mutations of interest. Against many mutations, however, there are no spontaneous immune responses in the patient and their potential as vaccination target is not easily assessable [19]. We address this by developing tools for *in silico* selection of targets. Research in murine tumor models revealed that, surprisingly, most immunogenic mutations are MHC class II restricted. Those $CD4^+$ T cells of a T_H1 subtype are attractive effectors as they were shown to exert a potent antitumoral effect [15, 19, 20]. By applying thresholds for MHC class II binding prediction and mRNA expression levels, we were able to enrich immunogenic MHC class II-restricted epitopes. Such purely *in silico* predicted mutations used for vaccination without further validation by immunogenicity testing result in efficient and sustained control of advanced tumors in mouse models. Vaccines based on mutations selected for abundant expression only did not succeed in controlling those tumors [19].

So far, the majority of groups focused on the selection of mutations for immunization based on their property to

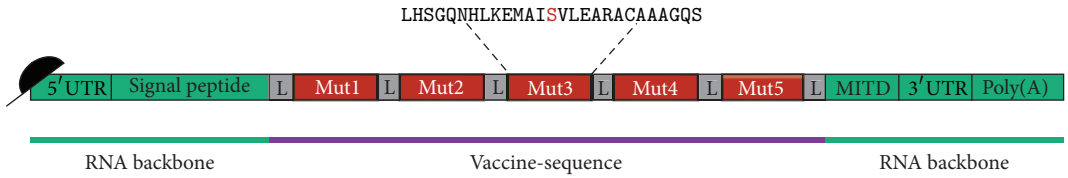

Figure 2: Structure of the pentatope RNA vaccine. Several modifications in the 5′ cap, 5′ and 3′ untranslated regions (UTR), poly(A) tail, and codon usage increased the translation efficiency and stability of the mRNA [30, 31]. Mutated sequences (Mut1–5) encoding 27 amino acids with the mutation in the center (red letter) are separated by nonimmunogenic 10 mer linkers. The antigen encoding sequences are flanked by a signal peptide and the MHC class I trafficking domain (MITD, transmembrane, and cytoplasmic domain of MHC class I) to ensure optimal antigen presentation [32].

be recognized by $CD8^+$ T cells, which appear to be rare [8, 21–23] as compared to $CD4^+$ T cell recognized ones. In comparison to cytotoxic $CD8^+$ T cells that directly kill tumor cells, $CD4^+$ T cells have a "catalytic" function within the immune system. $CD4^+$ T cells orchestrate the activity of many other cell types including macrophages, NK cells, B cells, DCs, and $CD8^+$ T cells or act on tumor cell survival by intratumoral secretion of inflammatory cytokines like IFN-γ [19, 24–26]. This further encourages approaches including mutations capable of inducing $CD4^+$ T cell responses into a mutation-based vaccine on top of MHC I binding ones.

2.3. Vaccine Format and Production. Effective personalized tumor vaccination requires a suitable vaccine format that bundles the following features: safety, cost-efficiency, and scalability under GMP conditions, stability, and, most importantly, a reliable induction of a proper T cell response that depends on the adjuvant capacity and targetability into antigen-presenting cells. In this regard, synthetic *in vitro* transcribed (IVT) mRNA is appealing for several reasons. A number of clinical studies proved the safety of mRNA vaccines per se [27, 28]. Furthermore, high amounts of IVT RNA can be manufactured at reasonable cost under GMP conditions, including synthesis of the DNA template, *in vitro* transcription, purification, and quality control. For clinical grade RNA as an investigational medicinal product, the correct identity, integrity, sterility, quantity, and functionality need to be monitored. mRNA can be stable for years in the absence of RNases. The rather short half-life of IVT mRNA, due to degradation by RNases, has so far compromised the direct *in vivo* use of RNA for vaccination. For this reason, mRNA vaccines are usually delivered via adoptive transfer of *in vitro* electroporated DCs [29]. Under GMP conditions and in large scale, this process is demanding and costly. In order to allow for direct use *in vivo*, our group introduced several improvements (e.g., 5′ cap modifications, stabilizing UTR sequences, and modified poly(A) tails). This leads to increased stability and translational efficacy of synthetic IVT mRNA by several thousandfold as compared to conventional mRNA resulting in expression of the respective antigen for days [30, 31]. Moreover, we introduced routing signals which enrich the encoded antigens in MHC I as well as MHC II loading compartment and thereby result in superior $CD8^+$ and $CD4^+$ T cell responses [32]. A further advantage of IVT mRNA is that several mutated epitopes can be easily encoded on the same RNA molecule. Multi-epitope formats could be beneficial for priming of $CD8^+$ T cells through improved $CD4^+$ T cell-mediated DC licensing [26]. Moreover, a polyclonal effector T cell response may act synergistically and allows addressing tumor heterogeneity and immune escape mechanisms. We therefore use synthetic RNA encoding five neoepitopes comprising selected MHC class I as well as MHC class II binders separated by nonimmunogenic 10 mer glycine/serine linkers (Figure 2). We demonstrated that such a "pentatope" RNA is more immunogenic than the mixture of equal amounts of the respective single epitopes encoded by RNA [19]. Synthetic mRNA as a class of drug was reviewed in more detail by Sahin and coworkers [33].

2.4. Vaccine Delivery and T Cell Priming. T cells are primed by "professional" antigen-presenting cells such as DCs in secondary lymphoid tissue like the spleen and lymph nodes. T cell priming requires the presentation of processed peptides of the antigen by MHC class I or class II molecules of a DC to a T cell receptor on T cells. Only in combination with a costimulatory signal by immunogenic DCs are T cells activated. In addition to the costimulatory signal, the cytokine milieu during the priming phase determines the fate of T cell differentiation [34]. Thus, delivery of the vaccine into sites of T cell priming and codelivery of an adjuvant that activates DCs to upregulate costimulatory molecules and secrete the appropriate cytokines are key for obtaining a meaningful vaccine response. IVT mRNA not only encodes the antigen but acts as a DC-maturating adjuvant as well. mRNA triggers inflammation by activation of several pattern recognition receptors such as Toll-like receptors (TLR) 3, 7, and 8, retinoic acid-inducible gene 1 (RIG-I), protein kinase R (PKR), and melanoma differentiation-associated protein 5 (MDA5) [33]. This results in the maturation of immunogenic DCs and secretion of the proper cytokines for priming of antitumor T_H1 cells and cytotoxic $CD8^+$ T cells.

In order to deliver the mRNA vaccine into DCs *in vivo*, we established two different methods. One is the direct injection of naked mRNA into the lymph node, where the RNA is taken up selectively and efficiently by DCs via macropinocytosis [35, 36]. We showed in mice that in terms of expansion of antigen-specific T cells, this approach was superior to subcutaneous, intradermal, and near nodal vaccinations [35]. The other is intravenous administration of a nanoparticle formulation [19]. This formulation encapsulates the synthetic RNA, thereby protecting it from degradation and thus optimizing its bioavailability. In addition, the IV route

ensures systemic delivery specifically in antigen-presenting cells, most importantly spleen-resident antigen-presenting cells. Alternatively, RNA can be administered intradermally [37] or intramuscularly [38]. All these administration routes are currently being tested in various preclinical and clinical studies (NCT01684241, NCT02410733).

Upon selective uptake by DCs into the cytosol [36], translation of the mRNA starts immediately [33]. Cytosolic proteins are usually C-terminally processed via the proteasome and transported by the TAP transporter into the endoplasmic reticulum (ER), where peptides can be further N-terminally truncated and loaded onto MHC class I molecules. $CD4^+$ T cell epitopes commonly derive from extracellular proteins loaded onto MHC class II molecules in the late endosome [39]. To ensure optimal antigen presentation of encoded proteins, not only for $CD8^+$ but also for $CD4^+$ T cell epitopes, we flanked the target sequences with a signal peptide and the trafficking domain (transmembrane and cytosolic domain) of MHC class I. The fusion protein is routed into the ER membrane from which it travels via the Golgi apparatus to the cell membrane and back, until it is degraded and loaded onto MHC class I or MHC class II molecules. This leads to increased antigen presentation, resulting in enhanced $CD4^+$ and $CD8^+$ T cell responses [32].

3. Preclinical and Clinical Proof of Concept

The first preclinical proof of concept for the mutanome engineered RNA immunotherapy (MERIT) integrating the above described aspects into one process was obtained in 2012 [6]. Sequencing of DNA, as well as RNA, of the C57BL/6-derived B16F10 melanoma cell line in comparison to healthy tissue revealed hundreds of targetable mutations. Immunogenicity and mouse tumor treatment studies with the mutations presented as peptide as well as mRNA vaccine format revealed that more than a third of the identified mutations were recognized by T cells (16/50) and that a fraction of these T cell responses were associated with tumor growth control and survival benefit in immunized mice. In CT26 and 4T1 tumor models of BALB/c background we confirmed that mutations are frequently immunogenic (21–45%) and capable of inducing meaningful control of advanced tumors in mice. Recently, MERIT entered trials in cancer patients and the lessons learned in the preclinical models were translated into the human setting. In the meantime, clinical trials exploring adoptive T cell transfer [15, 21, 40] or checkpoint blockade [11, 13, 14, 23, 40] data are supporting the notion that mutation recognizing T cells play a pivotal role in the therapeutic effect. Moreover, concepts similar to the MERIT approach, exploiting peptide or DC vaccines, proved successful as well [8, 9, 11, 22]. In a first-in-human clinical study, in which according to the MERIT approach an actively individualized mutation-based vaccine is manufactured for each and every patient ("IVAC mutanome," Phase I, NCT02035956) and administered intranodally, we are currently assessing the safety and tolerability, as well as the induction of cellular immune responses in melanoma patients. To bridge the time required for manufacturing of their individual mutanome vaccine, patients with positive tumors start with an mRNA vaccine encoding two shared antigens (NY-ESO-1 and Tyrosinase) and continue with the vaccine targeting ten of their mutations encoded on two penta-epitope (pentatope) RNAs [19]. As the assessment of treatment-emergent T cell responses is end-point relevant, scientifically sound and qualified bioassays (e.g., ELISpot, flow cytometric cytokine release, and tetramer technology) have to be used according to good clinical laboratory practice (GCLP) standards and documented according to MIATA [41].

4. Conclusions

Cancer therapy is moving from a drug-centered to a patient-centered paradigm. The MERIT approach integrates several highly innovative technologies into a process for a universally applicable, but truly personalized, tumor treatment and therefore initiates a paradigm shift in cancer therapy. Research in murine tumor models raises hope that this concept will be effective in humans as well and first clinical results seem promising. We believe that neoantigen-targeting immunotherapies, probably in combination with other therapies such as checkpoint blockade, will become a relevant part of cancer treatment in the near future.

Conflict of Interests

Ugur Sahin and Mathias Vormehr are employees at BioNTech AG (Mainz, Germany). Ugur Sahin is stock owner of BioNTech AG. Mathias Vormehr, Barbara Schrörs, Sebastian Boegel, Martin Löwer, Özlem Türeci, and Ugur Sahin are inventors on patents and patent applications, which cover parts of this review.

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Research Article

Electroporated Antigen-Encoding mRNA Is Not a Danger Signal to Human Mature Monocyte-Derived Dendritic Cells

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For therapeutic cancer vaccination, the adoptive transfer of mRNA-electroporated dendritic cells (DCs) is frequently performed, usually with monocyte-derived, cytokine-matured DCs (moDCs). However, DCs are rich in danger-sensing receptors which could recognize the exogenously delivered mRNA and induce DC activation, hence influencing the DCs' immunogenicity. Therefore, we examined whether electroporation of mRNA with a proper cap and a poly-A tail of at least 64 adenosines had any influence on cocktail-matured moDCs. We used 16 different RNAs, encoding tumor antigens (MelanA, NRAS, BRAF, GNAQ, GNA11, and WT1), and variants thereof. None of those RNAs induced changes in the expression of CD25, CD40, CD83, CD86, and CD70 or the secretion of the cytokines IL-8, IL-6, and TNFα of more than 1.5-fold compared to the control condition, while an mRNA encoding an NF-κB-activation protein as positive control induced massive secretion of the cytokines. To determine whether mRNA electroporation had any effect on the whole transcriptome of the DCs, we performed microarray analyses of DCs of 6 different donors. None of 60,000 probes was significantly different between mock-electroporated DCs and MelanA-transfected DCs. Hence, we conclude that no transcriptional programs were induced within cocktail-matured DCs by electroporation of single tumor-antigen-encoding mRNAs.

1. Introduction

During the last decade, immunotherapy has evolved as a new pillar of cancer treatment [1]. Therapeutic vaccination with dendritic cells (DCs) is a safe and well-established strategy [2–4]. A deeper understanding of DC maturation and activation together with efficient, GMP-compliant and reproducible antigen- (Ag-) loading strategies is the key to success. One technology that has proven suitable in this context is mRNA transfection [3, 5, 6], which can be utilized, on the one hand, to load mature DCs with tumor antigen [7–9], and on the other hand, to deliver maturation and activation signals to the DCs. The latter is usually achieved by using mRNA that encodes DC-activating proteins, like constitutively active inhibitor of kappa B kinase (IKK) [10] or CD40L, alone [11], or combined with a constitutively active TLR [12].

However, since DCs comprise a whole battery of nucleic acid receptors on their surface, in their endosomes, and in their cytoplasm [13, 14], the transfected mRNA itself, independently from the encoded protein, may deliver a maturation signal. Single-stranded (ss)RNA was reported to activate TLR7 and TLR8 on DCs [15–17] and TLR3 can be activated by short double-strand stretches in exogenous mRNA [18, 19]. RIG-I-like receptors (RLR) recognize various viral RNA species in the cytoplasm [20] and may be capable of sensing transfected RNA as well. Bacterial RNA is a potent DC maturation stimulus, but the specific receptors are yet unknown [21].

Table 1: mRNAs used for transfection.

Antigen	Description	Abbreviation
MelanA	MelanA (MART1) full length wild type	MelanA
NRAS	NRAS-fragment 40 AA around mutation site 61 with DC-LAMP signal and flag-tag	NRAS-DCL
NRAS Q61K	NRAS-fragment 40 AA around mutation Q61K with DC-LAMP signal and flag-tag	NRAS-DCL K
NRAS Q61R	NRAS-fragment 40 AA around mutation Q61R with DC-LAMP signal and flag-tag	NRAS-DCL R
BRAF	BRAF-fragment 67 AA around mutation site 600 with flag-tag	BRAF
BRAF V600E	BRAF-fragment 67 AA around mutation V600E with flag-tag	BRAF E
BRAF	BRAF-fragment 67 AA around mutation site 600 with DC-LAMP signal and flag-tag	BRAF-DCL
BRAF V600E	BRAF-fragment 67 AA around mutation V600E with DC-LAMP signal and flag-tag	BRAF-DCL E
GNAQ	GNAQ-fragment 47 AA around mutation site 209 with DC-LAMP signal and flag-tag	GQ-DCL
GNAQ Q209P	GNAQ-fragment 47 AA around mutation Q209P with DC-LAMP signal and flag-tag	GQ-DCL P
GNAQ Q209L	GNAQ-fragment 47 AA around mutation Q209L with DC-LAMP signal and flag-tag	GQ-DCL L
GNA11	GNA11-fragment 47 AA around mutation site 209 with DC-LAMP signal and flag-tag	G11-DCL
GNA11 Q209P	GNA11-fragment 47 AA around mutation Q209P with DC-LAMP signal and flag-tag	G11-DCL P
GNA11 Q209L	GNA11-fragment 47 AA around mutation Q209L with DC-LAMP signal and flag-tag	G11-DCL L
WT-1	Wilms tumor 1 full length wild type	WT1
GNAQ	Wilms tumor 1 full length with DC-LAMP signal and flag-tag	WT1-DCL
IKKβ[1]	Constitutively active stabilized IKKβ to activate NF-κB	IKKβ

[1]Not a tumor antigen, but a DC-activating protein.

In the design of clinical vaccination protocols, it is, however, pivotal to know which maturation program is induced in the DCs and whether and how any additional maturation stimulus might distort the intended mature phenotype of the vaccine DCs. When DCs (usually monocyte-derived ones) are generated, matured, and Ag-loaded for clinical application, one requires a well-defined product, and any remaining insecurity about any factors that influence the phenotype of the DCs should be resolved.

Hence, we took the effort to carefully compare monocyte-derived cytokine-matured DCs that were electroporated without RNA with DCs that were electroporated with various *in vitro*-transcribed mRNAs. We analyzed the DCs' phenotype and cytokine secretion and, for mRNA, encoding the tumor antigen MelanA, a transcriptome analysis was performed, to detect if any of these features would be changed by the introduced mRNA.

2. Materials and Methods

2.1. Cells and Reagents. Monocyte-derived dendritic cells (DCs) were generated from blood, obtained from healthy donors following informed consent and approval by the institutional review board as described before [9]. PBMCs were purified by density centrifugation, and monocytes were separated from the nonadherent fraction (NAF) by plastic adherence and differentiated to DCs over 6 days in DC medium (RPMI 1640 (Lonza, Verviers, Belgium) containing 1% heat-inactivated autologous plasma, 2 mM L-glutamine (Lonza), and 20 mg/L gentamicin (PAA, Pasching, Austria)) with GM-CSF (800 IU/mL; CellGenix, Freiburg, Germany, PeproTech, Hamburg, Germany, and Miltenyi Biotec, Bergisch Gladbach, Germany) and IL-4 (250 IU/mL; CellGenix, PeproTech, and Miltenyi Biotec) in the absence of fetal calf serum, as described before [9]. DCs were matured (mDCs) on day 6 for 24 h with 200 IU/mL IL-1β (CellGenix), 1000 U/mL IL-6 (CellGenix), 10 ng/mL TNFα (Beromun, Boehringer Ingelheim Pharma, Germany), and 1 μg/mL PGE_2 (Pfizer, Zurich, Switzerland). mDCs were used for electroporation with mRNA after maturation.

