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ORIGINAL RESEARCH

A poly-neoantigen DNA vaccine synergizes with PD-1 blockade to induce T cell-mediated tumor control

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ABSTRACT

The combination of immune-stimulating strategies has the potency to improve immunotherapy of cancer. Vaccination against neoepitopes derived from patient tumor material can generate tumor-specific T cell immunity, which could reinforce the efficacy of checkpoint inhibitor therapies such as anti-PD-1 treatment. DNA vaccination is a versatile platform that allows the inclusion of multiple neoantigen-coding sequences in a single formulation and therefore represents an ideal platform for neoantigen vaccination. We developed an anti-tumor vaccine based on a synthetic DNA vector designed to contain multiple cancer-specific epitopes in tandem. The DNA vector encoded a fusion gene consisting of three neoepitopes derived from the mouse colorectal tumor MC38 and their natural flanking sequences as 40 amino acid stretches. In addition, we incorporated as reporter epitopes the helper and CTL epitope sequences of ovalbumin. The poly-neoantigen DNA vaccine elicited T cell responses to all three neoantigens and induced functional CD8 and CD4 T cell responses to the reporter antigen ovalbumin after intradermal injection in mice. The DNA vaccine was effective in preventing outgrowth of B16 melanoma expressing ovalbumin in a prophylactic setting. Moreover, the combination of therapeutic DNA vaccination and anti-PD-1 treatment was synergistic in controlling MC38 tumor growth whereas individual treatments did not succeed. These data demonstrate the potential of DNA vaccination to target multiple neoepitopes in a single formulation and highlight the cooperation between vaccine-based and checkpoint blockade immunotherapies for the successful eradication of established tumors.

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Introduction

Tumor cells accumulate somatic point mutations that can alter wild-type protein sequences making them immunologically different from healthy cells. T cells can detect these alterations by virtue of recognition of MHC-presented peptides up to single amino acid modifications, named antigenic neoepitopes.[1] However, the initiation of spontaneous tumor-specific T cell responses is limited by the lack of proper immune stimulation and is often dampened by the immune suppressive activity exerted by the tumor.[2] Checkpoint inhibitor therapies unleash these T cells and result in the long-term survival of patients with previously untreatable cancers.[3] Still, only a minority of patients benefits from checkpoint inhibitor therapies, leaving room for complementary strategies.[4] These may include vaccination against neoantigens, which can not only boost pre-existing responses but also induce *de novo* priming of tumor-specific T cells.

The design of personalized cancer vaccines harboring tumor mutations is still in early stage and needs to meet several requirements.[5] Exact prediction of the neoantigens likely to generate a peptide epitope that will bind to the relevant MHC alleles and induce functional T cell responses is still not fully achievable by the current *in silico* systems used for epitope prediction. Therefore, it is required to include a sufficient number of candidate sequences to increase the chances of including actual T cell epitopes in the vaccine. Furthermore, the inclusion of multiple antigens could promote the generation of a broad immune response, which may enhance vaccine efficacy and contribute to counteract immune suppression. Another requisite for patient-tailored cancer vaccines is versatility in synthesis and production of several different sequences, as the heterogeneous array of antigen sequences varies across individual patients. This aspect is not trivial in classical peptide-based systems, as amino acid sequence dictates the physico-chemical properties of the vaccine, adding complications to the manufacturing process and formulation.[6] In short, an ideal neoantigen vaccine platform should be flexible enough to be able to incorporate a multitude of epitopes and allow fast and reliable production independently of the exact amino acid sequences of the selected epitopes.

In the last few years, efforts in refining neoantigen identification and formulation of cancer vaccines for therapeutic treatment have demonstrated the potential of this approach in preclinical models for synthetic peptide- and RNA-based vaccines.[7-10] These studies have led the way for the first in-

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human application in two independent pioneering trials in melanoma patients.[11,12] Vaccination with neoepitopes derived from single amino acid mutations selected upon sequencing of patients' material elicited tumor-specific T cell responses with clinical benefits both with peptide- and RNA-based vaccines.

Up until recently, DNA-based vaccines targeting neoantigen have been scarcely explored. DNA represents a versatile platform that can accommodate any sequence without affecting its stability or solubility. In addition, DNA is easily synthesized and production costs are relatively low. DNA vaccines were first shown to be immunogenic nearly 30 years ago.[13-15] Since then, numerous studies have explored the potential of gene immunization. Methods for optimizing administration routes, delivery, and plasmid design have been central in a variety of preclinical and clinical studies.[16] Several studies demonstrated that immune responses can be induced by intramuscular, intradermal or intravenous administration of DNA[14,17,18] and original administration devices such as gene gun,[19] electroporation[20] and tattooing[21] have been employed to improve transfection efficiency and induction of both humoral and cellular immune responses. A recent study using electroporation-mediated DNA delivery of multiple neoantigen constructs showed effective induction of anti-tumor CD8 T cell responses in mice.[22]

In this study, we show the efficacy of DNA vectors as a vaccine carrier for multiple neoantigens based on a string-of-bead design. Using regular intradermal injection without the need for specialized equipment or an adjuvant, the DNA vaccine induced multiple CD8 and CD4 T cell responses against both reporter epitopes and neoantigens. We demonstrate that vaccination enables T cell-mediated anti-tumor control in a prophylactic as well as in a therapeutic setting. Furthermore, we show that DNA vaccination can synergize with and improve the efficacy of checkpoint inhibitor therapy.