2.2. In Vitro RNA Transcription and Electroporation of DCs. *In vitro* transcription of mRNA from pGEM4Z64A vectors was performed as described previously [9] with Life Technologies mMESSAGE mMACHINE T7 ULTRA kits according to the manufacturer's instructions. DCs were electroporated with different mRNAs (Table 1) as described in [10, 22]. As a control, mDCs were electroporated without mRNA.

2.3. Cell Surface Marker Analysis. mDCs were electroporated as described above, incubated in DC medium at 37°C in a humidified incubator, and harvested 24 h after electroporation. The expression of distinct markers was analyzed by flow cytometry. For the determination of surface marker expression, the following antibodies and their respective isotype controls were used: IgG1-PE, anti-CD25-PE, anti-CD40-PE, anti-CD70-PE, anti-CD80-PE, anti-CD83-PE, anti-CD86-PE (all from BD), and IgG3-PE (eBioscience). Seventy-five to one hundred thousand cells were incubated with antibody for 30 minutes at 4°C in FACS solution, consisting of PBS supplemented with 1% FCS (PAA, GE healthcare) and 0.02% sodium azide (Merck). The cells were then washed once with FACS solution and immunofluorescence was measured using a FACScan cytofluorometer equipped with CellQuest software (BD Biosciences). mDCs were gated on in the forward and side scatter channels and the mean fluorescence

intensities (MFIs) were measured. Specific MFI was calculated by subtraction of the MFI of the isotype control.

2.4. Cytokine Secretion Analysis. mDCs were electroporated as described above and were incubated in DC medium at 37°C in a humidified incubator, and supernatants were taken 24 h after electroporation. Cytokine concentrations were analyzed with an Inflammation Cytometric Bead Array (BD, Heidelberg, Germany) following the manufacturer's instructions.

2.5. Statistical Analysis. We performed a 1-way ANOVA with multiple comparison test (according to Dunnet/Tukey) with a confidence level of 0.05 using GraphPad Prism V6.02 to determine statistically significant differences for the surface staining data (Figure 2) with the unadjusted mean fluorescence intensities, the percentage values of positive cells, and the cytokine concentrations (Figures 3 and 4). For the data in Figures 2 and 3, the multicomparison was performed according to Dunnet against the mock condition. For the data in Figure 4, all conditions were compared to each other according to Tukey.

2.6. Cryoconservation of Cell Pellets. mDCs were electroporated as described above, were incubated in DC medium at 37°C in a humidified incubator, and were harvested 4 h after electroporation. One to two hundred thousand cells were centrifuged for 10 min at 10.000 rpm at 4°C and the supernatant was removed. The cell pellet was frozen and stored in liquid nitrogen until microarray analysis.

2.7. Microarray Analysis. Cryoconserved electroporated mDCs were sent to Miltenyi Biotec for microarray analysis. Cells were lysed, mRNA was isolated and reverse-transcribed to cDNA, the cDNA was amplified, and Cy3 was labeled and then hybridized to Agilent Whole Human Genome Oligo Microarrays (8 × 60 K). Fluorescence signals of the hybridized Agilent Microarrays were detected using Agilent's Microarray Scanner System (Agilent Technologies). The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. The resulting text files produced by FES were then processed for quality control, removing control probes and probes flagged as unreliable by the scanning software. The raw data underwent background correction to eliminate background noise and local fluctuations. To this end, the normal-exponential convolution method was used (Normexp). Next, the data were normalized to correct chip-related variations in the signal intensity (e.g., labeling and hybridization inefficiencies). To this end, the quantile method with offset = 16 was applied to the data.

Unadjusted p values were calculated with Student's t-test and the Benjamini Hochberg method for False Discovery Rate was used to adjust the p value and find differentially expressed genes. Data processing and analysis were performed in the software R computing environment (version 3.0.2) using the Bioconductor (version 3.1) package "limma" (linear models for microarray and RNA-seq data) described in [23].

3. Results

3.1. mRNA Electroporation into Human Cocktail-Matured, Monocyte-Derived DCs Results in a High Transfection Efficiency. To formally show that mRNA electroporation results in protein expression in the cocktail-matured, monocyte-derived DCs, we generated these DCs and electroporated them either without RNA (mock) or with RNA encoding the tumor antigen MelanA (Figure 1). These DCs were produced by a highly standardized and validated process, which is approved for DC generation for clinical applications [24]. Hence, the product is very well known considering the phenotype of the DCs. Therefore, we did not include a typical DC-specific marker in these experiments but rather focused on maturation and activation markers on these DCs. The DCs displayed a mature phenotype, which was not altered by transfection with MelanA-encoding mRNA (Figures 1(a) and 1(b)). Four and twenty-four hours after electroporation, the intracellular MelanA expression was determined by flow cytometry. As shown in Figure 1(c), MelanA expression was detected at both time-points. The transfection efficiency at 4 h was >95% (Figure 1(c); left panel). Due to the transiency of mRNA transfection, the MelanA expression had decreased at the 24 h time-point (Figure 1(c); right panel). These data show that the electroporated mRNA enters the cytoplasm of the vast majority of the mature DCs very efficiently.

3.2. mRNA Electroporation into Human Cocktail-Matured, Monocyte-Derived DCs Has No Influence on the Phenotype of These Cells. As it was suggested that introduction of mRNA could trigger intracellular TLRs or other receptors [15–21], we examined whether mRNA electroporation has an influence on the phenotype of cocktail-matured, monocyte-derived DCs. We electroporated these DCs either without RNA (Figure 2; mock) or with a panel of 16 different RNAs encoding tumor antigens, or parts thereof (MelanA, NRAS, BRAF, GNAQ, GNA11, and WT1), either mutated or not, and either linked to the lysosomal targeting signal DC-LAMP or not (Table 1). Twenty-four hours after electroporation, the surface expression of CD25, CD40, CD86, CD70, and CD83 was determined by flow cytometry. When looking at mean fluorescence intensities (MFIs), electroporations with the 16 different RNAs encoding different tumor antigens resulted in differences in cell surface marker expression of less than 1.5-fold compared to mock-electroporated DCs (Figure 2(a)). No large differences in expression of these markers were observed when looking at percent positive cells (Figure 2(b)). According to a 1-way ANOVA with multiple comparison test, no statistically significant differences were present within the surface staining data ($p > 0.05$). We did not measure expression of MHC-class II, as we had observed before that there are no big changes on human monocyte-derived DCs, even after activation of NF-κB (data not shown).

From these data, we can conclude that the introduction of mRNA into human cocktail-matured, monocyte-derived

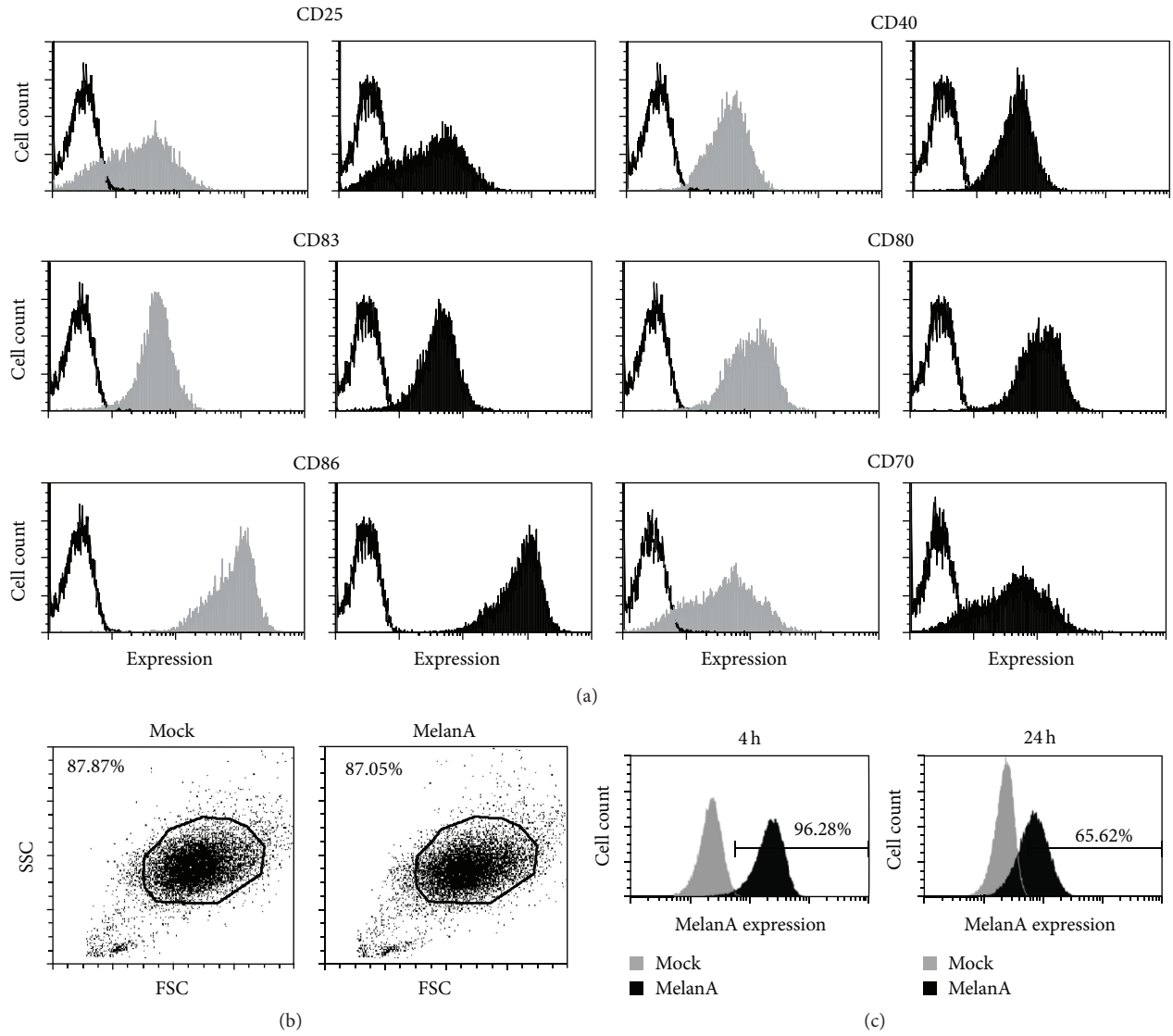

Figure 1: MelanA is expressed in cocktail-matured, monocyte-derived DCs after mRNA electroporation. DCs were either electroporated without mRNA (mock; gray histogram) or with mRNA encoding the tumor antigen MelanA (MelanA; black histogram). (a) Surface marker expression of CD25, CD40, CD83, CD86, CD70, and CD80 on mock-electroporated and MelanA-RNA-electroporated DCs 24 h after electroporation is shown (black lines; respective isotype controls). One representative of ≥4 experiments is shown. (b) Gating of mock-electroporated (Mock) or MelanA-RNA-electroporated (MelanA) DCs was performed according to forward and side scatter. (c) Four and twenty-four hours after electroporation, the intracellular MelanA expression was determined by flow cytometry. The percentage of positive cells is indicated. One representative of >10 experiments is shown.

DCs by electroporation did not result in a relevant change of the phenotype of these cells.

3.3. mRNA Electroporation into Human Cocktail-Matured, Monocyte-Derived DCs Has No Influence on the Cytokine Secretion by These Cells. Next, we investigated whether mRNA electroporation has an influence on the cytokine secretion of cocktail-matured, monocyte-derived DCs. We electroporated DCs either without RNA (Figure 3; Mock) or with the 16 different RNAs encoding tumor antigens and harvested the supernatants of the cells 24 h after electroporation to determine the cytokine secretion in a cytometric bead array. Within 24 h after electroporation, the DCs hardly any IL-1β, IL-10, and IL-12p70 (data not shown) but produced measurable quantities of IL-8, IL-6, and TNFα (Figure 3). However, mock-electroporated DCs also secreted these cytokines, and the electroporations with the 16 different RNAs, encoding different tumor antigens, resulted in a difference in cytokine secretion of maximum 1.5-fold compared to mock-electroporated DCs (Figure 3). According to a 1-way ANOVA with multiple comparison test,

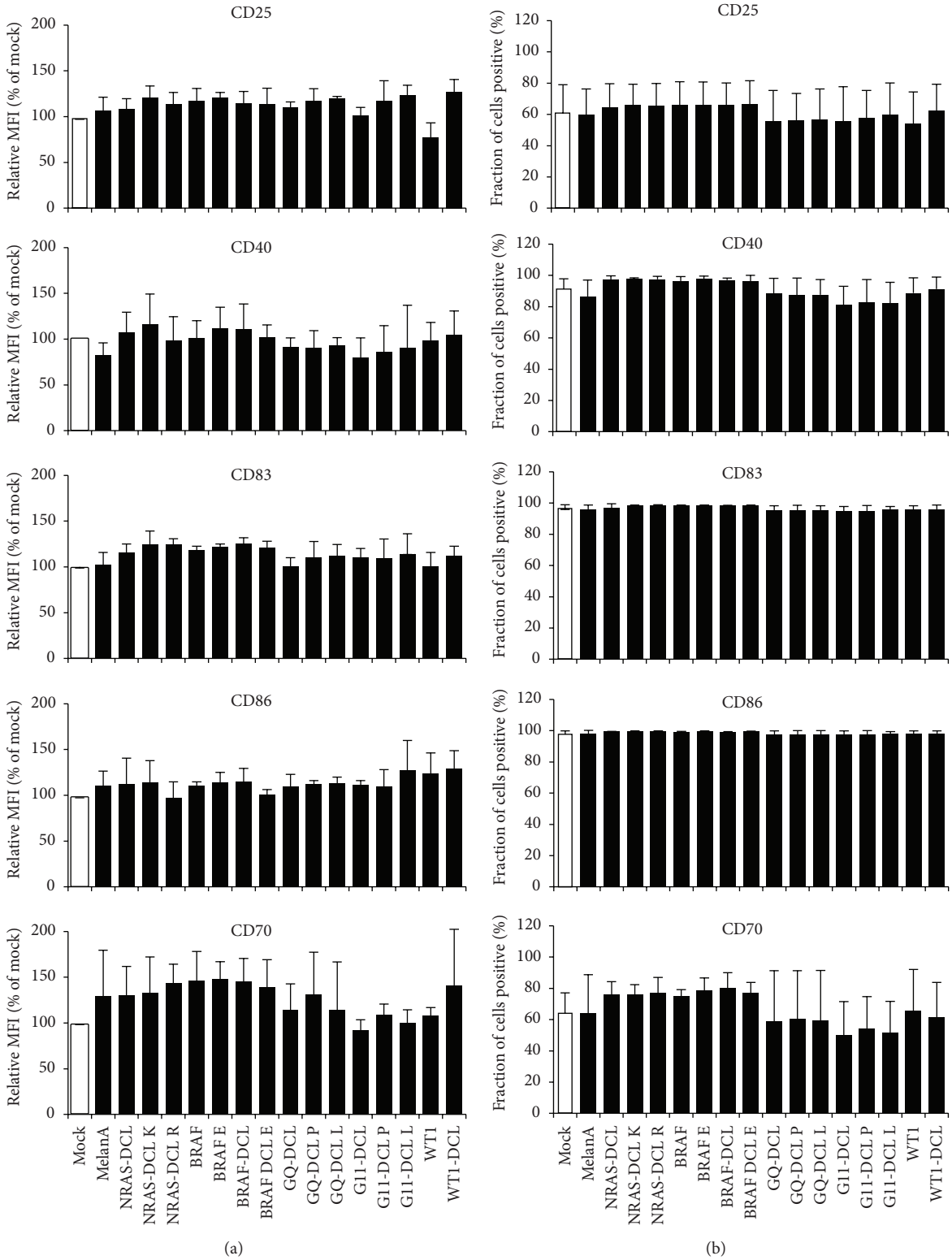

Figure 2: No difference in phenotype between mock- and mRNA-electroporated DCs. DCs were either electroporated without RNA (Mock) or a panel of 16 RNAs encoding different tumor antigens, either mutated or not and either linked to the lysosomal targeting signal DC-LAMP or not (see Table 1). Twenty-four hours after electroporation, the surface expression of CD25, CD40, CD86, CD70, and CD83 was determined by flow cytometry. Surface marker expression of mock-electroporated DCs was put at 100% and marker expression after electroporation of the mRNAs was put in relation to that (a), or percent positive cells are shown (b). Shown are averages of at least 3 independent experiments. Error bars indicate the standard deviation (SD). According to a 1-way ANOVA with multiple comparison test, no statistically significant differences were present within the surface staining data ($p > 0.05$).

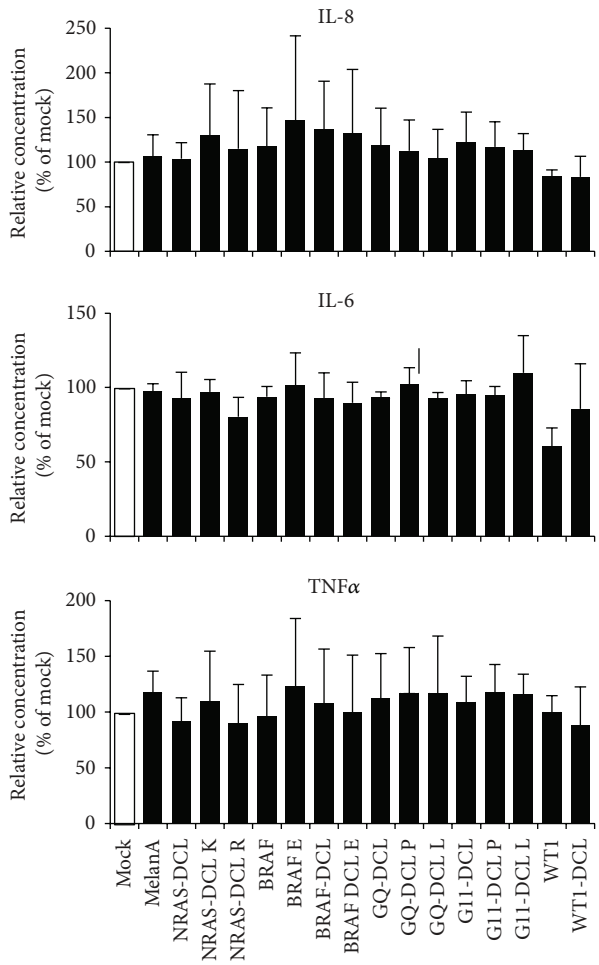

Figure 3: No difference in cytokine secretion between mock- and MelanA-RNA-electroporated DC. DCs were electroporated either without RNA (Mock) or with a panel of 16 RNAs encoding different tumor antigens, either mutated or not, and either linked to the lysosomal targeting signal DC-LAMP or not (see Table 1). Twenty-four hours after electroporation, supernatants of the cells were taken and the cytokine secretion by the cells was determined in a cytometric bead array. Cytokine secretion of mock-electroporated DCs was defined as 100% and concentrations after electroporation of the mRNAs were put in relation to that. Shown are averages of at least 3 independent experiments. Error bars indicate the standard deviation (SD). According to a 1-way ANOVA with multiple comparison test, no statistically significant differences were present within the cytokine secretion data ($p > 0.05$).

no statistically significant differences were present within the cytokine secretion data ($p > 0.05$).

These data show that the electroporated mRNAs also had no relevant influence on cytokine secretion by human cocktail-matured, monocyte-derived DCs.

Due to the fact that the secretion of IL-8, IL-6, and TNFα was clear but at low quantities, we wanted to formally prove that our DCs were able to produce these cytokines at higher quantities, when properly activated under similar conditions. Therefore, we electroporated the mature DCs either without RNA or with RNAs encoding MelanA combined or not with RNA encoding a constitutively active form of IKKβ, which is, on the protein level, able to activate the NF-κB pathway in the DCs [10]. Indeed, we saw that transfection with constitutively active IKKβ resulted in high IL-8, IL-6, and TNFα secretion (Figure 4), proving that our DCs can produce these cytokines at high quantities. In addition, there was no difference between the cytokine secretion by mock-transfected and MelanA-transfected DCs (Figure 4), again showing that mRNA transfection *per se* did not induce cytokine production in DCs. The 1-way ANOVA with multiple comparison test showed that the IKKβ-transfected DCs were highly significantly different from the mock and MelanA conditions but that the mock and MelanA conditions were not.

3.4. Microarray Analysis Reveals No Differentially Expressed Genes between Mock- and MelanA-Transfected DCs. Although we found no obvious differences in the expression of a handful of surface markers and the secretion of half a dozen of cytokines upon mRNA electroporation, we still could not exclude that the exogenous mRNA would induce signaling within the DCs, which, by chance, would regulate other target genes and modulate the expression of other factors. To explore in more detail whether mRNA electroporation has any effect on the transcriptome of the DCs, we performed GeneChip microarray analyses with matured DCs (mDCs) of 6 independent donors, which had either been mock-electroporated or electroporated with MelanA RNA. DCs were harvested and frozen 4 h after electroporation, and samples were hybridized to Agilent Whole Human Genome Oligo Microarrays (8×60 K). Fluorescence signals of the hybridized Agilent Microarrays were determined, preprocessed, and normalized. Afterwards, differentially expressed genes were calculated and significance was examined by Student's t-test and subsequent adjustment using the Benjamini Hochberg method for False Discovery Rate. These calculations were performed using the limma (linear models for microarray and RNA-seq data) software package for the Bioconductor R computing environment [23] (see Section 2). The expression levels of all the microarray probes are compared directly in Figure 5 to depict the degree of difference between the two sample groups. The scatter plot shows that the individual values are close to the identity function (Figure 5), except for a small number of outliers, of which none was significant (Figure 5). Indeed, none of the probes showed a significant difference between mock-electroporated DCs and MelanA-electroporated DCs, and the adjusted p values were all above 0.99995, indicating that no differentially expressed genes (DEGs) were present. This suggests that the difference at the transcriptional level between mock-electroporated DCs and MelanA-electroporated DCs is negligible.

4. Discussion

In this study, we have shown that electroporation of antigen-encoding RNA into matured monocyte-derived dendritic cells (moDCs) had no influence on the phenotype of these

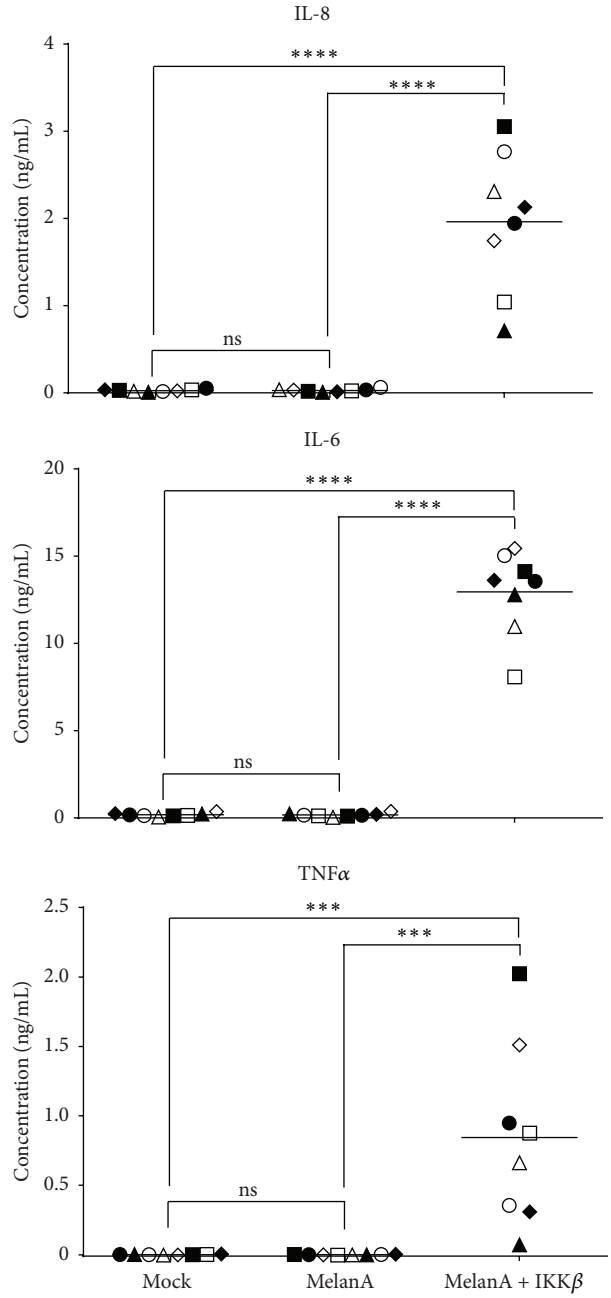

Figure 4: Massive cytokine secretion after electroporation with mRNA encoding a DC-activating protein. DCs were electroporated either without RNA (Mock) or with RNA encoding the tumor antigen MelanA (MelanA) or MelanA and constitutively active stabilized mutated IKKβ. Twenty-four hours after electroporation, supernatants of the cells were taken and the cytokine secretion by the cells was determined in a cytometric bead array. The cytokine concentrations in the supernatants are depicted. Each symbol represents an individual donor, tested in an independent experiment ($n = 8$). The horizontal bars show the average values. p values were calculated by the 1-way ANOVA with multiple comparison test (according to Tukey) with a confidence level of 0.05; ns: $p > 0.05$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$.

DCs and on the cytokine secretion by these DCs and even that there was no influence of the RNA on the transcriptome of the DCs. This is pivotal information for the use of mRNA-electroporated moDCs in a clinical setting, since it is necessary to generate vaccines of consistent quality by a stable production process, no matter what antigen-encoding RNA is used for electroporation, and it shows that our matured moDCs are of a robust phenotype. Since we [9] and others [8, 11, 25] observed that DCs, which were antigen-electroporated after cytokine-maturation, seemed to perform better, we limited our analysis to DCs matured and electroporated in that order. Hence, we cannot say anything about the influence of mRNA electroporation into DCs prior to maturation and can only speculate that immature

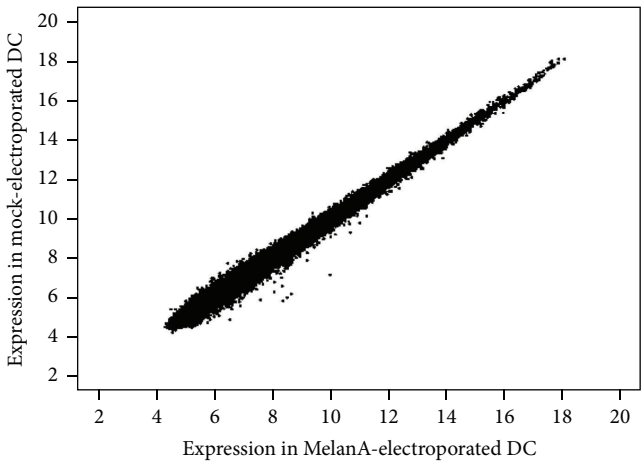

Figure 5: No difference in microarray analysis between mock- and MelanA-RNA-electroporated DCs. Scatter plot of the probe set expression levels for MelanA-RNA-electroporated DCs versus Mock-electroporated DCs samples. Data from 6 samples per experimental condition were processed for background correction and normalization. In the figure, the average probe intensity for each experimental condition is visualized.

DCs might be more susceptible for mRNA-mediated signals. However, this should be investigated in separate studies and is beyond the scope of this paper.

Other researchers have indeed observed mRNA-induced maturation of DCs, however under different experimental conditions [19, 26]. Ceppi and coworkers, who worked with porcine monocyte-derived DCs, observed that DC activation can occur after exogenous delivery of mRNA. Lipofection of mRNA induced maturation of immature porcine DCs, that is, MHC class II and CD80/CD86 upregulation [19]. An important element therein is the lipofection-induced production of type I IFN by the DCs, which also showed evidence of maturation. The DC activation was caused by the double-stranded secondary structures formed by the transfected mRNA, and the effect depended on the quantity of lipofected mRNA [19]. It is well established that viral or synthetic double-stranded RNA (dsRNA) acts as a danger signal to DCs, inducing them to produce IFNα/β and to mature [27]. Furthermore, it was reported that mRNA lipofection has the capacity to activate DCs (human moDCs) [26]. These authors noted the upregulation of activation markers, like CD25, CD80, CD83, CD86, MHC class I, and MHC class II, and cytokine production, like IL-12, IFNα, and TNFα [26].

Although our different antigen-encoding mRNAs can fold and form dsRNA stretches according to the Mfold Web Server (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form) [28] and thus in theory can also stimulate dsRNA-sensing receptors, we did not observe any DC activation. This can be explained by two main differences of our experiments compared to the experimental setup used by Ceppi et al. and Ni et al.

We used moDCs, which had been matured with a cocktail containing IL-1β, TNFα, IL-6, and PGE_2 before the mRNA was introduced, while in the other publications the mRNA was introduced into immature DCs. It might be that the weak stimulus of the introduced mRNA is just not able to change the robust mature phenotype of the cytokine-matured DCs. However, sensing of RNA by receptors should still induce a difference in the transcriptome.

Therefore, it is more plausible that our antigen-encoding RNAs are simply not sensed by the corresponding receptors, because they do not reach the compartments containing these receptors. Indeed it was shown that the RNA must reach the active TLRs in the endolysosomal compartment to be recognized by TLR7, TLR8, and TLR9 and that self-nucleic acids do not enter the TLR-sensing compartment under normal physiological conditions (reviewed in [13]). Only after entering the endolysosomal compartment, the TLRs are activated upon cleavage by resident pH-dependent proteases. This mechanism prevents that self-nucleic acids at different locations in the cell are recognized by the TLRs. Once activated, the TLRs themselves cannot distinguish between foreign and self-nucleic acids; however, the latter do not encounter the active receptors [29]. Normal "naked" mRNA is rapidly degraded by endolysosomal RNas before the receptor is activated. However, if the RNA is protected by lipids, which is the case with lipofection in the publications of Ceppi et al. and Ni et al., or stabilized by protamine [30], or protected by virus particles (reviewed in [13]), it is stable enough to enter the endolysosomal compartment where it is recognized by the activated TLRs resulting in an activation of the DCs.

5. Conclusion

Taken together, our data show that electroporation of mature monocyte-derived DCs with antigen-encoding RNA does not deliver a danger signal to the DCs and does not result in a change of the DCs. This is important knowledge for the scientific community using these DCs in vaccination trials, where a stable and robust cell type is needed.

Disclosure

Stefanie Hoyer, Kerstin F. Gerer, Isabell A. Pfeiffer, Sabrina Prommersberger, Sandra Höfflin, and Tanushree Jaitly share first authorship. Niels Schaft and Jan Dörrie share senior authorship.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Towards Targeted Delivery Systems: Ligand Conjugation Strategies for mRNA Nanoparticle Tumor Vaccines

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The use of nanoparticles encapsulating messenger RNA (mRNA) as a vaccine has recently attracted much attention because of encouraging results achieved in many nonviral genetic antitumor vaccination studies. Notably, in all of these studies, mRNA nanoparticles are passively targeted to dendritic cells (DCs) through careful selection of vaccination sites. Hence, DC-targeted mRNA nanoparticle vaccines may be an imminent next step forward. In this brief report, we will discuss established conjugation strategies that have been successfully applied to both polymeric and liposomal gene delivery systems. We will also briefly describe promising DC surface receptors amenable for targeting mRNA nanoparticles. Practicable conjugation strategies and receptors reviewed in this paper will provide a convenient reference to facilitate future development of targeted mRNA nanoparticle vaccine.

1. Introduction

Messenger RNA (mRNA) has achieved great success in an increasing number of biological applications. Apropos, the notion of nonviral genetic vaccination is also increasingly associated with mRNA instead of DNA. Given a mature drug and gene delivery field, mRNA nanoparticle delivery science is often deferred or closely compared with DNA and siRNA systems [1, 2]. However, as various reports have shown, unique properties of mRNA delivery exist [3, 4] and continue to be a relevant research focus today. mRNA delivery science has made significant progress since the first demonstration of cell based mRNA tumor vaccine delivery via RNA loaded DCs [5]. They include the optimization of the mRNA molecular structure [6, 7], direct *in vivo* administration of mRNA [8, 9], delivery routes [3, 4], evaluation of rationally designed gene carriers [10–14], and, recently, self-replicating RNA [15].

Along this developmental trajectory, DC-targeted nanoparticle gene delivery systems may be an imminent next step forward for nonviral tumor vaccine delivery. In this brief report, established conjugation strategies for both polymeric and liposomal gene delivery systems will be described. This will be followed by a brief discussion on three promising DC receptors that are suitable for targeted delivery of mRNA nanoparticles for tumor vaccination.

2. Ligand Conjugation Strategies for Gene Delivery Systems

Ligands targeting surface receptors on DCs are molecules grafted onto surfaces of formulated nanoparticles, recognizable by DC-specific uptake mechanisms, and endow nanoparticles with the ability to be taken up exclusively by them. This has the benefit of reducing effective doses of vaccine required through nonspecific uptake by other cell types. In the case of vaccines, which typically contains proinflammatory adjuvant molecules, a decreased dose also has the benefit of reducing undesired side effects. Since a wide variety of nanoparticle delivery systems exist, different ligand conjugation strategies have been developed. In this section, we will discuss three conjugation strategies that are most often applied to gene delivery systems.

First, nanoparticles with solid cores such as poly(lactic-co-glycolic acid) (PLGA) and inorganic nanoparticles (e.g., gold nanospheres, calcium phosphate) possess excellent colloidal stability such that ligands can be covalently conjugated directly onto particles surfaces without aggregation. In PLGA systems, nanoparticles are formulated by emulsion techniques [16–18] using PLGA-PEG-COOH copolymer, which can be synthesized by grafting PEG-COOH onto the ends of PLGA [19]. The resultant mRNA infused

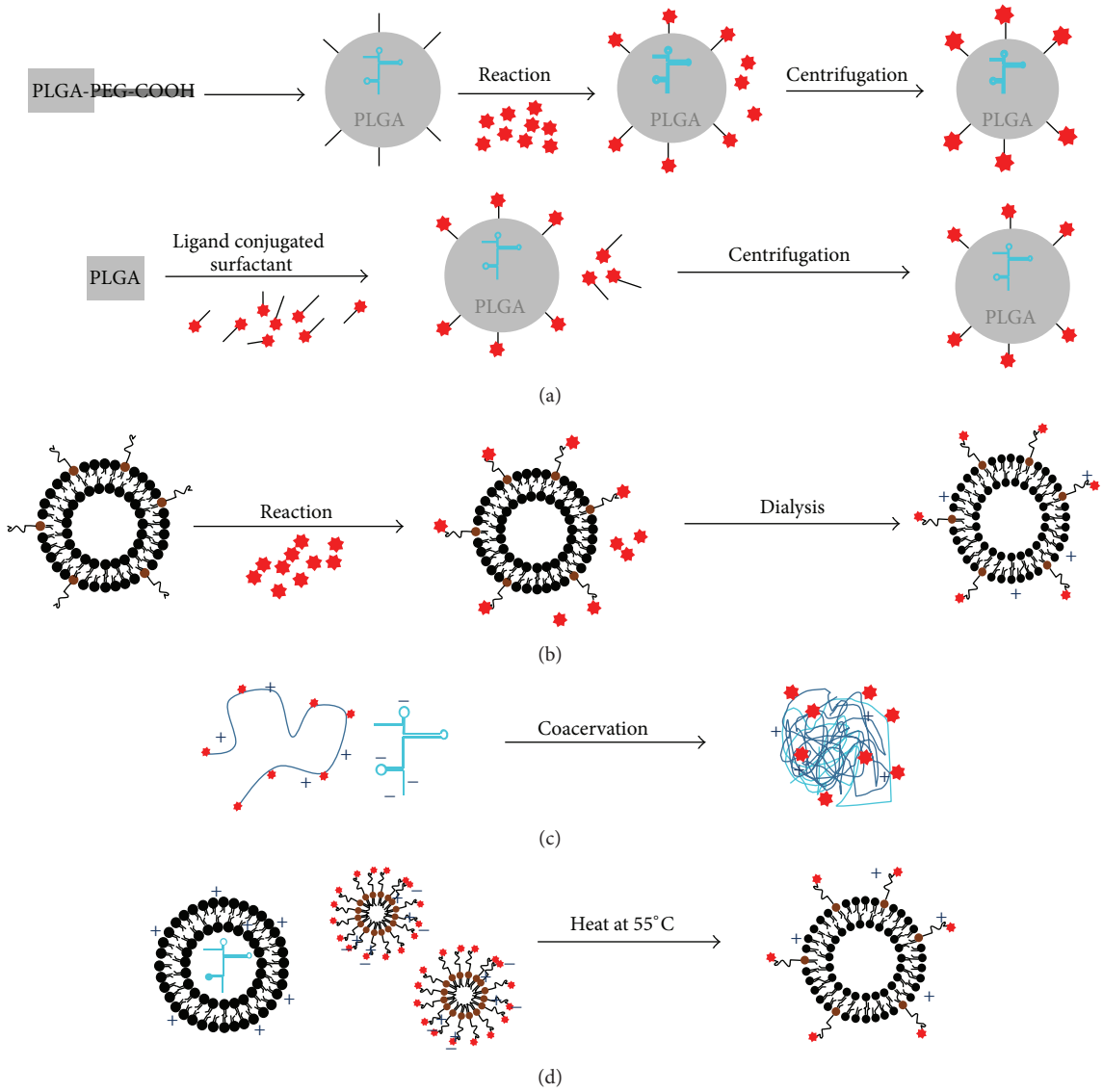

Figure 1: Established strategies for the conjugation of ligands onto polymeric and liposomal nanoparticles. (a) (Top) PLGA (poly(lactic-co-glycolic acid)) nanoparticles formed by copolymer PLGA-PEG-COOH are stabilized with normal surfactant and subsequently reacted with ligands bearing compatible linking groups. (Bottom) PLGA nanoparticles are stabilized with amphiphilic surfactants containing functionalizable molecules. PLGA nanoparticles, susceptible to hydrolysis, are purified by centrifugation to reduce water exposure time. (b) DC-targeting antibodies bearing compatible cross-linkers (e.g., -SH) are reacted with preformed liposomes to form immunoliposomes, which are purified by dialysis. (c) Electrostatically neutral ligands (mannose) are covalently conjugated to cationic polymers and directly used to formulate targeted nanoparticles. (d) Postinsertion functionalization of liposomes/lipopolyplexes. Formulated liposomes/lipopolyplexes are heated with micelles bearing targeting ligands at 55°C for at least 15 mins. The resultant ligand conjugated liposomes/lipopolyplexes can be used without further purification.