Methods

Animals: For vaccination and tumor experiments, 6–8 weeks old female C57BL/6 were purchased from Charles River Laboratories. The TCR transgenic OT-I and OT-II mouse strains were obtained from Jackson Laboratory and maintained on CD45.1$^+$ C57BL/6 background. Mice were housed in specific pathogen-free (SPF) conditions at the LUMC animal facility. All animal experimentations were approved by and according to guidelines of the Dutch Animal Ethical Committee.

Cell lines: The B3Z hybridoma cell line was cultured in IMDM medium (Lonza) supplemented with 8% FCS (Greiner), penicillin and streptomycin, glutamine (Gibco), β-mercaptoethanol (Merck), hygromycin B (AG Scientific Inc) to maintain expression of the beta-galactosidase reporter gene. B16-OVA and MC38 tumor cell lines were cultured in IMDM medium supplemented with penicillin/streptomycin, glutamine, and 8% FCS. B16-OVA were cultured in the presence of G418 (Life Technologies) for maintenance of OVA expression.[23] For *ex vivo* stimulation of lymphocytes, the dendritic cell line D1 was used and cultured as previously described.[24]

DNA construct and peptides: Codon-optimized antigen sequences, fuzed by alanine linkers were synthesized and cloned into a CMV-driven expression vector containing a rabbit beta-globin poly-A signal and kanamycin resistance marker (ATUM). As control, DasherGFP was cloned in the same plasmid vector. Plasmids were propagated in *E. coli* cultures and purified using Nucleobond Xtra maxi EF columns (Macherey-Nagel) according to manufacturer's instructions. For vaccination, plasmids were column-purified twice, each time using a fresh column, and dissolved at 3 mg/ml in TrisEDTA buffer (1:0.1 mM).

Synthetic long peptides for the five **epitopes** were synthetized by LUMC peptide facility **SIIVFNLL**ELEGDYR (Dpagt), LFRA**AQLANDVVL**QIM (Reps1), EL**ASMTNMELM**SSIV (Adpgk), ISQAVHAAHAEINEAGR (OVA CD4), DEVSG LEQLE**SIINFEKL**AAAAAK (OVA CD8) and used as peptide controls for all experiments.

In vitro transfection and antigen recognition assay: 3′000 MC38 cells were seeded overnight in 96-well flat-bottom plates. Next day, cells were transfected using the SAINT-DNA transfection kit (SD-2001, kindly provided by Synvolux). In brief, a solution of plasmids and cationic lipids was mixed in a ratio 1:20 (μg DNA: μl Saint-DNA) in titrating quantities. SIINFEKL presentation by H2-K^b was detected with 25-D1.16 antibody[25] in-house conjugated to Alexa 647. After 48 h, 50′000 B3Z cells per well were added and incubated with transfected cells overnight. The following day, TCR activation triggered by recognition of the SIINFEKL epitope was detected by measurement of absorbance at 570 nm upon color conversion of chlorophenol red-β-D-galactopyranoside (Calbiochem®, Merck).

In vivo proliferation of adoptively transferred OT-I and OT-II cells: Naïve C57BL/6 mice received an intradermal injection of lipoplexes comprising vaccine or control plasmids complexed to cationic lipid SAINT18 (kindly provided by Synvolux)[26] 7, 4, 2 or 0 days prior transfer of ovalbumin-specific OT-I and OT-II cells. CD8$^+$ cells or CD4$^+$ cells were isolated from spleens and lymph nodes of CD45.1$^+$ OT-I or OT-II mice with enrichments sets (BD Biosciences), labeled with 5 μM CFSE (Invitrogen) and intravenously injected in vaccinated mice. Three days after transfer, proliferation of OT-I and OT-II cells was measured in lymph nodes and spleens by CFSE detection in CD45.1$^+$/CD8$^+$ or CD45.1$^+$/CD4$^+$ T cells.

Vaccination with peptide mix: Peptide vaccination was used as a positive control for priming and tumor experiments *in vivo*. It consisted of a mix of 50 μg of the five long peptides containing the five epitopes encoded in the DNA vaccine. The formulation was adjuvanted with 20 μg of poly(I:C) (Invivogen)

Priming of endogenous T cells: Naïve C57BL/6 mice were injected with plasmid-SAINT18 complexes in a 1:0.75 ratio (μg DNA: nmole SAINT18) in 0.9% NaCl[26] either intradermally (30 μl), subcutaneously (30 μl), intramuscularly (30 μl), intraperitoneally (100 μl) or intravenously (100 μl) and boosted after 14 and 28 days. The level of SIINFEKL-specific CD8 T cells was monitored in blood with labeled tetramers. Twelve days after second booster injection, splenocytes were harvested and expanded for 1 week with D1 dendritic cells loaded with long peptide pools. Intracellular staining was performed upon stimulation with individual long peptides overnight in presence of 2 μg/ml Brefeldin A (Sigma Aldrich). T cells and cytokines were detected by antibody staining and analyzed with FlowJo software. The following antibody mix was used: eFluor450 anti-CD3, PE-Cy7

anti-CD4 (eBioscience), BV605 anti-mouse CD8α, APC anti-IFNγ (Biolegend), FITC anti-TNFα (eBioscience), PE IL-2 (eBioscience).

In vivo specific killing: Naïve C57BL/6 were primed and boosted after 21 days with 10 or 100 µg of plasmid-SAINT18 complexes. Twenty-one days after boost, vaccinated mice received peptide-loaded splenocytes to measure the cytotoxic activity of endogenously primed T cells. To this end, splenocytes were harvested from CD45.1^{+} or WT C57BL/6 naïve mice, labeled with 5, 0.25 or 0.0025 µM CFSE and differentially loaded for 1 h at 37°C with 1µM SIINFEKEL, Adpgk or Reps1 epitopes or an irrelevant peptide epitope derived from the E6 protein of Human Papilloma Virus (sequence: RAHYNIVTF). 4′000′000 splenocytes per peptide-loaded group were injected intravenously in vaccinated mice. One day after transfer, mice were sacrificed and single-cell suspension was analyzed by flow cytometry. Specific killing was calculated according to the following equation:

$$\text{Specific killing} = 100 - \left[100 * \frac{\left(\frac{CFSE\ target\ peptide}{CFSE\ irrelevant}\ immunized\ mice \right)}{\left(\frac{CFSE\ irrelevant}{CFSE\ target}\ naïve\ mice \right)} \right]$$

Prophylactic vaccination and B16-OVA tumor challenge: Naïve C57BL/6 female mice were vaccinated intradermally with 10 or 90 µg of plasmid-SAINT18 complexes. At day 42 (21 days after booster injection) 50′000 B16-OVA cells were injected subcutaneously in the flank and tumor growth was monitored. Mice were sacrificed when the tumor volume surpassed 1000 mm^3.

MC38 tumor challenge and therapeutic vaccination: Naïve C57BL/6 female mice were injected subcutaneously in the flank with 350′000 MC38 cells and tumor growth was monitored. When tumors reached a palpable size with an estimated volume of 1 to 2 mm^3 (day 5), mice were vaccinated with 10 µg of plasmid-SAINT18 complexes. Three and 7 days after vaccination, 50 µg of anti-PD-1 (Clone RMP1-14, InvivoPlus, BioXCell) antibody was injected subcutaneously next to the tumor mass.[27] Mice were sacrificed when the tumor volume surpassed 1′000 mm^3.

Statistical analysis: Results are expressed as mean ± SD. Statistical significance among groups was determined by multiple comparisons using the Graphpad software after ANOVA or non-parametric Kruskal–Wallis test. Cumulative survival time was calculated by the Kaplan–Meier method, and the log-rank test was applied to compare survival between two groups. *P*-values of ≤0.05 were considered statistically significant.

Results

Development of a poly-neoantigen DNA vaccine for in vitro and in vivo antigen presentation to T cells.

We aimed to include multiple antigenic sequences in a single DNA vaccine construct, and therefore we designed a plasmid encoding five epitopes in tandem in a single open reading frame (Figure 1(a)). Three epitopes (Dpagt, Reps1, Adpgk) are described neoantigens containing specific point-mutated MHC class I binding sequences present in the mouse colon carcinoma cell line MC38.[7] The other two epitopes are, respectively, the helper (Help) and the cytotoxic T lymphocyte (CTL) epitopes from the model antigen chicken ovalbumin (OVA), and were included as control reporter epitopes for CD4 and CD8 T cell responses, respectively. Every epitope is flanked by its natural amino acid sequence for a total length of approximately 40 amino acids, and is linked to the next epitope by a linker encoding four alanines. Transcription is driven by the strong viral promoter of the immediate early gene 1 (IE1) of human cytomegalovirus (HCMV).

We first analyzed whether these five artificially connected sequences lead to the generation of the expected peptide epitopes and their presentation on MHC molecules. Upon transfection of the designed DNA construct, the translated protein product needs to be processed in such a way that the T cell epitopes are generated and presented by MHC molecules. MC38 cells, which do not express the ovalbumin gene, were transfected with the neoantigen construct and the presentation of the ovalbumin CTL epitope SIINFEKEL was detected by staining the SIINFEKL/H2-K^b complex with the 25-D1.16 antibody (Figure 1(b), upper panel). Transfection with the poly-neoantigen construct, but not with a control GFP-encoding construct, displayed positive staining for SIINFEKL/H2-K^b complexes. Moreover, after transfection with the neoantigen construct, cells were recognized by the hybridoma T cell line B3Z, which express a TCR specific for SIINFEKL/H2-K^b (Figure 1(b), lower panel).

Next, we tested the ability of the neoantigen DNA construct to transfect cells and present the expected reporter ovalbumin epitopes *in vivo*. The plasmid was injected intradermally in mice 7, 4, 2, or 0 days prior transfer of CFSE-labeled OT-I and OT-II T cells, which possess transgenic TCRs specific, respectively, for the CTL and the helper epitopes of ovalbumin. Antigen induced proliferation of these cells was analyzed 3 days after transfer in draining lymph nodes and spleen (Figure 1(c,d), Fig. S1 A and B). Injection of the construct was able to induce both OT-I and, to a lesser extent, OT-II proliferation, confirming successful transfection and presentation of the epitopes also *in vivo*. OT-I and OT-II proliferation upon DNA vaccine injection exhibited different kinetics compared to traditional synthetic peptide vaccine (Figure 1(d)). DNA vaccination presents a slower onset of T cell proliferation compared to peptide vaccination, with optimal induction between 5 and 7 days after DNA vaccination (Figure 1(d), *left panel*), as opposed to 3 days for peptide vaccination (Figure 1(d), *right panel*). Finally, we evaluated whether the position of the epitopes or the artificial linker sequence between them could influence the efficiency of antigen presentation *in vivo*. To test this, we created a variant of the original neoantigen DNA construct in which the reporter SIINFEKL epitope was positioned as the first epitope at the N-terminal end of the polypeptide and additional variants in which the epitopes were connected via different amino acid linkers. We evaluated OT-I and OT-II proliferation upon vaccination *in vivo* (Figure 1(e)). Overall, we observed no significant differences in OT-I or OT-II proliferation between the variants tested. Altogether these results demonstrate efficient MHC surface presentation of the CD8 and CD4 reporter