PLGA nanoparticles bearing surface carboxylate groups (COOH) can be further functionalized with any ligands bearing amine groups (e.g., peptides, antibodies, nanobodies, and aptamers) via N-hydroxysuccinimide (NHS) chemistry, which proceeds with good efficiencies under physiological conditions if NHS bearing ligands are applied in excess [20] (Figure 1(a), top). However, this conjugation strategy will require the colloidal nanoparticles to remain stable through every step of the conjugation process (surface chemistry modifications, purification and lyophilization). Ligand conjugated nanoparticles are normally purified from the reaction mixture via centrifugation, and hence this strategy is compatible with formulations bearing a solid core because they can withstand compression without aggregation. Apart from centrifugation, dialysis is another common technique used to remove unconjugated ligands. However, dialysis is not compatible with PLGA (as well as other polyesters, e.g., poly-β-amino esters) as ester bonds in these polyesters undergo hydrolysis. Conversely, formulations that are chemically inert (e.g., gold nanoparticles, immunoliposomes, and polyamide-based nanoparticles) but aggregate upon centrifugation can be purified by dialysis (Figure 1(b)). A similar approach

uses functionalized amphiphilic surfactants commonly used to stabilize the PLGA nanoparticles in colloidal suspension (Figure 1(a), bottom). These surfactants, which bear reactive chemical moieties (e.g., COOH, NH_2, and OH), are optimally incorporated on particle surfaces and amenable for subsequent conjugation with targeting ligands bearing compatible linkers [21]. In particular, avidin-fatty acid surfactants have been applied to stabilize PLGA nanoparticles [22, 23]. The resulting nanoparticles can be subsequently functionalized with biotinylated ligands such as antibodies, which are easily available, to render user defined DC surface receptor targets such as DEC-205 and DC-SIGN [22, 24, 25]. This formulation is relatively attractive because DC receptors are very often targeted by antibodies. However, notwithstanding the immunological consequences of antibodies, the sheer size of antibodies may result in low surface coverage due to steric hindrance. This can be mitigated with more advanced ligands such as single chain fragment variable (scFv) [26, 27] or aptamers [28], making this an attractive conjugation method.

Second, targeting moieties can instead be incorporated as part of the carrier molecule (polymer or lipid). The ligand conjugated carrier is directly used to formulate the nanoparticles via coacervation between positively charged gene carriers and negatively charged mRNA, and hence no additional step is needed to affix the ligands. This strategy is typically applicable for electrostatically neutral, low molecular weight ligands to ensure that they do not interfere with the carrier molecule during nanoparticle formulation (Figure 1(c)). Mannan/Mannose, a sugar that interacts with C-type lectin/lectin-like receptors, is the most commonly applied DC-targeting ligand incorporated into nanoparticles using this approach. A large number of mannosylated lipids and polymers have been developed hitherto for the purpose of vaccination [29–36]. For liposomal systems, mannose are grafted onto the head groups of lipids [29–31], while, for polymeric systems, they are normally covalently attached along the backbone of polymeric carriers [32–36]. Most of these systems are tested for delivery of different vaccine molecules including peptides, DNA, and siRNA with a consistent improvement in uptake efficiencies over nonmannosylated nanoparticles, which translates to an improved immunization outcome. Notably, Midoux group elegantly demonstrated, as a proof-of-concept, that mRNA-loaded mannosylated lipophosphoramides target DCs *in vivo* and translate into a better survival outcome based on a B16-F10 prophylactic tumor model [31, 37].

Third, another tried and tested strategy for ligand conjugation primarily in liposomal systems exploits the use of hydrophobic interaction (Figure 1(d)). It is well known that liposomes/lipopolyplexes are not thermodynamically stable colloids that aggregate slowly over time [38–40]. Aggregation is a fusion process when hydrophobic interactions between the lipid tails are stronger than the repulsive forces on the surfaces of the liposomes. Factors determining this balance include temperature, ionic concentration of the buffer, and amphiphilic property (surface charge of the lipids versus length and number of the lipid tails). Exploiting effects of temperature on lipid fusion, liposomes or lipopolyplexes encapsulated with mRNA or other payloads can be incubated with ligand-micelles (e.g., DSPE-PEG-2000-X, where X = ligand) at a temperature of 55°C for at least 15 minutes. Due to increased hydrophobic interaction at a higher temperature, ligand conjugated lipids from these micelles can be transferred to the liposomes, effectively decorating them with the desired targeting ligands. These ligand conjugated micelles can be prepared by reacting thiol (SH-) or amine (NH_2-) bearing ligands with DSPE-PEG-NHS or DSPE-PEG-maleimide (DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine) available commercially with different PEG molecular weight. This so-called "postinsertion" strategy is a facile approach to functionalizing liposomes with any desired ligands. Unlike PLGA system, DSPE-PEG-ligand can be prepared separately and conveniently incorporated into formulated liposomes on demand [41–43]. The amount of PEG coverage over a 100 nm liposome needed to prevent aggregation in serum is determined to be >8 mole% (based on total lipid content) in the liposome formulation [44, 45]. A caveat to postinsertion strategy is that if the amphiphilicity of the micelles is significantly affected by an excessively hydrophilic head (e.g., highly charged aptamer, long PEG chain), postinsertion method may fail because the increased hydrophobic interaction induced at a higher temperature may not be sufficient to trigger micelle fusion with the liposomes/lipopolyplexes.

3. Targeting mRNA Nanoparticles via Selective Endocytic Pathways

When particles are administered into the body, unless the injected site is already the lymph node (e.g., intranodal administration) or has a high density of antigen presenting cells (e.g., intradermal or intranasal administration), nanoparticles need to be passively transported from the site of administration to the lymph nodes via the body's circulatory system such as the lymphatics or the systemic circulation [28, 46–48]. During passive transport from the site administration to the lymphoid tissues, nanoparticles may be taken up nonspecifically by bystander cells based on a range of physiochemical factors such as size, surface charge, and chemical structure of surface molecules. Targeting ligands may reduce such occurrences due to incompatible surface chemistries while increasing uptake efficiencies of nanoparticles when reaching the target site [49–51].

There are different interpretations of "targeted delivery." While generally it means selective delivery of the vaccine to DCs bearing specific surface receptors, direct outcome of receptor binding depends on what receptors are being targeted. Targeting ligands can, amongst other functions, help increase the uptake by binding to receptors designed to endocytose larger particles [50], mitigate repulsive forces [51], or improve surface compatibility between the particles and the cell membrane [49]. Since intracellular fate of the particles taken up by endocytosis [52] is determined largely by the mechanism through which they are being taken up, targeting ligands may help direct endosomes into specific intracellular trafficking pathways that are less degradative so that gene delivery efficiencies are increased.

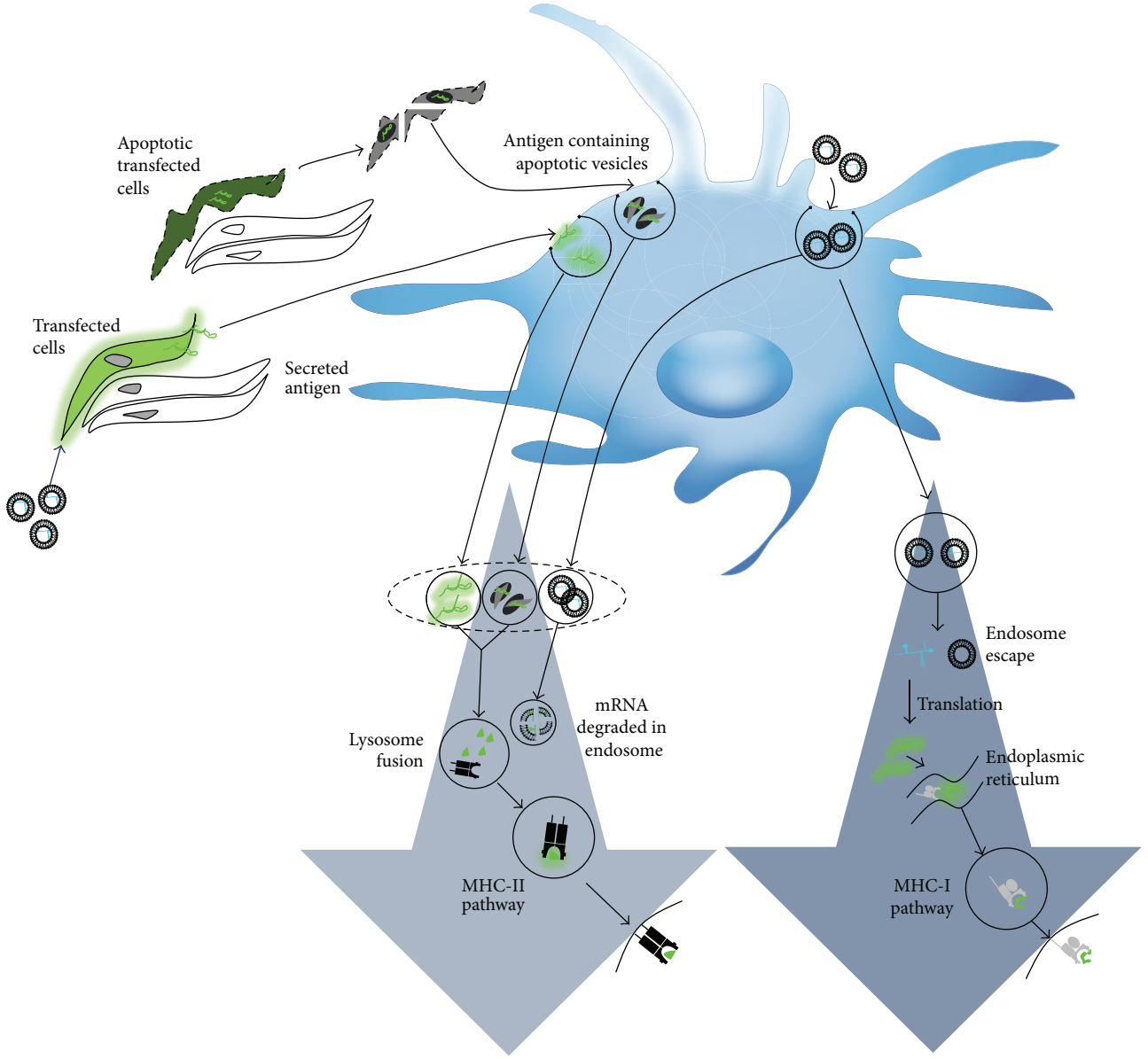

Figure 2: The genetic vaccination model. Antigen presentation occurs directly by transfected DCs through gene expression of the antigen. DCs also cross present antigens secreted by transfected bystander cells, or derived from phagocytosis of apoptotic cells. Cross presentation mechanisms in DCs may facilitate delayed lysosomal delivery leading to higher delivery efficiencies.

DCs, unlike other somatic cells, possess unique endocytic receptors catered to antigen uptake and processing. These receptors are special because they not only trigger particle uptake, but also mediate cross presentation and the development of the immune response. Although cross presentation in DCs influenced the development of subunit nanoparticle vaccines, its impact on genetic vaccination is less conclusive.

The genetic vaccination delivery model has been described as a process where both bystander and antigen presenting cells are transfected [53, 54]. According to this model, as illustrated in Figure 2, antigen presentation occurs through direct transfection of DCs and also through indirect transfer by transfected bystander cells. When the mRNA nanoparticles are targeted to DCs directly, those that escape the endosomes will have a higher chance of being expressed. In DCs, endosome escape not only depends on the efficiency of the gene carrier, but also depends on the trafficking mechanisms. For example, cross presentation mechanisms in DCs can disrupt lysosome trafficking pathways via mediation of endosomal pH leading to higher delivery efficiencies [55]. But, on the other hand, intracellular trafficking pathways of nonprofessional antigen presenting cells often terminate at the lysosomes. When mRNA nanoparticles are delivered without specific DC-targeting ligands, they will also transfect bystander cells. The latter provide an alternative source of antigens by secreting them (if the antigens are secretory in nature or designed with a secretory signal) into the extracellular space for capture by DCs. Finally, according to

the consensus genetic vaccination, the other indirect delivery mechanism occurs when transfected bystander cells become apoptotic due to significant stress caused by viral or tumor infection. DCs then acquire antigen through phagocytosis of these apoptotic cells.

Sufficient literature exists to suggest that indirect delivery mechanisms via bystander cells does not play a significant role in targeted delivery systems since targeted genetic nanoparticle vaccines consistently improve immunization outcomes [32–34, 56–58]. DC-specific receptors that not only increase uptake but also enhance transfection via less degradative intracellular trafficking pathways will be attractive for mRNA nanoparticle tumor vaccination [59]. While a long list of DC receptors has been discovered to possess immune modulating function, only a few may benefit mRNA delivery beyond uptake enhancements because they are also targeted towards less degradative intracellular trafficking pathways [60]. They are type I C-type lectins such as CD205 (DEC-205) and CD206 (macrophage mannose receptor) and type II C-type lectins such as CD370 (CLEC9A/DNGR-1). These will be briefly described.

3.1. DEC-205. DEC-205 is ubiquitous receptor found on almost every conventional dendritic cell [61]. It is a type I C-type lectin-like molecule consisting of a single polypeptide chain that functions as recycling endocytic receptor and caters for a wide range of cargos that include, notwithstanding lectin-like molecules, apoptotic cells [62], necrotic cells [63], and CpG [64]. DEC-205 is an attractive target receptor because antigens delivered via this receptor are presented on both MHC-I and MHC-II molecules [63]. Furthermore, engagement of DEC-205 does not lead to proinflammatory response, making it an attractive receptor target for tolerance immunization [65]. The anti-DEC-205 ligand is one of the most developed ligands in immunotherapy. While ligands targeting most of the other DC-specific receptors continue to manifest in antibody molecules, anti-DEC-205 ligands in form of scFv [26, 27] and aptamer [66] have been reported.

Functional properties of DEC-205 will benefit mRNA vaccination via higher transfection efficiencies. For example, being a cognate endocytic receptor for apoptotic cells, DEC-205 will efficiently uptake both nano- and microparticles it comes into close contact with. Hence, given mRNA nanoparticles tendency to aggregate *in vivo* (increased particle sizes), administered dose will have higher bioavailability when targeted towards DEC-205. In addition, cross presenting properties of DEC-205, thought to be results of "leaky endosomes" or less degradative endocytic pathway, will facilitate endosome escape of mRNA nanoparticles into the cytoplasm and avoid the lysosomes.

3.2. Mannose Receptor. The mannose receptor, another type I C-type lectin receptor with a well-established role in tissue homeostasis [67], recognizes sulfated carbohydrates, collagen, and oligosaccharides through its cysteine-rich domain [68, 69], fibronectin domain [70], and C-type lectin domains [71, 72], respectively. The mannose receptors have been well-known endocytic receptors for decades in part because they are extensively studied as scavenging receptors in macrophages, which were initially thought to be the major antigen presenting cells before DCs were discovered. The ligand for this receptor is mannose residue grafted on the gene carrier [29–36] as previously described. Its role in antigen presentation was conclusively determined through the use of DCs derived from mannose receptor negative transgenic mice [73]. This study confirmed that DCs' mannose receptors not only serve as uptake receptors [74–77], but also mediate cross presentation of soluble mannosylated antigens [78–80]. Since payload taken up via mannose receptor stably accumulates in the early endosome and is excluded from lysosomes for up to 6 hours [78, 79], this intracellular trafficking pathway is expected to be less degradative and highly attractive for mRNA nanoparticle delivery.