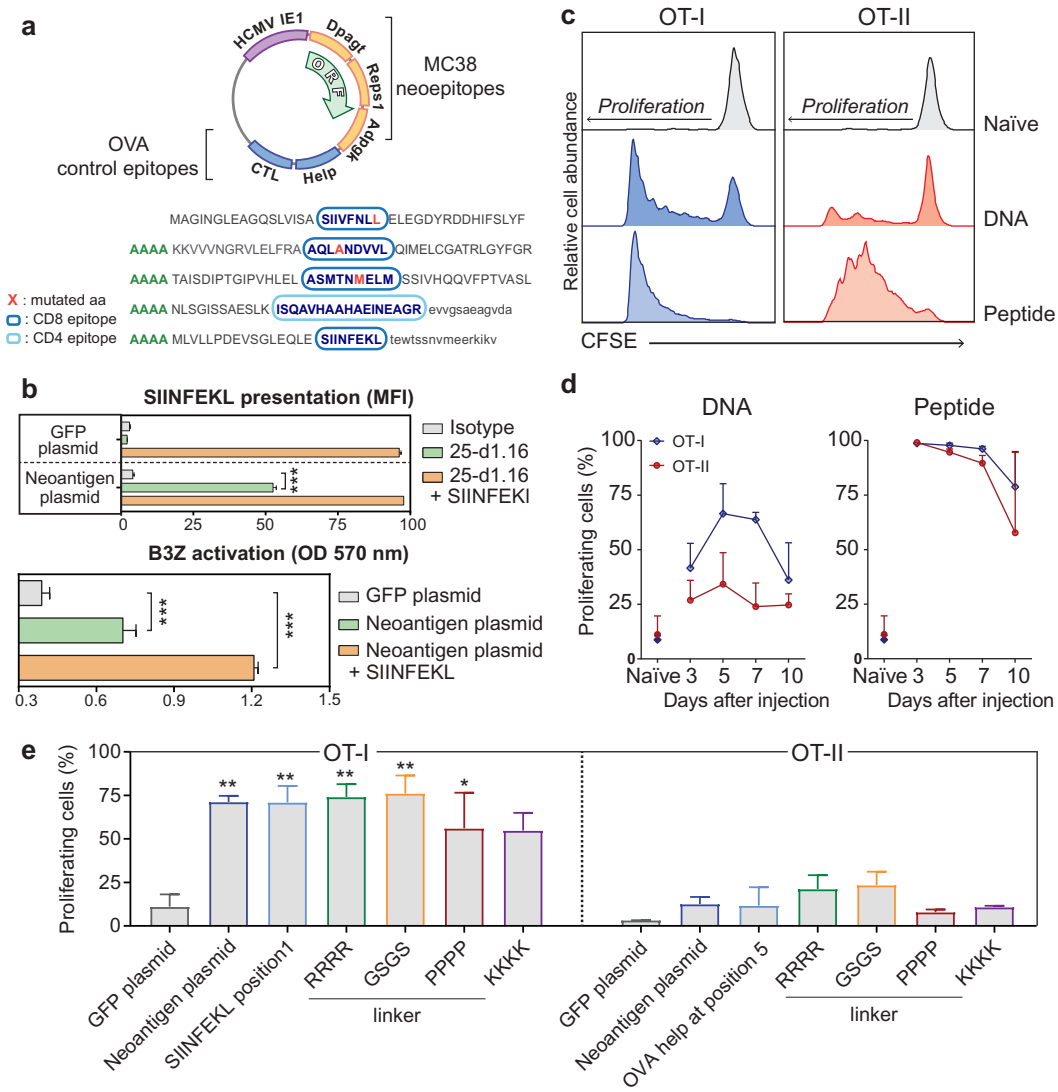

Figure 1. The poly-antigen DNA vaccine activates antigen-specific CD8 and CD4 T cells *in vivo*. (a) Schematic representation of the neoantigen DNA vaccine and the resulting poly-epitope peptide sequence. Direction of the open reading frame (ORF) is indicated. The individual CD8 and CD4 epitopes in the peptide are encircled in dark or light blue, respectively. For each of the three neoepitopes, the amino acid (aa) change resulting from somatic mutation is highlighted in red. (b) *Upper panel*: Quantification of the mean fluorescence intensity (MFI) using 25-D1.16 antibody of the SIINFEKL peptide presentation on MHC I molecule after transfecting MC38 cells *in vitro* with either the GFP plasmid (negative control) or the neoantigen plasmid. *Lower panel*: Activation of SIINFEKL-specific T cell hybridoma B3Z cells by MC38 cells transfected with the neoantigen DNA vaccine. The SIINFEKL synthetic peptide (1 µM) was added as a positive control (orange bars), and a plasmid coding for GFP was used as a negative control. Statistical significance was determined by one-way ANOVA followed by multiple comparison, *** $p < 0.001$ (c) Proliferation of adoptively transferred OT-I and OT-II cells, 3 days after intradermal injection of 10 µg of the neoantigen DNA vaccine. (d) Kinetics of *in vivo* antigen presentation to OT-I or OT-II cells after injection of the DNA construct. Proliferation of the OT-I (blue lines) and OT-II cells (red lines) measured by CFSE dilution in the inguinal lymph nodes of C57BL/6 mice immunized with either neoantigen DNA vaccine (*left panel*) or 50 µg of OVA CTL and helper peptides (*right panel*), used as positive controls for OT-I and OT-II cells proliferation, respectively. Error bars indicate mean ± SD, N = 2. (e) Proliferation of adoptively transferred OT-I and OT-II cells, 3 days after intradermal injection of 10 µg of different variants of neoantigen DNA vaccine. Error bars indicate mean ± SD, N = 2. Significance in relation to the GFP plasmid negative control was determined by one-way ANOVA followed by multiple comparisons. * $p < 0.05$, ** $p < 0.01$.

epitopes of ovalbumin, irrespective of the position and linker sequence in the poly-antigen encoding DNA construct. This shows the feasibility of the string-of-bead design as a method to target multiple antigens in a single vaccine construct.

The DNA vaccine primes neoantigen-specific t cell responses in vivo

Next, we evaluated the ability of our DNA vaccine to generate all five encoded epitopes *in vivo* and its ability to induce *de novo* priming of antigen-specific T cells in wild-type C57BL/6 mice. Previous studies have highlighted an influence on vaccine efficacy depending on its formulation, methods, and routes of administration.[14,17,18,28] To determine the optimal delivery route of our designed DNA vaccine, the construct was administered to mice via different routes (intradermal, subcutaneous, intramuscular, intraperitoneal and intravenous) and the immune response was boosted twice in intervals of 2 weeks (**Fig. S2A**). Tetramer staining in blood at several time points revealed effective priming of SIINFEKL-specific CD8 T cells for the groups that intradermally and intravenously received the DNA vaccines (**Fig. S2B** and **S2C**). In addition, splenocytes of vaccinated mice restimulated *ex vivo* with peptide-loaded dendritic cells displayed responses for all five

epitopes encoded by the DNA vaccine (**Fig. S2D** and **S2E**). We concluded that intradermal injection of the neoantigen DNA vaccine was able to induce *de novo* priming of T cells upon classical needle-mediated administration *in vivo*.

To evaluate the cytotoxic function of the DNA vaccine-induced CD8 T cells, we analyzed the kinetics of the SIINFEKL-specific CD8 T cells and their capacity to specifically kill target cells presenting the epitopes. We also explored the dosing of plasmid administration to optimize the T cell response. C57BL/6 mice were vaccinated and boosted with two different doses of a DNA vector or with peptides, and the ability to kill splenocytes loaded with either SIINFEKL, Adpgk or Reps1 peptide was determined (Figure 2(a)). The kinetics of DNA vaccination was slower compared to the synthetic peptide vaccination, as the peak of the priming response appeared 3 days later (Figure 2(b)). After the boost, T cell responses to DNA and peptide vaccination were similar. Considering the dose, 100 µg of DNA appeared to be more effective mostly in the priming phase.

CD8 T cells primed by DNA were effective in killing SIINFEKL and Adpgk peptide-loaded T cells at day 44. Cytotoxicity against the Reps1 epitope was not detected upon DNA vaccination (Figure 2(c,d)). Vaccination with a higher dose of DNA marginally improved the killing capacity of the SIINFEKL-specific CD8 T cells and correlated with the levels of tetramer-specific cells present in blood two days before injecting target cells (see Figure 2 (b), day 42). Based on these results we concluded that the poly-neoantigen DNA vaccine is able to induce functional CD8 T cells against multiple epitopes and we proceeded to evaluate its efficacy in immune control of cancer.

Prophylactic and therapeutic DNA vaccination elicits tumor control

We next investigated whether the T cell responses induced by DNA vaccination were able to provide immune control of tumors *in vivo* for both the OVA reporter epitopes and the neoantigens.

First, we evaluated anti-tumor efficacy for the reporter ovalbumin epitopes. Mice were prophylactically vaccinated with DNA or peptides before being challenged with the OVA-expressing melanoma cell line B16-OVA (Figure 3(a)). To explore the impact of DNA dosing on the induction of T cell responses, two different amounts of DNA were tested and the induction of ovalbumin-specific CD8 responses was monitored by tetramer staining in blood samples (**Fig. S3**). Unvaccinated control mice developed tumors within 20 days from challenge. Mice vaccinated with DNA developed tumors later than unvaccinated controls, and a significant number of mice were fully