3.3. CLEC9A/DNGR-1. CLEC9A (C-type lectin domain family 9, a.k.a. DNGR-1 or CD370) is a recently discovered endocytic receptor that is implicated in the clearance of damaged [81] and dead [82, 83] cells. This receptor, currently targeted via antibody, is restricted to a very small population of blood BDCA3$^+$ DCs [84] (in humans) and its equivalent in mice models is CD8$^+$ DCs. Due to its endocytic nature, antigen delivery properties of CLEC9A are rapidly investigated [85, 86]. Recent reports show that CLEC9A are effective in cross presenting antigens for cell mediated immunity [83, 87] and can be as effective T cell activators compared to Langerin and DEC-205 [88]. Similar to other receptors capable of cross presenting soluble antigens, nanoparticles targeted to CLEC9A are expected to enter a less degradative intracellular trafficking pathway, leading to higher transfection efficiency. Restricted expression of CLEC9A to blood DCs may limit it as a practical receptor compared to the mannose receptor and DEC-205 for targeted delivery to conventional DCs. Nevertheless, CLEC9A remains an attractive receptor for targeting plasmacytoid DCs [86].

4. Conclusion

As a late bloomer, development of mRNA therapeutics benefits from a plethora of related knowledge on similar delivery systems. Advancing from passive targeting strategies employed for most mRNA nanoparticle tumor vaccine to date, active targeting of mRNA nanoparticles to DCs will further improve current therapeutic outcome for the treatment of cancer. Practicable conjugation strategies as well as target receptors reviewed in this paper will provide a convenient reference to facilitate future development of targeted mRNA nanoparticle vaccine.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Review Article

RNA-Based Vaccines in Cancer Immunotherapy

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RNA vaccines traditionally consist of messenger RNA synthesized by *in vitro* transcription using a bacteriophage RNA polymerase and template DNA that encodes the antigen(s) of interest. Once administered and internalized by host cells, the mRNA transcripts are translated directly in the cytoplasm and then the resulting antigens are presented to antigen presenting cells to stimulate an immune response. Alternatively, dendritic cells can be loaded with either tumor associated antigen mRNA or total tumor RNA and delivered to the host to elicit a specific immune response. In this review, we will explain why RNA vaccines represent an attractive platform for cancer immunotherapy, discuss modifications to RNA structure that have been developed to optimize mRNA vaccine stability and translational efficiency, and describe strategies for nonviral delivery of mRNA vaccines, highlighting key preclinical and clinical data related to cancer immunotherapy.

1. Introduction

Cancer immunotherapy seeks to stimulate a host antitumor immune response, leading to tumor shrinkage and improved clinical outcomes in patients. In recent years, this field has exploded with the development of many different classes of agents aimed at enhancing immune responses against tumors. These include cytokines, immune checkpoint inhibitors, adoptive T cell therapies, and numerous vaccine strategies [1–3]. Several of these new immunotherapies, particularly the immune checkpoint inhibitors, including ipilimumab in metastatic melanoma [4] and nivolumab in non-small cell lung cancer [5], have demonstrated impressive survival benefits in large phase III trials, leading to the FDA approval of these agents and solidifying immunotherapy as a new modality for the treatment of cancer.

Compared to some other types of cancer immunotherapy, vaccines can be more cumbersome to produce and, for the most part, have shown more modest clinical responses in patients [6–12]. However, they remain an attractive cancer treatment approach because they represent a specific, safe, and well-tolerated therapy that also offers the potential to avoid drug resistance and obtain durable treatment responses due to immunologic memory. There are four main categories of cancer vaccines: (1) peptide vaccines, (2) cellular vaccines, including tumor cell and immune cell vaccines, (3) viral vector vaccines, and (4) nucleic acid vaccines, including DNA and RNA vaccines. This review will begin with a brief overview of nucleic acid vaccines for context and will then focus on RNA cancer vaccines. We will provide a discussion of RNA structure, as it relates to RNA vaccine therapy. We will then describe key preclinical and clinical data for the different types of RNA cancer vaccines, highlight advantages and disadvantages of various methods of RNA vaccine delivery, and discuss potential combinations of RNA vaccines with other therapies.

2. Nucleic Acid Vaccines: Definition, Appeal of the Platform, and Advantages of RNA over DNA

By definition, nucleic acid vaccines are vaccines containing antigens encoded by either DNA or RNA. More specifically, DNA vaccines consist of antigen-encoding gene(s) inserted into a bacterial plasmid under the control of a eukaryotic

promoter. The DNA plasmid is administered to the host and internalized by host cells, where it is transcribed in the nucleus and translated in the cytoplasm by host cellular machinery. The resulting proteins are processed into peptides, which are ultimately presented on the surface of host antigen-presenting cells (APC) in the context of major histocompatibility complex (MHC) molecules. This can occur by APC being directly transfected with the DNA or by cross-presentation from non-APC to APC. The peptide-MHC complex is recognized by antigen-specific T cells, resulting in a cellular host immune response [13]. Alternatively, RNA vaccines involve messenger RNA (mRNA) synthesized by *in vitro* transcription (IVT) using a bacteriophage RNA polymerase and template DNA that encodes the antigen(s) of interest. Once administered and internalized by host cells, the mRNA transcripts are translated directly in the cytoplasm and then, like DNA vaccines, the resulting antigens are presented to APC to stimulate an immune response [14]. It is important to recognize that mRNA-encoded products are degraded by proteasomes and presented on MHC class I molecules to CD8+ T cells and do not reach the MHC class II processing pathway to induce CD4+ T helper cell responses. However, several studies have demonstrated that addition of a lysosomal targeting signal to the antigen-encoding sequence can result in a productive T helper cell response [15, 16]. Additionally, tumor antigen mRNAs fused to a signal peptide and an HLA class II sorting can result in HLA class I and II presentation [17].

The nucleic acid vaccine platform is appealing because it allows easy delivery of multiple antigens with one immunization and induces both humoral and cellular immune responses, which makes tumor escape less likely. Additionally, unlike peptide-based vaccines, nucleic acid-based vaccines do not require prior knowledge and are not restricted by the patient's HLA type. Finally, like other vaccine types, nucleic acid-based vaccines have proven to be safe and tolerable [14, 18–20].

Despite the promising features of DNA vaccines, in general, they have been found to elicit less of an immune response than other types of vaccines, including peptide vaccines, cellular vaccines, viral vector vaccines, and RNA vaccines. The reasons for this are not completely clear, but possible explanations include inefficient delivery of DNA into human cells, the need for DNA to cross both cell and nuclear membranes and be transcribed in the nucleus in order to transfect a cell, low expression of DNA-sensing machinery, and differing expression of nucleic acid sensing pattern recognition receptors [11, 21]. The relatively poor immunogenicity of DNA vaccines combined with concerns about their potential for oncogenesis via integration into the host genome has driven a shift away from DNA vaccines and towards RNA vaccines.

RNA vaccines are attractive because they retain the same appealing characteristics as DNA vaccines but also offer some additional benefits. Unlike DNA, RNA only needs to gain entry into the cytoplasm, where translation occurs, in order to transfect a cell. Moreover, RNA cannot integrate into the genome and therefore has no oncogenic potential. In addition to *in vitro* transcription, RNA can also be isolated from a limited tumor sample and amplified using techniques such as polymerase chain reaction (PCR), yielding large amounts of patient-specific antigens [11, 14]. Finally, RNA can act as an adjuvant by providing costimulatory signals, for example, via toll-like receptors TLR3, TLR7, and TLR8 [22]. For these reasons, there is a growing interest in the research and development of RNA vaccines.

3. RNA Structure: 5′ Cap, Poly(A) Tail, UTR, and Chemically Modified Nucleosides

Eukaryotic mRNA is composed of a coding region flanked by 5′ and 3′ untranslated regions (UTR), as well as a 5′ 7-methylguanosine triphosphate (m^7G) cap and a 3′ poly(A) tail. The m^7G cap, poly(A) tail, and UTR are all critical for mRNA stability and translation [23–25]. A keen awareness of this mRNA biology is vital when developing RNA vaccines since mRNA stability and translational efficiency dictate the amount of antigen produced, which impacts the degree of immune response generated.

5′ m^7G Cap. A 7-methylguanosine triphosphate (m^7G) cap is added to the 5′ end of almost all eukaryotic mRNA transcripts during transcription. The cap protects the 5′ end of the mRNA transcript from 5′ to 3′ exonucleases [26] and is recognized by the eukaryotic translation initiation factor eIF4E [27], thus playing a key role in both mRNA stability and translation.

Capping of IVT mRNA transcripts can be achieved using cap analogues. However, it has been shown that cap analogues are often incorporated in the reverse orientation with the methylated G proximal to the RNA [28], resulting in an inability to translate a substantial number of mRNA transcripts [29]. To address this issue, antireverse cap analogues (ARCA) have been designed that cannot be incorporated in the reverse orientation because they contain only one 3′-OH group, rather than the two 3′-OH groups contained on the initial cap analogues. Importantly, the translational efficiency of ARCA-capped mRNA transcripts is more than twice that of mRNA capped with conventional cap analogues [29]. Additionally, a higher level of protein expression, maintained for a longer duration of time, is achieved in cells transfected with ARCA-capped IVT mRNA transcripts, compared to cells transfected with mRNA transcripts capped with regular cap analogues [30].

Adding a cap analogue, conventional or ARCA, during IVT is not 100% efficient; thus a portion of the resulting transcripts is not capped at all. These uncapped RNAs are not efficiently translated. A method of adding a cap structure posttranscriptionally is gaining favorability among those developing RNA-based therapies.

Poly(A) Tail. A poly(A) tail is added to the 3′ end of the majority of eukaryotic mRNA transcripts during transcription. The poly(A) tail regulates mRNA stability and translation synergistically with the m^7G cap by binding poly(A) binding protein (PABP) [31], which interacts with eukaryotic translation initiation factor eIF4G, which in turn forms a complex with the m^7G cap and eIF4E [32].

There are two ways to add a poly(A) tail to IVT mRNA: (1) encoding the poly(A) tail on the DNA template from which the IVT mRNA is transcribed or (2) using recombinant poly(A) polymerase to extend the IVT mRNA after transcription. In contrast to enzymatic polyadenylation with recombinant poly(A) polymerase, which yields mRNA transcripts with poly(A) tails of varying lengths, mRNA transcribed from a DNA template yields transcripts with a defined poly(A) tail length and is therefore preferred [33]. Studies have shown that increasing the length of the poly(A) tail increases the efficiency of polysome formation [34] as well as the level of protein expression. The optimal length of the poly(A) tail in IVT mRNA appears to be between 120 and 150 nucleotides [33, 35, 36].

5′ and 3′ Untranslated Regions. Eukaryotic mRNA transcripts include 5′ and 3′ untranslated regions (UTR), which contain important regulatory elements.

IVT mRNA can be optimized by incorporating 5′ and 3′ UTR known to enhance RNA stability and translational efficiency. The most well recognized examples of such UTR in IVT mRNA are the alpha- and beta-globin mRNAs. Beta-globin 5′ and 3′ UTR improve translational efficiency, and alpha-globin 3′ UTR stabilize mRNA [37–39]. These globin UTR are used in many preclinical and clinical studies involving IVT mRNA [40–42].

Chemically Modified Nucleosides. Finally, IVT mRNA can be created by incorporating chemically modified nucleosides, which are known to reduce immunogenicity. Natural nucleosides are added to mammalian RNA during posttranslational RNA processing in eukaryotes [43]. This has been explored as a potential method to render IVT mRNA less immunogenic.

IVT mRNA containing modified nucleosides such as pseudouridine possesses increased stability and translation [44, 45]. This is thought to occur as a result of nucleosides rendering IVT mRNA undetectable by cytoplasmic TLRs such as TLR3, TLR7, and TLR8, as well as RIG-I and PKR [43, 44, 46]. Moreover, further processing of IVT mRNA by high-performance liquid chromatography purification to remove dsRNA contaminants results in reduced type I interferons and proinflammatory cytokines production [47], thus leading to increased and prolonged mRNA translation.

4. Nonviral Strategies for Delivery of mRNA Vaccines

The previously described modifications to the 5′ m^7G cap, poly(A) tail, 5′ and 3′ UTR, and nucleosides are fundamental to optimize the stability and translational efficiency of all IVT mRNA for all RNA vaccines. Once an IVT mRNA transcript has been generated, it must be administered and ultimately must reach the cytoplasm of target cells. In general, nonviral delivery methods are preferred over viral vectors for their low cost, ease of large-scale production, and potential for improved safety [48, 49]. We will now review different nonviral strategies for delivery of mRNA vaccines, with a focus on their role in cancer immunotherapy.

4.1. "Naked" mRNA Vaccines. The initial data for the use of mRNA as a vaccine platform emerged 25 years ago when Wolff et al. demonstrated that intramuscular injection of mRNA coding for reporter genes induced *in vivo* expression of those reporter genes in mice [50]. Five years later, Conry et al. developed the first mRNA cancer vaccine by showing that mice immunized with mRNA coding for carcinoembryonic antigen (CEA) mounted an anti-CEA antibody response when challenged with CEA expressing tumor cells [51]. Both of these accomplishments represent examples of "naked" RNA vaccines, meaning the mRNA is injected directly, formulated only in buffer and without a carrier. Since then, numerous studies in animal models have confirmed that naked mRNA can transfect host cells and induce antigen-specific antibody and T cell immune responses [41, 52–56]. However, despite some encouraging early results, naked mRNA vaccines remain limited by the short extracellular half-life of naked mRNA due to rapid degradation by ubiquitous RNAases [57, 58]. Moreover, RNA vaccines induce transient protein expression, thus limiting the time for treatment effectiveness. This would in turn increase the number of times a patient visits the clinic for treatment. In addition to the previously described techniques to optimize the structure of IVT mRNA, different strategies, including two key techniques described below, have been tried in order to stabilize the naked mRNA, improve translational efficiency, and overcome this barrier.

Gene Gun. The gene gun is an alternative delivery method, in which IVT mRNA is injected directly into the target cell cytoplasm, thereby limiting exposure to extracellular exonucleases that might degrade it. More specifically, in the gene gun technique, IVT mRNA is coated onto gold particles, which are then accelerated toward a stopping plate by a pressurized helium pulse. The gold particles penetrate into the cytoplasm of target cells, serving as carriers for the mRNA [59, 60]. The gene gun has been shown to be an effective delivery mechanism for IVT mRNA in animal models. For example, gene gun-based immunization using IVT mRNA coding for the melanocyte self-antigen TRP2 linked to the immunogenic protein EGFP induced antigen-specific cellular and humoral immunity in mice and was protective against B16 melanoma lung metastases [53]. However, despite this success in animal models, the gene gun delivery method has not yet been translated into clinical trials in humans.

Protamine Condensation. Another method to improve the stability of naked IVT mRNA is to condense it in order to provide protection from RNA degradation. This can be accomplished by incubating IVT mRNA with protamine, a small arginine-rich polycationic protein, normally involved in DNA condensation [56]. Protamine condensation is also attractive because, in addition to stabilizing IVT mRNA, protamine acts as a danger signal and stimulates an immune response through MyD88, TLR7, and TLR8 dependent pathways [61–63]. Protamine-condensed IVT mRNA has been shown to induce specific cellular and humoral immune responses *in vivo* in both mice and humans. For example, the injection of protamine-protected naked mRNA into mice

stimulated production of antigen-specific IgG antibodies as well as activation of a specific cytotoxic T lymphocyte response and effective lysis of target cells [56]. In a phase I/II clinical trial in 21 patients with metastatic melanoma, intradermal injection of protamine-condensed naked mRNA encoding six melanoma-associated antigens was feasible and safe, increased vaccine-directed T cells in two of the four evaluable patients, and yielded a complete response in one of the seven patients with measurable disease [64].

4.2. Adjuvants to mRNA-Based Vaccines. An adjuvant can be broadly defined as a component that is added to a vaccine to enhance the immunogenicity of the vaccine. Naked IVT mRNA possesses inherent self-adjuvanticity. Additionally, several other molecules, including protamine, as described previously, poly I:C RNA, and CpG containing motifs can be combined with naked IVT mRNA to augment an mRNA-based vaccine's ability to induce an adaptive immune response. However, even with the addition of an adjuvant, a vaccine may not be potent enough to overcome the powerful immunosuppressive effects of tumors. To address this issue, mRNA encoding costimulatory molecules, such as CD40L, CD70, OX40L, GITR, and CD83, can be incorporated into mRNA-based vaccines to further boost their immunogenicity [65–67].

4.3. Encapsulated mRNA Vaccines. Despite the previously described strategies to improve the stability of naked IVT mRNA, RNA degradation remains a significant concern with naked RNA vaccines. Because of this, carriers have been developed to encapsulate IVT mRNA, thereby protecting it from degradation and improving vaccine delivery [68]. To date, many such carriers have shown great potential in mRNA delivery to mammalian cells [69]. Cationic liposomes, specifically N-[1-(2,3-dioleoloxy)propyl]-N,N,N-trimethyl ammonium chloride 1(DOTAP), have been the most widely used encapsulating agents [69–72]. Despite these discoveries, the search continues for delivery agents that provide effective cytosolic mRNA delivery and are associated with limited *in vivo* cytotoxicity. Recent studies have shown that encapsulating mRNA in nanoparticles protects the mRNA from nuclease degradation and enhances cell uptake and delivery efficiency [73]. Moreover, these nanoparticles can be engineered to be fully degradable, a desired property in vaccine delivery. These particles contain a biodegradable core-shell structured nanoparticle with a pH responsive poly-(b-amino ester) (PBAE) core enveloped by a phospholipid shell [74]. Preclinical studies have shown that these particles are efficient in delivering mRNA *in vivo* and eliciting an antitumor immune response [66, 75, 76].

5. mRNA Transfected DC Vaccines

mRNA transfected dendritic cell (DC) vaccines represent a distinct type of vaccine strategy involving RNA. DCs are professional antigen presenting cells that play an essential role in bridging innate and adaptive immune responses. When used as a vaccination platform, dendritic cells (DCs) are transfected with mRNA encoding a desired tumor antigen and then delivered to the host in order to elicit an immune response against the antigen of interest. DCs can be transfected with tumor associated antigen (TAA) mRNA or total tumor RNA. Both methodologies have their advantages and drawbacks.