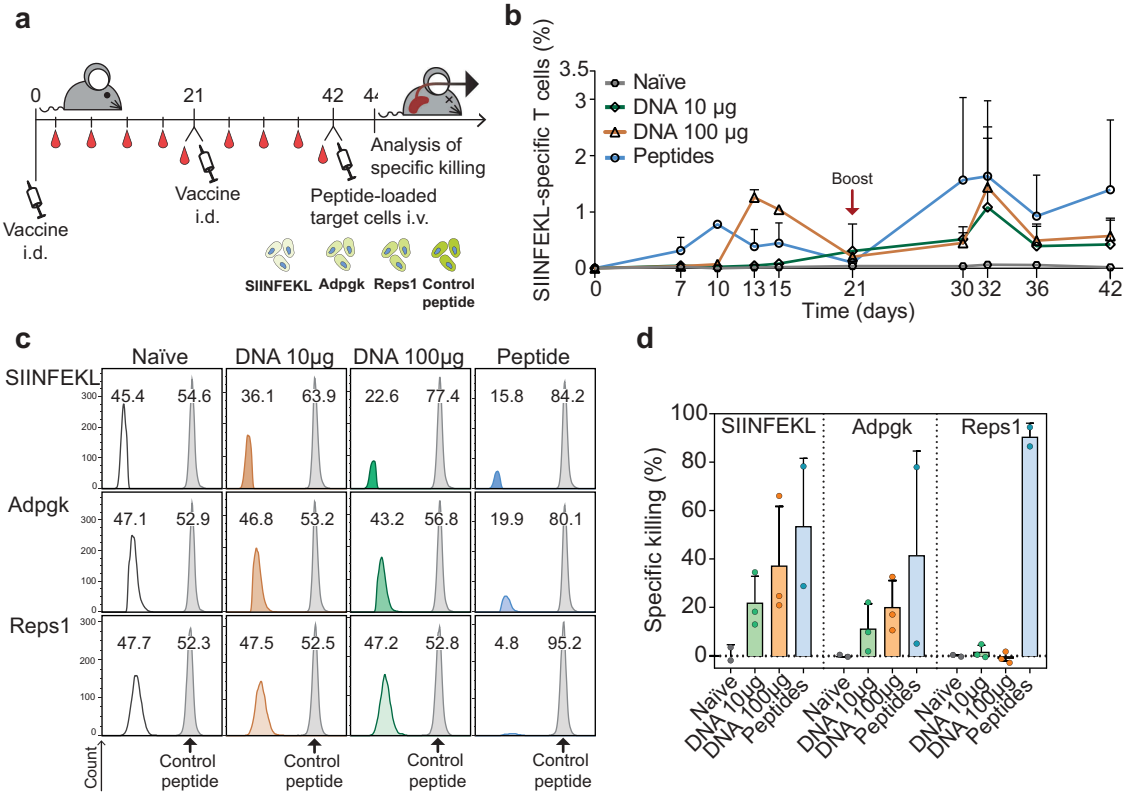

Figure 2. DNA vaccine primed T cells are functional. (a) Schematic representation of the vaccine administration, tetramer staining, target cells injection and specific killing analysis schedule in C57BL/6 mice. Mice were vaccinated intradermally with 10 or 100 µg of DNA (or a mix of peptides as positive control). SIINFEKL-specific responses were monitored in blood at different time points. To evaluate the killing capacity of the responses induced after vaccination, mice were injected with CFSE-labeled splenocytes loaded with minimal peptides and specific killing was analyzed two days later. (b) Kinetics of the SIINFEKL-specific $CD8^+$ T cells responses induced by the vaccines, measured by SIINFEKL-H2-K^b tetramersand reported as percentage of total $CD8^+$ T cells. (c). Representative flow cytometry histograms of CFSE-labeled antigen- or control peptide-loaded splenocytes detected in naïve and vaccinated mice. Two days after transfer, these target cells were detected in the spleen and specific killing was calculated. Percentages represent the relative proportions between cells loaded with an irrelevant control peptide (in gray) and target cells (white, orange, green or blue). (d) Specific killing by T cells in naïve mice versus mice vaccinated with DNA (10 or 100 µg) or peptide after transfer of antigen-loaded splenocytes. Error bars indicate mean ± SD.

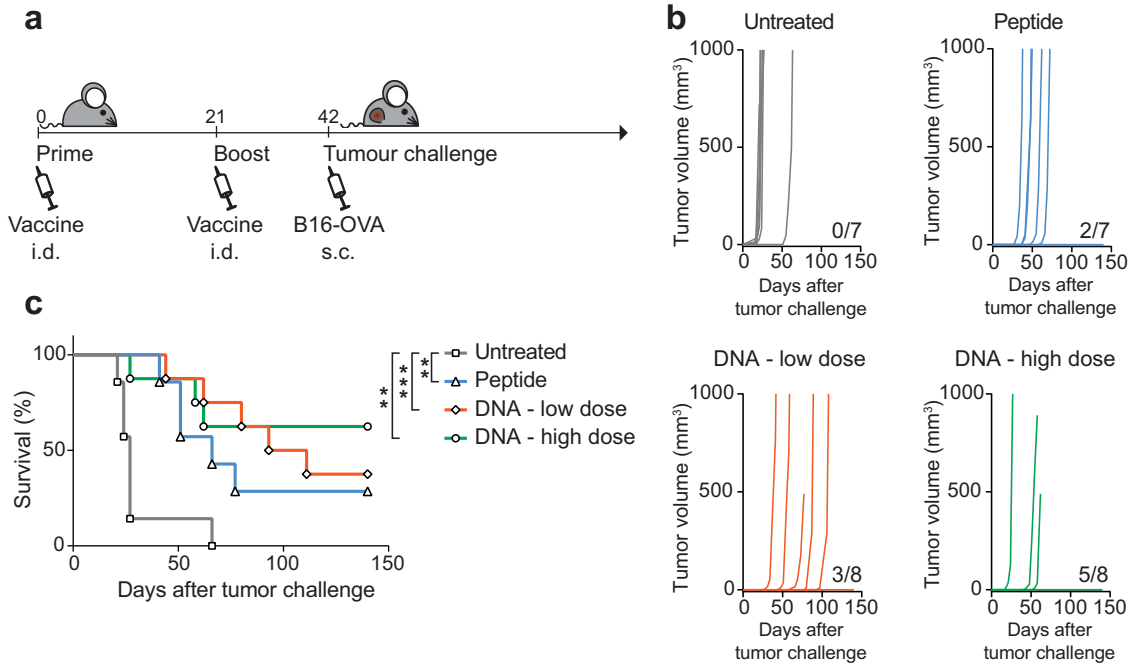

Figure 3. DNA vaccination protects from challenge with B16-OVA. (a) Schematic representation of the schedule followed for vaccine administration and tumor challenge in C57BL/6 mice. Mice were vaccinated with a low (10 µg) or a high (90 µg) dose of DNA or with peptide and subsequently challenged with B16-OVA melanoma cells. Tumor growth was monitored for 150 days after challenge. (b) Tumor growth curves (represented in mm^3) of individual mice in non-vaccinated versus vaccinated groups. The number of tumor-free mice for each vaccination group is indicated. Shown is one of two independently performed experiments which resulted in similar outcomes. (c) Overall survival of mice either untreated or vaccinated with peptide or DNA vaccines. Statistical significance was determined via Log-rank Mantel-Cox test. **$p < 0.01$, ***$p < 0.0001$.

protected from this aggressive tumor. A lower dose of vaccine corresponded to a lower protection but was still effective to prevent tumor growth in ~40% of the animals (Figure 3(b,c)). Vaccination with a higher dose of DNA resulted in full protection of 60% of the mice (Figure 3(b,c)). Hence, DNA vaccines were effective in inducing protective antitumor T cell responses, comparable to or better than the mice that received synthetic peptide vaccination. Moreover, a higher dose of vaccine corresponded to stronger protection, and this dose was used for further studies in a therapeutic setting.

After demonstrating the potential for antitumor activity of the DNA vaccine in the B16 melanoma model, which expresses the ovalbumin antigen but not the MC38-specific neoantigens, we evaluated the therapeutic efficacy of the same DNA vaccine in the MC38 tumor model. Mice were first inoculated with the MC38 colon carcinoma cell line, expressing the three neoantigens Dpagt1, Reps1 and Adpgk but not the ovalbumin epitopes. Mice with established tumors were vaccinated therapeutically on day 5 followed by a booster vaccination at day 26. As MC38 is known to exert a strong immunosuppressive effect,[29] we combined the vaccine with the immunomodulatory anti-PD-1 antibody treatment on day 8, 12, 22 and 29 (Figure 4(a)). Without any treatment, tumors progressed rapidly and all mice succumbed within 21 days from tumor inoculation. Vaccination with DNA or peptides gave little or no delay and eventually all mice showed rapid tumor outgrowth, except for one mouse in the DNA vaccinated group. Anti-PD-1 treatment induced some delay in tumor growth but was not sufficient to prevent tumor outgrowth. Remarkably, when anti-PD-1 treatment was combined with DNA vaccination, tumor growth was significantly delayed and 25% of mice were able to clear the tumor and survive long term (Figure 4(b,c)). Notably, this effect was only observed with DNA vaccination but could not be achieved with the synthetic peptide vaccine.

In addition, a single dose of the DNA vaccine combined with anti-PD-1 treatment (Figure 4(d)) also resulted in a substantial delay of tumor outgrowth and complete tumor clearance was observed in some mice, resulting in a 25% cure rate. (Figure 4(e,f)). Importantly, this effect was not observed when vaccination was performed with a GFP-coding plasmid and was abolished when CD8 T cells were depleted right after vaccination, indicating the implication of neoantigen-specific CD8 T cell responses in tumor growth control. Altogether these data demonstrate that the designed poly-neoantigen DNA construct is an effective vaccine vector and that this design holds potential for neoantigen vaccination for specific immunotherapy of cancer.

Discussion

In this study, we demonstrate that a poly-neoantigen DNA vaccine not only provides prophylactic protection against tumor challenge but also synergizes with PD-1 blockade for tumor control in a therapeutic setting. The versatile DNA platform presented here allows the inclusion of multiple epitopes in tandem derived from multiple antigenic sequences, increasing the chances of triggering relevant T cell responses to improve the overall effectiveness of neoantigen-specific immunotherapy.

Our vaccine vector was able to induce functional responses without any additional adjuvant. DNA vectors may act as self-adjuvating vaccines as the innate immune system possesses