Tumor Associated Antigens. Vaccine strategies utilizing DCs transfected with defined TAA mRNA circumvent the need for growth of patient specific tumor cells and/or isolation of patient specific tumor antigen [77–79]. Moreover, the antigen preparation is homogenous and highly pure and the majority of the loaded DCs present the same epitope(s) on the surface. This vaccination strategy also lowers the risk of autoimmunity, which can be induced in patients by the inclusion of nonmutated, normally expressed endogenous proteins. However, TAA vaccination strategies come with many limitations. For many cancers the TAAs are not identified and significant investigation is still required. Vaccine development is costly and TAA selection can be difficult as not all identified TAAs elicit an antitumor immune response. Finally, when targeting a single antigen, there exists the possibility that the tumor itself will downregulate the TAA and allow escape.

Several studies to date have utilized TAA mRNA-loaded DCs to stimulated antitumor responses [80–84]. In a study by Heiser et al., DCs were transfected with prostate-specific antigen (PSA) TAA and administered into prostate cancer patients [85]. In this trial, the DC immunization elicited a PSA-specific T-cell response, which was accompanied by a significant decrease in PSA levels in six of seven patients. Additional studies have utilized CEA mRNA-loaded DCs to vaccinate patients with CEA expressing tumors [86, 87]. Although vaccination itself was well-tolerated, the antitumor response was limited to six (one complete response, two minor responses, and three stable diseases) out of 24 patients. Although we list many studies utilizing TAA mRNA vaccination strategies, this review cannot include all of the publications on this topic. Other articles in this issue will discuss additional vaccination outcome reports.

Total Tumor RNA. An alternative to the use of TAA to generate an anticancer immune response is the use of patient derived total tumor RNA [88–93]. This method utilizes cancer specific RNA and eliminates the need for identification of antigens expressed by the patient's tumor. Through this methodology the entire spectrum of tumor specific antigen is displayed, thus allowing the immune system to utilize the most effective antigens while reducing the risk of escape mutants. The advantage of using tumor-derived RNA as a source of whole-tumor antigen is that it can be quickly and easily amplified by RT-PCR from even a small amount of tumor. This allows for an unlimited supply of antigen, which differs from strategies using tumor lysate or cells [85].

Clinical studies utilizing total tumor RNA vaccination strategies have been tested in several tumor models including brain cancer, lung adenocarcinoma, melanoma, renal cell carcinoma, and ovarian cancer [94–100]. In the renal cell carcinoma study by Su et al., patients displayed no evidence

of dose-limiting toxicity or induction of autoimmunity [100]. Similarly, brain tumor and neuroblastoma studies conducted in nine and seven patients, respectively, showed a clinical response in a total of three of the patients. Despite many advances in RNA-DC immunotherapies, clinical responses remain modest and new strategies on how to best prepare and administer these vaccinations are being explored.

Of note, loading DCs with total tumor RNA can lead to expression of self-proteins and induction of autoimmunity. However, this has not been an issue in the studies conducted thus far.

Costimulatory Molecules and Checkpoint Inhibitors as Part of mRNA Transfected DC Vaccine Strategies. The benefit of DC based vaccinations has been limited primarily due to the presence of regulatory T cells as well as upregulation of checkpoint molecules. Recent studies have focused on new strategies to enhance the efficacy of RNA transfected DC vaccines. Cotransfection of DCs with mRNA encoding OX40-ligand in addition to tumor antigen improves mRNA-DC vaccine efficacy in preclinical models [101]. Moreover, DCs activated through electroporation with tumor mRNA as well as mRNA encoding CD40 ligand and constitutively active TLR4 and CD70 (TriMix-DCs) are potent antigen presenting cells. They induce effector T cells that do not respond to regulatory T cell suppression, an important strategy to generate a broad and robust immune response [102, 103].

In addition, studies where expression of checkpoint inhibitors, such as PD-1, is silenced utilizing siRNA have shown improved vaccine efficacy [104, 105]. Other strategies have focused on increased DC function by cotransfecting tumor antigen mRNA with mRNA encoding for checkpoint molecules such as CTLA-4 and GITR [106]. These engineered DCs have the ability to not only present the tumor antigen of interest but also secrete anti-CTLA4 and anti-GITR, thus locally modulating immune checkpoints and the tumor microenvironment. Taken together these strategies have potential to prevent or reduce cancer recurrence.

5.1. Route of Vaccine Delivery. Antigen-specific immune responses have been achieved in animal models and in humans by administration of RNA vaccines via various routes, including intramuscular, intradermal, subcutaneous, intravenous, intrasplenic, intranodal, intratumoral, and intranasal methods. These strategies each have their own advantages and disadvantages. However, they have not all been compared directly, and therefore the optimal route of RNA vaccine delivery is not known. For a more detailed discussion of RNA vaccine routes of administration, please refer to the following review papers: intramuscular [50, 52]; intradermal [107]; subcutaneous [108]; intrasplenic [109] and intranodal [41].

6. Conclusion

The field of cancer immunotherapy has undergone many changes in recent years, and immunotherapy has now been clinically validated as an effective way to treat many types of malignancies [1–3]. RNA vaccines represent an attractive form of cancer immunotherapy because they enable delivery of large amounts of patient-specific antigens derived from a small tumor sample, are not HLA-restricted, induce humoral and cellular immune responses, provide costimulatory signals, have no oncogenic potential, and are well-tolerated. As discussed in this review, several techniques have been developed to improve IVT mRNA stability and translational efficiency and to optimize RNA vaccine delivery. Despite these advances, clinical responses to RNA vaccines remain modest.

Moving forward, combining RNA vaccines with other therapies that have distinct mechanisms of action could potentially result in better treatment outcomes. For example, the vaccination methods described above could be used simultaneously or sequentially with immune checkpoint inhibitors such as anti-CTLA-4, anti-PD-1, and anti-PD-L1 [110]. In such a combination strategy, RNA vaccines could prove important in specifically targeting tumors, reducing tumor burden, and causing tumor cell lysis and antigen spread, while immune checkpoint blockade could be essential in perpetuating the immune responses and leading to better tumor cell clearance.

Moreover, in this review we discuss the importance of RNA strategies to directly block immune checkpoints and engineer the tumor microenvironment, similarly to antibodies against immune checkpoints. These strategies would perhaps limit the toxicities and side effects associated with systemic delivery of antibodies against immune checkpoints.

Further studies are needed to specifically investigate combinations of RNA vaccines with other immunotherapies as well as targeted and cytotoxic agents, with the overall goal of improving clinical outcomes and cancer care.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Prophylactic mRNA Vaccination against Allergy Confers Long-Term Memory Responses and Persistent Protection in Mice

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Recently, mRNA vaccines have been introduced as a safety-optimized alternative to plasmid DNA-based vaccines for protection against allergy. However, it remained unclear whether the short persistence of this vaccine type would limit memory responses and whether the protective immune response type would be maintained during recurrent exposure to allergen. We tested the duration of protective memory responses in mice vaccinated with mRNA encoding the grass pollen allergen Phl p 5 by challenging them with recombinant allergen, 3.5, 6, and 9 months after vaccination. In a second experiment, vaccinated mice were repeatedly challenged monthly with aerosolized allergen over a period of 7 months. Antibody and cytokine responses as well as lung inflammation and airway hyperresponsiveness were assessed. mRNA vaccination induced robust TH1 memory responses for at least 9 months. Vaccination efficiently suppressed TH2 cytokines, IgE responses, and lung eosinophilia. Protection was maintained after repeated exposure to aerosolized allergen and no TH1 associated pathology was observed. Lung function remained improved compared to nonvaccinated controls. Our data clearly indicate that mRNA vaccination against Phl p 5 induces robust, long-lived memory responses, which can be recalled by allergen exposure without side effects. mRNA vaccines fulfill the requirements for safe prophylactic vaccination without the need for booster immunizations.

1. Introduction

Due to a constant rise in incidence of type I allergic diseases the need for effective treatment options is apparent. However, specific immunotherapy (SIT), the only treatment currently available, is time-consuming and entails many disadvantages such as the potential to create new sensitizations and serious side effects, including anaphylaxis. Moreover, the inevitable transition from extract-based SIT to component-resolved diagnosis and therapy of allergic diseases with recombinant molecules seems to be a lengthy process. An alternative concept to SIT with recombinant molecules includes DNA immunization with allergen genes, an approach which meanwhile has entered the clinical study phase [1–3].

In the past years, the urgent need to fight the worldwide increasing incidence of allergies also drew attention to "true vaccination" against allergic diseases, that is, prophylactic immunization of healthy individuals [4, 5]. The identification of children at high risk to develop allergy has improved significantly [6, 7], thus facilitating the selection of target groups for prophylactic interventions. However, allergen extracts licensed for treatment of established allergies will not be applicable for prophylactic immunization due to safety issues and the risk to induce *de novo* sensitizations [8–10]. Only modified (hypoallergenic) allergen derivatives and gene vaccines can be considered as suitable candidates for prophylactic allergy vaccines. Among gene vaccines, mRNA conforms best to the stringent requirements for vaccines against type I allergy. Due to its short *in vivo* persistence mRNA acts in an "immunize and disappear" way, thus limiting expression of encoded allergens [11]. Furthermore, and in contrast to DNA vaccines, mRNA vaccines lack control sequences and cannot integrate into the host genome. These properties led to the classification of non-replicative mRNA as non-gene therapy by regulatory authorities [12]. Application of mRNA has so far proven its effectiveness for vaccination against infectious diseases and tumors in animal models [13, 14] and also in clinical studies with

mRNA encoding tumor-associated antigens [15, 16]. With regard to type I allergies we have demonstrated that mRNA vaccines induce a protective TH1-type response against a panel of different allergens, leading to inhibition of specific IgE production and prevention of lung inflammation and airway hyperresponsiveness (AHR) in mice [17]. Despite the proof that mRNA vaccines are effective and protect against allergic sensitization in murine models, doubts about their long-term efficacy remained. There have been concerns that short-term antigen expression might result in weak memory responses unable to protect from future encounters [18].

Therefore, in the present paper, one set of experiments investigates the long-term protection after mRNA vaccination (up to nine months after vaccination). A second approach deals with the robustness of the protective response. The immune system of patients under real-life conditions is exposed to allergen repetitively over weeks and months, or even perennial, depending on the allergen. This is in contrast to typical experimental setups which usually perform a few allergen challenges within a short time period. Hence we simulated the human situation of seasonal pollen exposure by a repeated challenge of vaccinated mice with aerosolized grass pollen allergen (up to seven months after vaccination).

2. Materials and Methods

2.1. Preparation of mRNA Vaccines. The plasmid encoding Phl p 5, pTNT-P5, has been described [17]. Plasmids for RNA transcription were purified using an EndoFree Plasmid Giga Kit (Qiagen, Düsseldorf, Germany). For RNA transcription, plasmids were linearized and templates were purified via phenol-chloroform-isoamyl alcohol extraction, followed by a single chloroform-isoamyl alcohol extraction. Plasmids were precipitated by adding a 1/10 volume of 3 M sodium acetate (pH 5.2) and two volumes of 100% ethanol on ice and washed three times with 70% ethanol. All transcription reactions were performed with T7 or SP6 RiboMAX Large Scale RNA Production Systems (Promega, Mannheim, Germany). Residual template DNA was removed by means of digestion with RNAse-free DNAse (Promega, Mannheim, Germany). After transcription, the RNA was precipitated by ammonium acetate precipitation (addition of 1 volume 5 M ammonium acetate, 15 min on ice) followed by centrifugation, washed with 70% ethanol, and resuspended in nuclease-free H_2O. Capping was performed *in vitro* by using a ScriptCap m^7G Capping Kit (Epicentre Biotechnologies, Madison, USA), following the manufacturer's instructions.

2.2. Animals and Immunizations. BALB/c mice, aged between 6 and 14 weeks, were obtained from Charles River Laboratories (Sulzfeld, Germany) and were maintained according to the local guidelines for animal care. All animal experiments were approved by the Austrian Ministry of Science.

To evaluate the duration of the protective effect of mRNA immunization, 5 mice per group were immunized intradermally (i.d.) three times in one-week intervals with 100 μg of capped-Phl p 5 mRNA. Non-vaccinated mice served as a control group. 3.5, 6, or 9 months after vaccination the animals were sensitized by two subcutaneous (s.c.) injections of 200 μL PBS containing 1 μg recombinant Phl p 5 (Biomay, Vienna, Austria) and 100 μL Alu-Gel-S (1.3% suspension with an aluminium content of 5.9–7.1 mg/mL, Serva Electrophoresis GmbH, Heidelberg, Germany) in a 10-day interval. Seven days after the second sensitization, mice were exposed to nebulized recombinant Phl p 5 in PBS for 30 min on three consecutive days. Therefore, 5 mL recombinant Phl p 5 in PBS (0.2 mg/mL) was nebulized using a PARI BOY SX nebulizer with a PARI LL nebulizer head (PARI, Starnberg, Germany) in a 25 × 25 × 25 cm nebulization chamber.

To analyze the effect of repeated allergen challenge, 5 animals per group were vaccinated i.d. three times in one-week intervals, with either 100 μg of capped-Phl p 5 or capped-Bet v 1 control (mock) mRNA (encoding the irrelevant allergen Bet v 1). Non-vaccinated mice served as a control group. All groups were sensitized twice by s.c. injection of 200 μL PBS containing 1 μg rPhl p 5 and 100 μL Alu-Gel-S 33 and 42 days after the last vaccination. Seven days after the second sensitization, the mice were challenged three times with 1 mg nebulized rPhl p 5 in PBS. On the next day, Penh was measured to assess AHR. These challenges were repeated monthly over a period of seven months. Sera were collected at regular intervals during the course of the experiments.

2.3. Serology: ELISA and Mediator Release Assay. Antigen-specific IgG1 and IgG2a antibody levels in sera were determined by using a luminescence-based ELISA, as described [19]. Sera were diluted 1 : 100,000 for IgG1 and 1 : 10,000 for IgG2a determination. Functional IgE levels were assessed by using a rat basophil leukemia (RBL) cell release assay as described previously [20]. For the determination of IgE levels, sera were diluted 1 : 100 (Figure 1) or 1 : 150 (Figure 4).

2.4. Lymphocyte Cultures and Cytokine Detection. To determine cytokine secretion by splenocytes, spleens were isolated and single cell suspensions were prepared as described [19]. Cells were restimulated *in vitro* in the absence or presence of 10 μg/mL recombinant Phl p 5 for 48 h. Cytokine expression in the culture supernatants was analyzed with a FlowCytomix Kit (eBioscience, Schwechat, Austria), following the manufacturer's instructions.

2.5. Bronchoalveolar Lavage. Bronchoalveolar lavage (BAL) was performed as described [21]. In short, cytokines were determined by FlowCytomix assay and cells were stained for FACS analysis with the following markers: anti-CD19-PE/Cy7, anti-CD45-PerCP/Cy5.5, anti-CD4-APC/Cy7, anti-Gr1-APC (all BioLegend, London, UK), anti-CD8-FITC (eBioscience, Schwechat, Austria), and anti-CD25-PE (BD Biosciences, Schwechat, Austria). Red blood cells were lysed and cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). The eosinophil population was distinguished by $CD45^{med}Gr1^{low}$side-$scatter^{high}$ phenotype. Neutrophils were identified as a $CD45^{high}$, $Gr1^{high}$ cell population.

2.6. Whole-Body Plethysmography. To measure the overall airway obstruction, non-invasive unrestrained whole-body

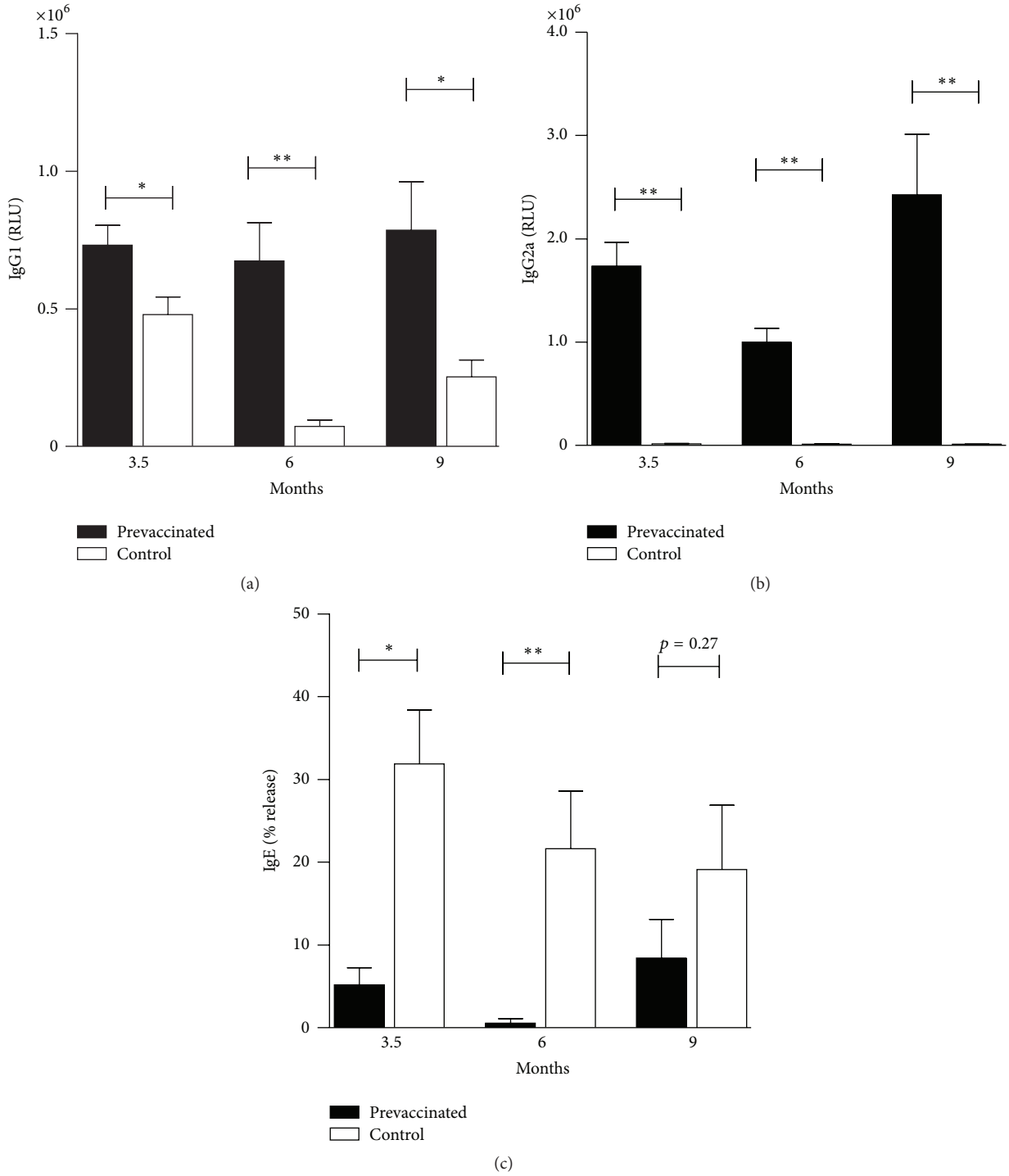

Figure 1: Phl p 5-specific IgG1 (a), IgG2a (b), and IgE (c) antibodies one week after sensitization. BALB/c mice were prevaccinated or left untreated and sensitized with recombinant Phl p 5 after the indicated time interval. Data are shown as relative light units of a luminometric ELISA or as percentage of total release induced by addition of 10% Triton X-100 and presented as means ± SEM ($n = 5$). $^{*}p < 0.05$; $^{**}p < 0.01$.

plethysmography (WBP) was performed using a Buxo WBP system consisting of a Bias Flow Regulator, 6 WBP chambers, a MAX II preamplifier unit, and BioSystem XA Software (Buxco, Winchester, UK). The animals were put into individual chambers and exposed to nebulized 0.9% NaCl followed by increasing concentrations of nebulized methacholine (5 mg/mL; 10 mg/mL dissolved in 0.9% NaCl) followed by 0.9% NaCl for a recovery phase. 50 μL of methacholine or NaCl per chamber was applied to the nebulizer head and enhanced pause in breathing (Penh) was measured for 5 min

at each concentration. Data were analyzed as the area under the curve [22].

2.7. Dynamic Lung Resistance and Compliance Measurement. Resistance and dynamic compliance were measured with a FinePointe Series RC site (Buxco, Winchester, UK), according to the manufacturer's instructions. Mice were anesthetized by means of intraperitoneal ketamine/xylazine injection, and the trachea was surgically exposed, cannulated, and connected to the ventilator. Transpulmonary pressure was measured by inserting an esophageal cannula. Baseline signals for resistance and dynamic compliance were recorded, and mice were exposed to aerosolized PBS containing increasing amounts of methacholine (5 mg/mL; 10 mg/mL). Values for each dose are expressed as raw values or percentage of baseline values.

2.8. Statistical Analysis. Differences between means of vaccinated versus control sample groups were analyzed by unpaired t-test (Figures 1–3). Comparisons between multiple groups (Figures 5 and 6) were done by one-way ANOVA followed by Tukey's post hoc test. All statistical analyses were performed using GraphPad Prism 5.

3. Results

3.1. RNA Vaccination Induces Long-Term Protective Memory Responses. To evaluate the long-term memory and duration of the protective effect after mRNA immunization, animals were vaccinated three times in one-week intervals with 100 μg of capped-Phl p 5 mRNA i.d. and sensitized with recombinant allergen after 3.5, 6, and 9 months, respectively. Sensitization was performed by s.c. injection of 1 μg recombinant Phl p 5 in alum. Seven days later, mice received three challenges with nebulized recombinant Phl p 5, on three consecutive days, in order to induce TH2-mediated lung inflammation. The effect of mRNA vaccination on the humoral immune response was determined by measuring IgG1 and IgG2a levels after the last aerosol challenge. We found antigen-specific IgG1 significantly elevated in prevaccinated mice compared to control animals even 9 months after the initial vaccination (Figure 1(a)), suggesting the presence of B cell memory. More strikingly, only prevaccinated mice showed Phl p 5-specific IgG2a, indicating the maintenance of an RNA vaccine-induced TH1 memory for at least 9 months (Figure 1(b)). Levels of functional Phl p 5-specific IgE were assessed by RBL release assay and data shows that mRNA vaccination reduced allergen-specific IgE levels at all three time points (Figure 1(c)). This data clearly demonstrates that RNA vaccination induces a long-lasting humoral immune response and maintains a TH1-biased memory, which prevents IgE induction for at least 6 months following prophylactic immunization.

To evaluate the long-term effects of mRNA vaccination on T cell responses, splenocytes of prevaccinated and control animals were harvested at each time point and cytokine secretion upon restimulation with antigen was measured. In contrast to cells from prevaccinated mice, splenocytes from control animals displayed elevated secretion of TH2 cytokines, including IL-5, IL-6, and IL-13 (Figures 2(a)–2(c)). In return, prevaccination significantly increased the expression of the TH1-type cytokines IL-2 and IFN-γ even 9 months after vaccination, compared to cells from control mice (Figures 2(d)-2(e)). This data confirms that RNA vaccine-induced TH1 memory responses are robust and long-lasting. No significant differences in IL-10 expression could be detected between prevaccinated and control groups (Figure 2(f)). IL-17 production was below the detection limit, whereas levels of IL-21 and IL-22 were detectable but not influenced by vaccination (data not shown).

Exposure to inhalant allergens, such as the grass pollen allergen Phl p 5, can cause the emergence of inflammation in the lung and the development of asthma. To test the efficacy of mRNA vaccination to protect from lung inflammation, BAL fluids were analyzed. The levels of IFN-γ were found to be significantly higher in the BAL fluids of prevaccinated mice (Figure 3(d)) and correlated with reduced levels of TH2-type cytokines. BAL fluids of prevaccinated mice contained less IL-4, IL-5, and IL-13 (Figures 3(a)–3(c)) compared to control groups. Eosinophil recruitment, one of the hallmarks of allergic lung inflammation, could be measured in all groups after the challenge with aerosolized allergen but was significantly reduced in prevaccinated mice (Figure 3(e)), whereas the percentage of infiltrating neutrophils in the lung was increased in the vaccinated groups (Figure 3(f)).

An important characteristic of allergic asthma is increased AHR. We assessed the effects of mRNA vaccination on AHR by measuring resistance and dynamic compliance in response to increasing concentrations of aerosolized methacholine. No statistically significant differences between prevaccinated and control mice could be detected, concerning neither resistance nor compliance of the lungs. However, by trend, vaccinated groups showed reduced resistance and increased compliance. This also indicates that the increase in neutrophils during the acute phase had no detrimental effect on lung function (Supplementary Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/797421).

3.2. RNA Vaccination Maintains Long-Term Protection over Repeated Aerosol Challenges. To determine the robustness of the protective immune response and whether repeated aerosol exposure would either abrogate the TH1-mediated protective effect by reconversion to an allergic TH-2 response or induce TH1-driven side effects, BALB/c mice were prevaccinated with an mRNA vaccine encoding Phl p 5 or the irrelevant antigen Bet v 1 (mock control) and sensitized with recombinant Phl p 5 one month later. Subsequently, mice were challenged monthly by exposure to aerosolized recombinant Phl p 5 on three consecutive days over a period of seven months. IgG1 and IgG2a levels were determined by ELISA after each challenge with aerosolized allergen.

Elevated levels of antigen-specific IgG2a were present in sera from prevaccinated mice throughout the experiment, indicating the maintenance of TH1-type memory induced by mRNA vaccination (Figure 4(b)). Prevaccinated mice also showed higher Phl p 5-specific IgG2a titers compared to the mock-RNA control groups, clearly pointing to the specificity

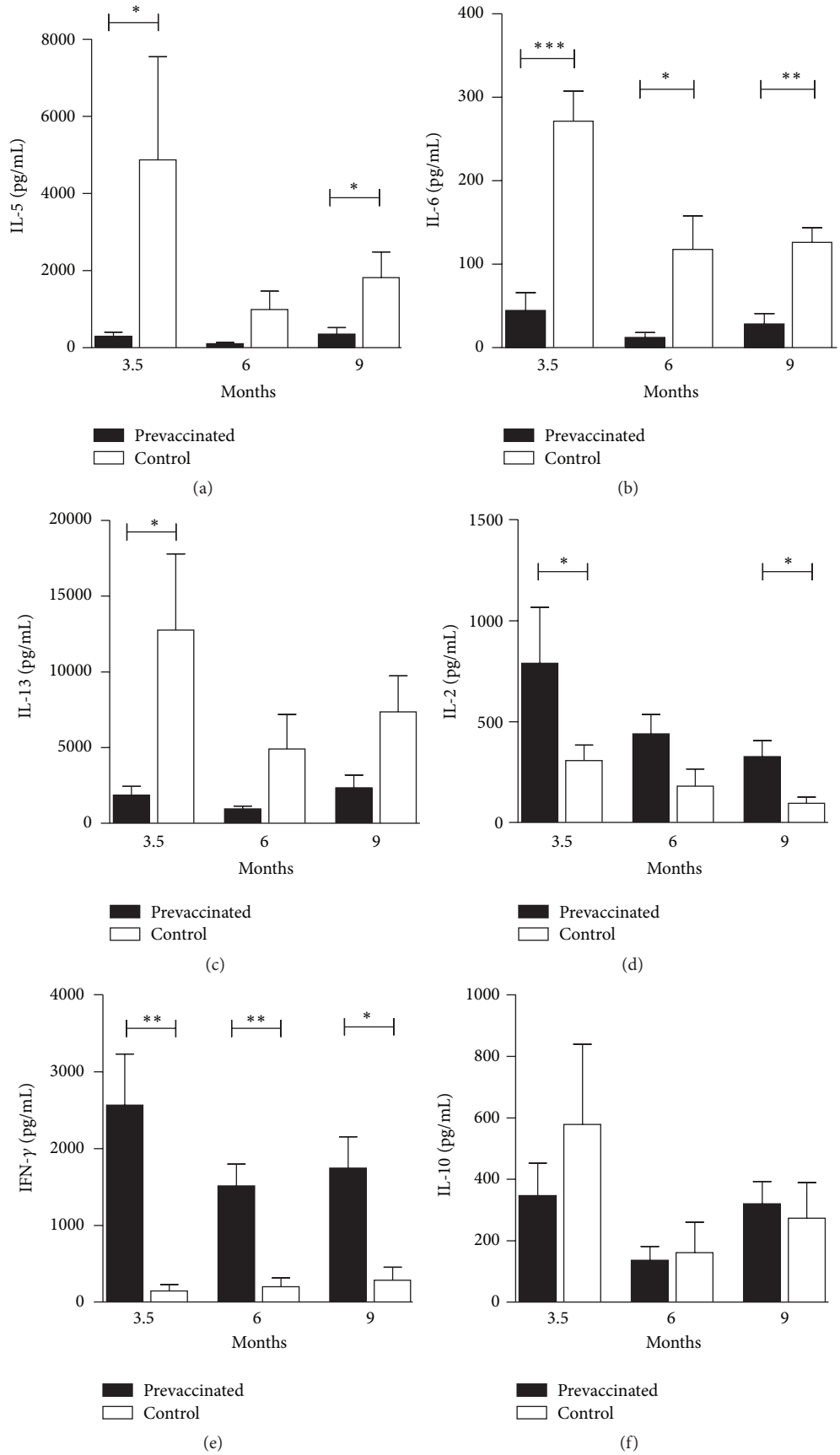

Figure 2: Levels of IL-5 (a), IL-6 (b), IL-13 (c), IL-2 (d), IFN-γ (e), and IL-10 (f) were determined in culture supernatants after *in vitro* restimulation of splenocytes with rPhl p 5. Data are displayed as means ± SEM ($n = 5$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

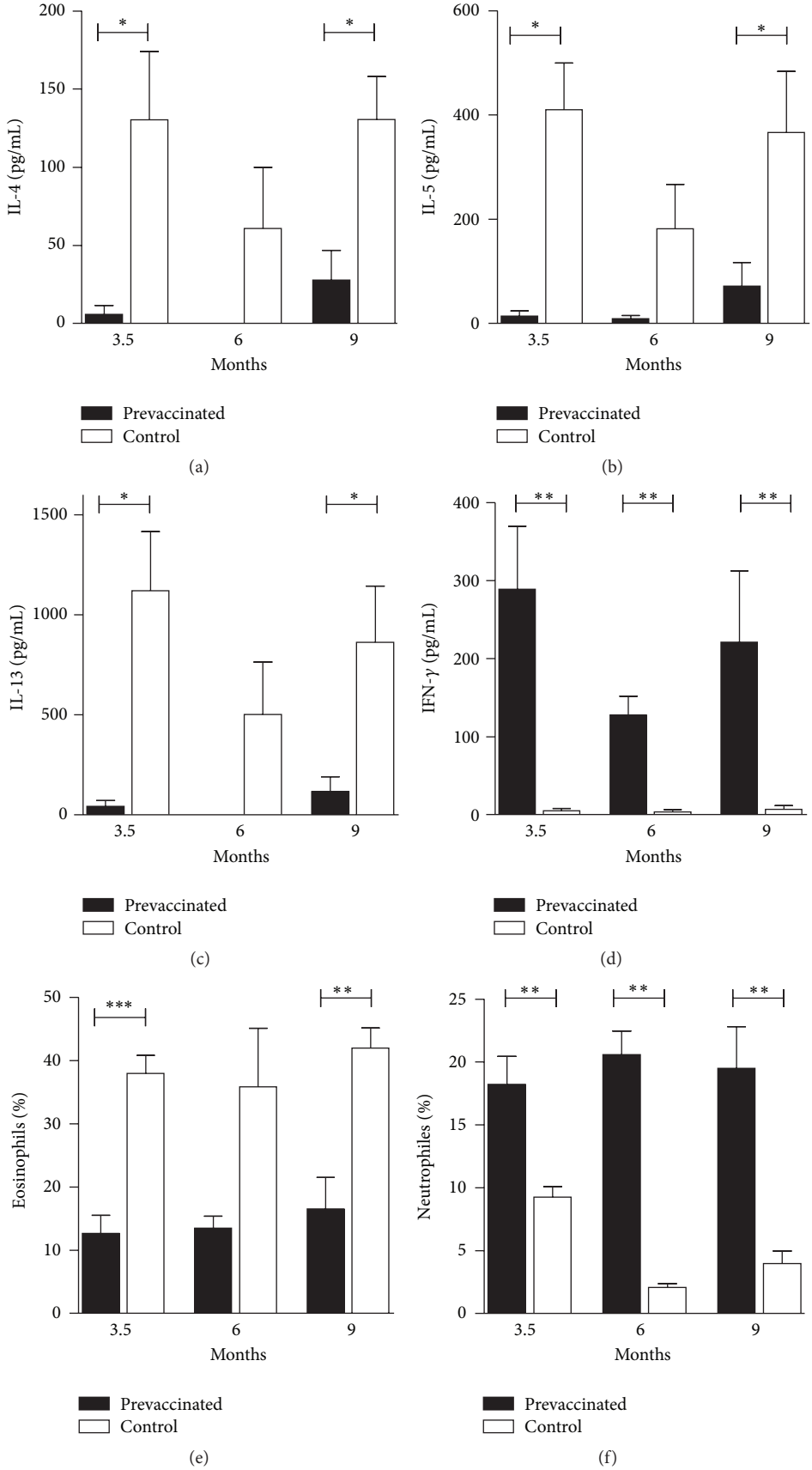

Figure 3: Levels of IL-4 (a), IL-5 (b), IL-13 (c), and IFN-γ (d) as well as the percentage of eosinophils (e) and neutrophils (f) of total leukocytes in BAL fluids were assessed. Data are shown as means ± SEM ($n = 5$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

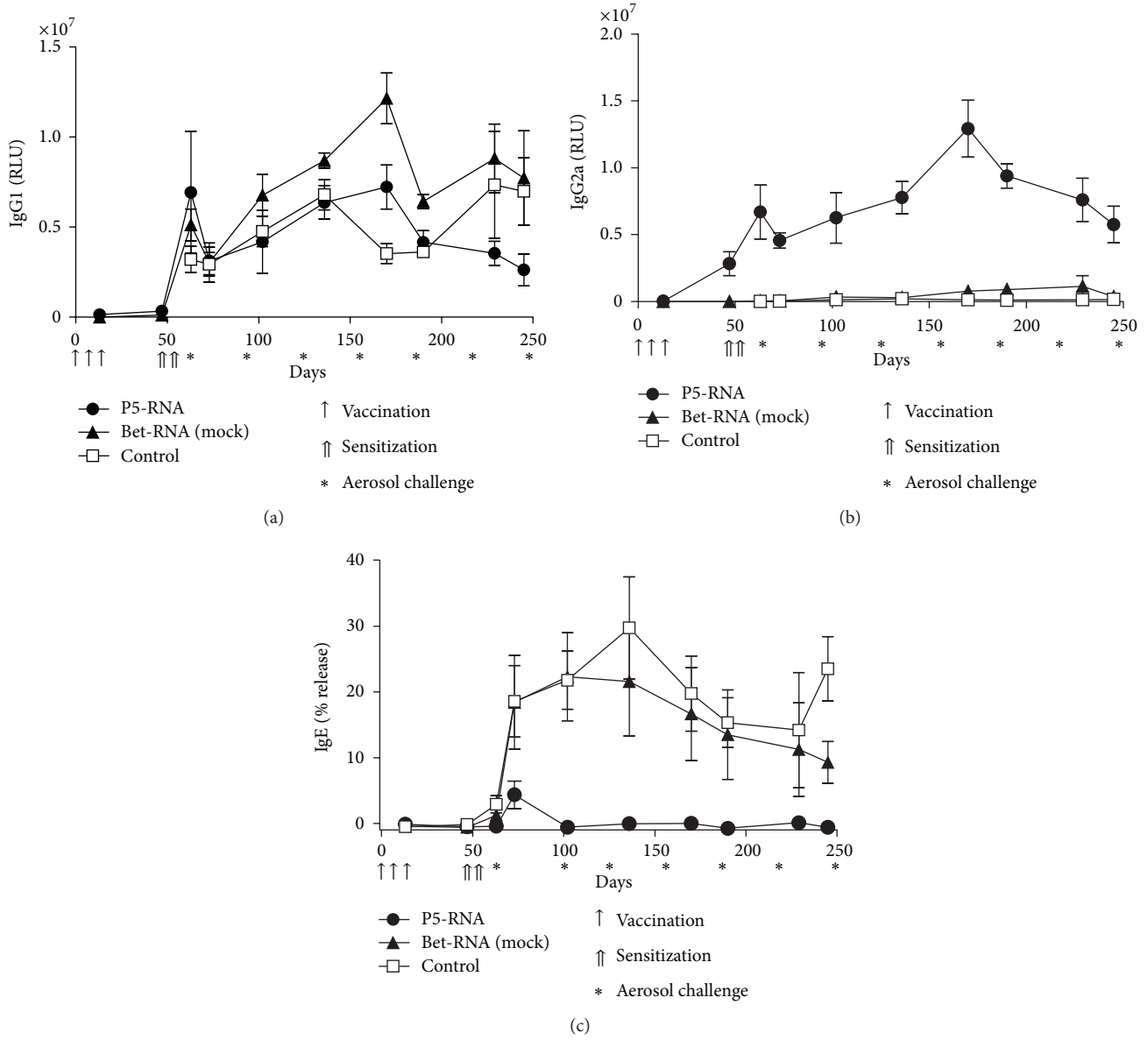

Figure 4: Time course of Phl p 5-specific IgG1 (a), IgG2a (b), and IgE (c) antibody levels as measured by a luminometric ELISA or RBL assay, respectively. BALB/c mice were prevaccinated and sensitized one month later, followed by monthly exposure to aerosolized rPhl p 5. Data are displayed as means ± SEM ($n = 5$).

of the mRNA vaccination. Phl p 5-specific IgG1 antibodies did not differ between the groups (Figure 4(a)). Importantly, vaccination with mRNA strongly suppressed the induction of Phl p 5-specific IgE antibodies. Levels of IgE were significantly reduced in the prevaccinated group even after mice had been challenged 21 times with aerosolized recombinant Phl p 5 over a period of seven months (Figure 4(c)). This correlated with significantly reduced secretion of TH2 cytokines IL-5, IL-6, and IL-13 by splenocytes from prevaccinated mice, which had been restimulated *in vitro* (Figures 5(a)–5(c)). In contrast, IL-2 and IFN-γ were increased in prevaccinated mice (Figures 5(d)-5(e)), indicating the maintenance of a TH1-biased response. We further confirmed that vaccination does not induce Tr1 cells, as shown by similar levels of IL-10 secretion in all groups (Figure 5(f)).

In addition to the systemic immune response, we also investigated the response in the target organ of the aerosol challenge, the lung. Similar to the cytokine expression by *in vitro* stimulated splenocytes, levels of IFN-γ were higher in BAL fluids from prevaccinated mice (Figure 6(b)), and this IFN-γ induction correlated with reduced IL-5 secretion, as prevaccinated mice had significantly decreased IL-5 levels in BAL fluids compared to control groups (Figure 6(a)). Furthermore, antigen specificity of protection was confirmed as levels of IFN-γ and IL-5 expression in mock-RNA vaccinated groups were similar to the control group

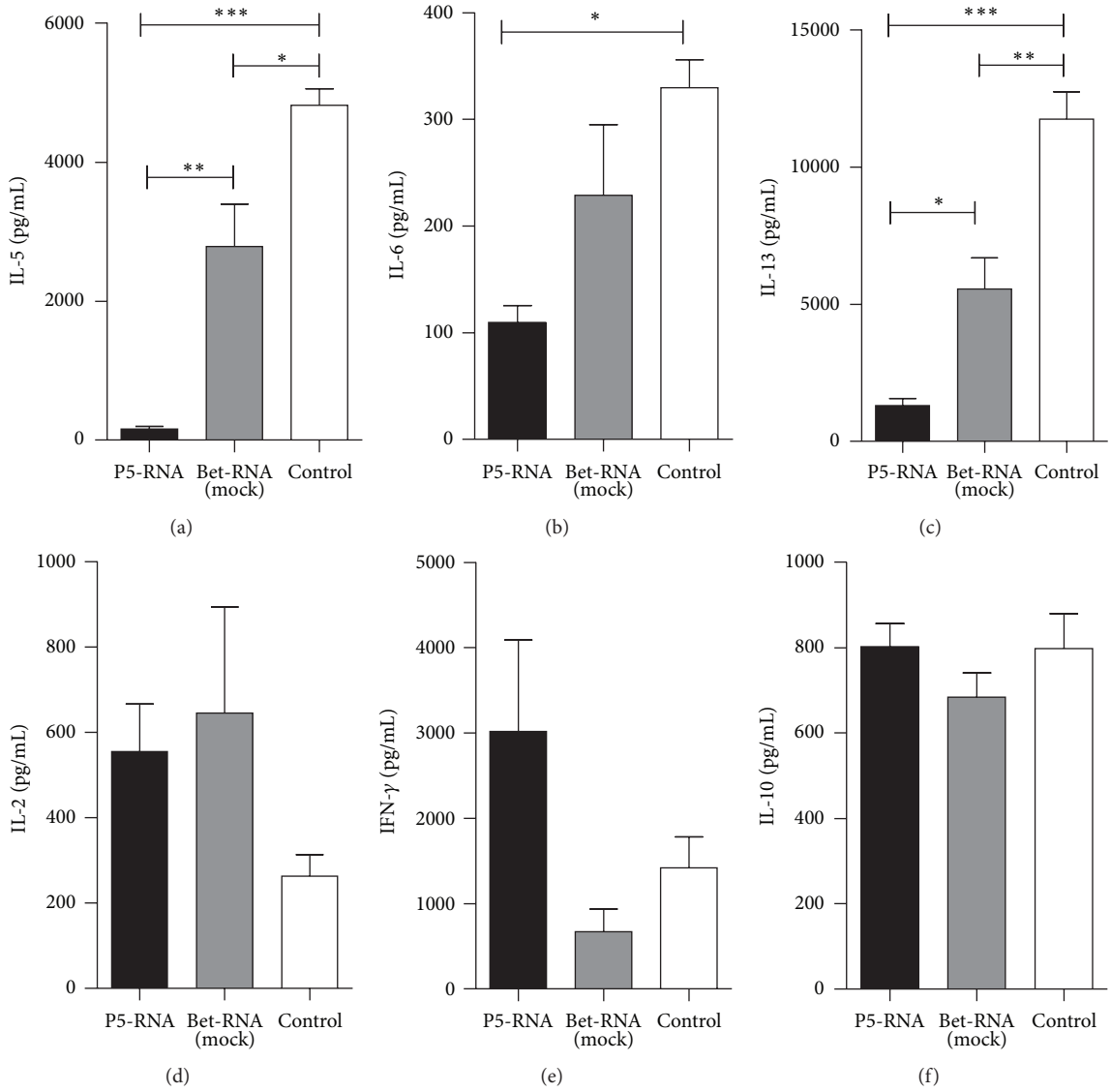

Figure 5: Levels of IL-5 (a), IL-6 (b), IL-13 (c), IL-2 (d), IFN-γ (e), and IL-10 (f) in supernatants of *in vitro* restimulated splenocyte cultures. Cells were obtained from mice immunized 9 months before, sensitized, and repeatedly exposed to aerosolized rPhl p 5. Data are displayed as means ± SEM ($n = 5$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

(Figures 6(a) and 6(b)). As seen in the acute phase response (Figure 3), the percentage of eosinophils infiltrating into the lung was significantly reduced in prevaccinated mice despite repeated aerosol challenges (Figure 6(c)). The percentage of neutrophils in the lung infiltrate was relatively high. However, this increased infiltration of neutrophils was not dependent on prevaccination and was present in all groups (Figure 6(d)).

To assess AHR during the course of this experiment we measured enhanced pause (Penh) with noninvasive whole-body plethysmography. Mice pretreated with the mRNA vaccine overall showed lower AHR compared to mock-RNA treated or control animals (Figure 6(e)). Additionally, after the final airway challenge, AHR was directly assessed using invasive R/C measurement. In agreement with the immunological results, these data also indicate a protective effect of the prevaccination with mRNA, which improved lung function compared to the nonvaccinated group (Figures 6(f) and 6(g)).

4. Discussion

We have previously demonstrated that mRNA vaccination can protect against a broad range of allergens in a mouse model of allergic asthma [17]. However, concerns have been raised that the short persistence of mRNA vaccines might induce insufficient memory responses. In the current study, we show for the first time that the protective, antiallergic TH1 memory is long-lasting and sufficient to prevent allergic sensitization up to 9 months after the initial vaccination. Sensitization, that is, induction of a TH2-biased immune response, is inhibited with respect to both branches of the immune system. Vaccination with mRNA encoding the

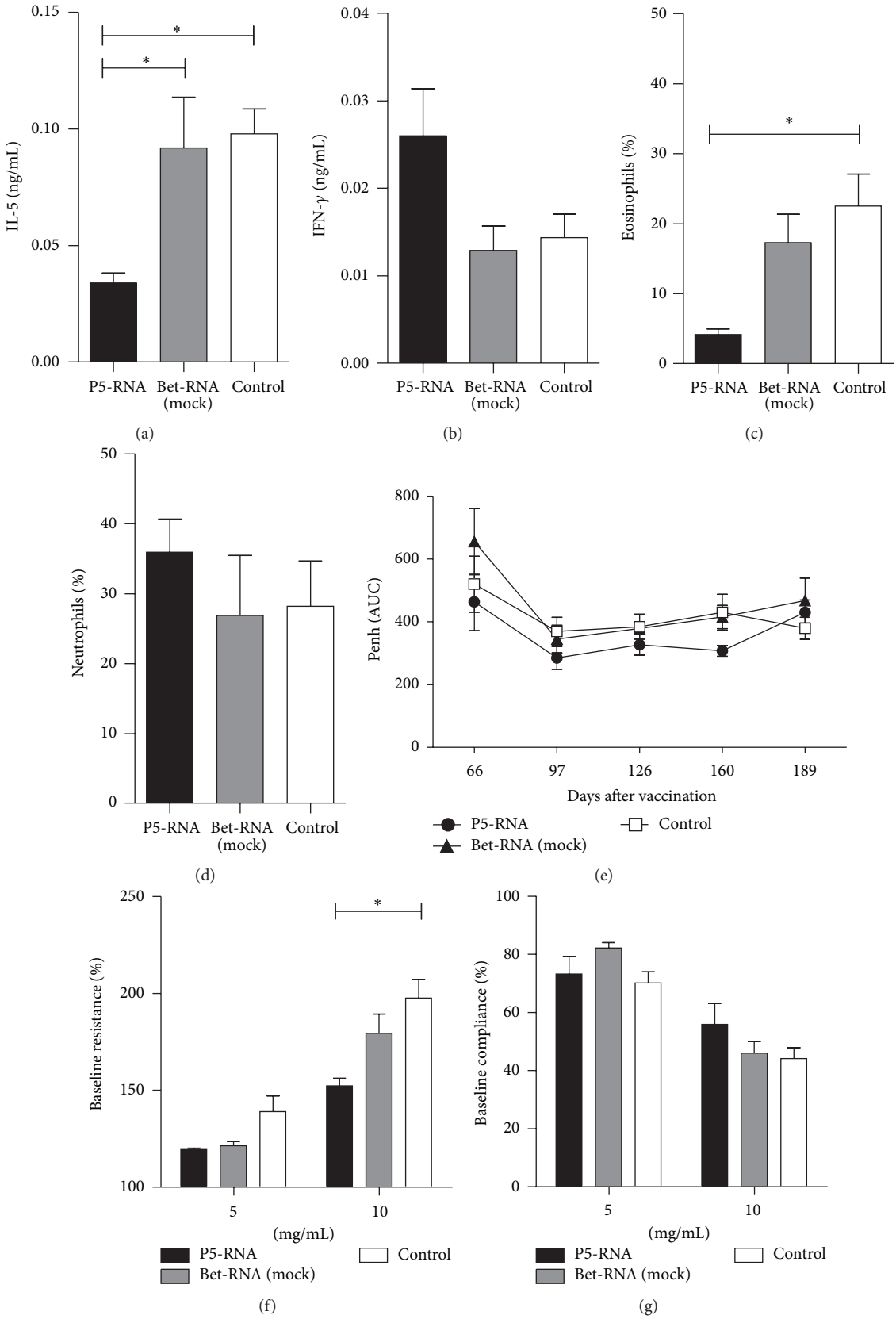

Figure 6: Levels of IL-5 (a) and IFN-γ (b) as well as the percentage of eosinophils (c) and neutrophils (d) of total leukocytes in BAL fluids determined 9 months after mRNA immunization. Effects of prevaccination on lung function were determined by measuring airway hyperresponsiveness via noninvasive whole-body plethysmography throughout the experiment (e) or invasive measurement of lung resistance (f) and dynamic compliance (g) at the endpoint. Mice were exposed to nebulized 0.9% NaCl followed by increasing concentrations of nebulized methacholine. Data are shown as means ± SEM ($n = 5$). $^{*}p < 0.05$.

grass pollen allergen prevents expression of TH2 cytokines, such as IL-5 and IL-13, and proinflammatory IL-6 by *in vitro* restimulated splenocytes, thus reflecting the systemic effect. It also inhibits the induction of a TH2-mediated lung inflammation as indicated by reduction of TH2 cytokines in the lung. IFN-γ in BAL fluid was elevated in vaccinated mice, especially in the acute phase of the response. IFN-γ plays an ambiguous role in allergic lung inflammation. On the one hand, it directly inhibits TH2 cells, induces apoptosis in eosinophils, reduces levels of lung IgE and airway hyperresponsiveness, and directly acts on lung epithelial cells, thus blocking mucus production [23–25]. On the other hand, chronic expression of IFN-γ has also been shown to enhance allergen induced eosinophilia, IL-5, and IL-13 expression [24] and has induced side effects when administered to allergic patients [25]. These data illustrate that IFN-γ has potent immunomodulatory capacities that can be highly beneficial but also induce side effects at high dosages. In our model, no detrimental effects were observed.

The induction of regulatory T cells has been shown to play an important role in keeping or restoring a nonallergic balanced status of the immune system against allergens [26]. However, in our model no significant differences in IL-10 expression could be detected between prevaccinated and control groups indicating no crucial role of IL-10-secreting Treg cells (Tr1) in the mechanisms underlying protection from an allergic immune response by mRNA immunization (Figure 2(f)).

Furthermore, recruitment of eosinophils to the lung was significantly reduced in the vaccination groups. Interestingly, the percentage of infiltrating neutrophils in the lung was increased in the vaccinated group during the acute phase of the lung response (Figure 3(f)). Similarly, Duechs et al. observed that application of various TLR agonists in an asthma model reduced airway eosinophilia and airway resistance but at the same time increased neutrophil influx [27]. It has been shown that IL-17 derived from TH17 cells [28] or iNKT cells [29] can induce recruitment of neutrophils to the lung. TH17 cells have also recently been found to be involved in the pathology of allergy [30]. However, in our model IL-17 production was below the detection limit, whereas levels of IL-21 and IL-22 were detectable but no alterations could be seen in the vaccination groups (data not shown). IFN-γ has also been shown to boost trafficking of neutrophils into the lung [31], and the elevated levels of this cytokine during the acute phase in the vaccinated groups may therefore contribute to the enhanced influx of neutrophils. During the chronic phase of inflammation, due to repeated exposure to aerosolized allergen, the percentage of neutrophils in the lung infiltrate was relatively high. However, this increased infiltration of neutrophils was not dependent on prevaccination but was present in all groups (Figure 6(d)). TNF-α, a cytokine secreted by TH1 cells, may also be responsible for recruitment of eosinophils to the lung [32]. In the acute phase of the response we found low levels of TNF-α in splenocytes which were higher in vaccinated mice compared to control animals, at least at the earlier challenge time points (Supplementary Figure S2A). TNF-α levels were higher after chronic allergen exposure in all groups (Supplementary Figure S2B). Neutrophil invasion therefore most likely represents a general side effect of chronic exposure to allergen and is only induced by mRNA vaccination during the acute sensitization phase. Nevertheless, it must be emphasized that even in the acute phase this vaccine-induced influx of neutrophils does not impair lung function, as AHR is not increased in these groups.

Our data further ascertained that mRNA vaccination induces a robust protection and inhibits induction of a TH2-type response even after repeated exposure to a high dose of allergen. Thus, repeated allergen exposure during the pollen season does neither lead to mitigation of the established TH1-biased response nor does it induce TH1-driven lung pathology. In contrast, it acts like booster immunizations and thus resembles the mechanisms by which lifelong specific immunity can be maintained against certain pathogens, after a single vaccination. Likewise one or two injections of an mRNA vaccine would be sufficient to trigger the allergen-specific recruitment of protective TH1 memory cells and, in the case of seasonal allergens, the natural exposure acts as boost and refreshment of the protective response type. Moreover, prophylactic mRNA vaccination against allergens does not need as strong immune responses as classical vaccination approaches against pathogens or tumors. An almost nondetectable primary immune response induced by the mRNA vaccine is sufficient to set an immunological bias, which prevents subsequent sensitization against the allergen [17, 21, 33].

5. Conclusions

mRNA vaccination prevents an allergen-specific TH2-type response by suppressing TH2 cytokines, eosinophils, and IgE expression, while increasing TH1-type parameters such as IFN-γ expression. Collectively, our data indicate that mRNA vaccines are effective in inducing a protective, robust, and long-lasting TH1-biased immune response.

mRNA vaccines therefore combine effective prevention of allergic sensitization with a commendable safety profile.

Abbreviations

AHR: Airway hyperresponsiveness
BAL: Bronchoalveolar lavage
SIT: Specific immunotherapy
Penh: Enhanced pause.

Conflict of Interests

E. Roesler, R. Weiss, S. Scheiblhofer, and J. Thalhamer are inventors of an RNA vaccine related patent held by BioNTech RNA Pharmaceuticals GmbH.

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