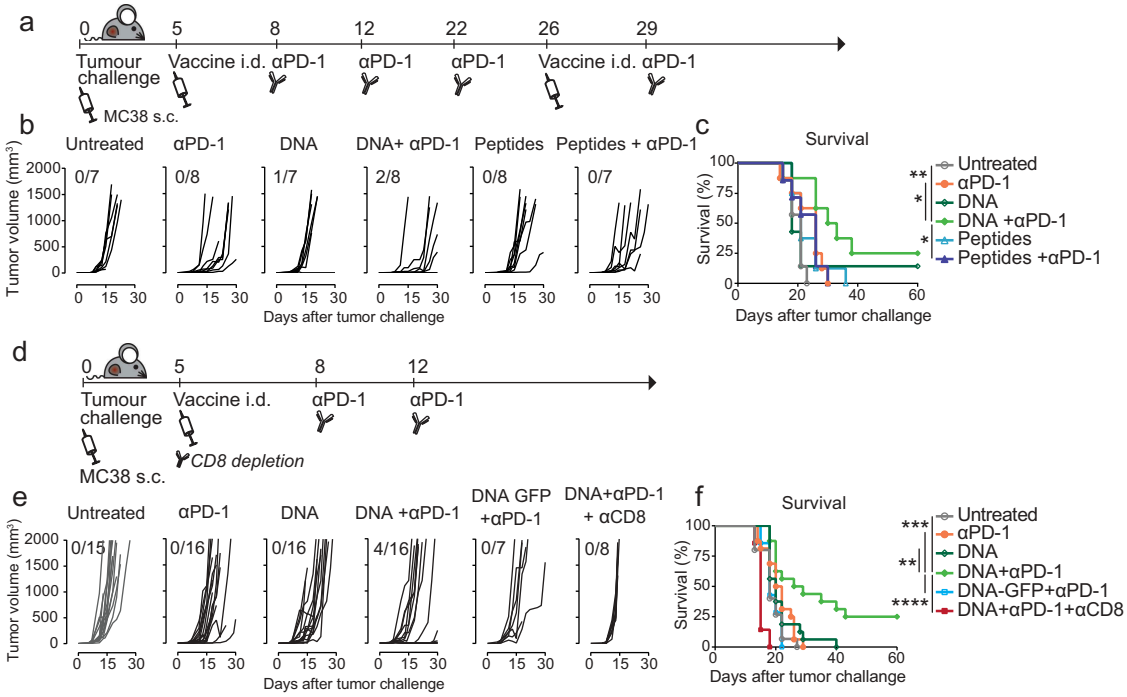

Figure 4. Therapeutic DNA vaccination combined with anti-PD-1 treatment promotes tumor eradication in CD8 T cell-dependent manner. (a) Schematic representation of the MC38 tumor challenge experiment in C57BL/6 mice in therapeutic setting. Mice were injected subcutaneously with MC38 cells at day 0 and vaccinated with the DNA construct at days 5 and 26. Vaccination was combined with anti-PD-1 treatment at days 8, 12, 22 and 29. Tumor growth was monitored for 60 days after challenge. (b) Tumor growth curves (measured in mm^3) of individual mice in untreated or treated with single or combined anti-PD-1, DNA vaccine and peptide mix. The number of tumor-free mice for each group is indicated. (c). Overall survival of mice represented in (b).(d). Schematic representation of the MC38 tumor challenge experiment in C57BL/6 mice and depletion of CD8 cells during vaccine and anti-PD-1 combination treatment. Mice were injected subcutaneously with MC38 cells at day 0, injected with anti-CD8 antibody at day 5, vaccinated with DNA construct and injected with anti-PD-1 antibody at days 5, 8 and 12. (e) Tumor growth curves (measured in mm^3) of individual mice untreated or treated with single or combined anti-PD-1, DNA vaccine and anti-CD8. The number of tumor-free mice for each group is indicated. Vaccination with a control DNA construct (DNA-GFP) that does not contain the three MC38-specific neoantigens is used as a negative control. (f) Overall survival of mice represented in (e). Statistical significance was determined via Log-rank Mantel-Cox test. * $p < 0.05$, **$p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

various means to sense foreign or cytoplasmic DNA and activates an inflammatory response. Low-unmethylated CpG-rich regions linked to bacterial production of the DNA vaccine may contribute to immunogenicity via TLR9 signaling.[30,31] Furthermore, cytoplasmic sensors in the STING axis were also reported to play a role in DNA-mediated immunization.[32] Nevertheless, efforts in optimizing the immunogenicity of DNA vaccines demonstrated that the inclusion in the sequence of pro-inflammatory cytokines such as IL-12[33] and GM-CSF[34-36] and costimulatory-molecules such as B7-1, B7-2[37,38] or CD40L[39] have a beneficial effect in generating effective immune responses. As we report significant but partial tumor control, it will be of interest to improve the cure rate by including such genetic adjuvants, which can readily be incorporated in the vaccine sequence.

The induction of CD8 as well as CD4 T cell responses is critical for cancer immunotherapy, as the ability of cytotoxic CD8 T cells to effectively attack and kill tumor cells depends on the presence of concomitant help provided by CD4 T cells.[40,41] This is especially important given the high frequency of CD4 neoepitopes in tumor cells,[9,11,12] and given recent observations that CD4 T cells can control tumors independently of CD8 T cells.[42,43] In a recent report, applying neoantigen DNA vaccination with electroporation resulted preferentially in the induction of CD8 responses.[22] In contrast, our intradermal DNA vaccination approach efficiently induced both CD8 and CD4 responses. Our data indicate that both MHC I and MHC II presentations occur; however induction of CD8 T cell responses appears more pronounced than induction of CD4 responses. This may suggest that antigen presentation is performed mainly by directly transfected cells and consequently cytosolic antigen is more efficiently presented. MHC II presentation occurs mainly on exogenously acquired antigen by specialized antigen-presenting cells (APCs). Previous reports investigating the working mechanism of intradermal DNA vaccination have highlighted that transfection takes place both in epidermal cells and, to a lesser extent, directly in professional APCs.[44,45] It is still controversial whether antigen presentation upon DNA vaccination occurs by directly transfected cells or antigen is indirectly acquired from transfected cells by APCs.[46]We believe that it will be important to elucidate the mechanism in the context of intradermal DNA vaccination in order to control and elicit optimal MHC II presentation.

The chosen colon cancer cell line MC38 tumor model represents a clinically relevant tumor both in light of neoantigen vaccination studies and immunomodulatory treatments. MC38 is known to induce spontaneous-CD8-mediated immune responses in mice with growing tumors, but, due to its highly immunosuppressive microenvironment, these T cells are apparently inactive and not able to eradicate tumor cells.[29] Treatment of MC38 tumor-bearing mice at

early stages with immunomodulating antibodies against PD-1 or PD-L1, elicit effector T cell responses which can mediate tumor regression.[27] Here we show that vaccination against the selected neoantigens[7] in combination with anti-PD-1 can mediate tumor regression in a CD8 T cells-dependent fashion, while anti-PD-1 antibody by itself could not effectuate tumor clearance. Physiologically, the PD-1 axis contributes to negatively regulate peripheral-activated CD8 T cells, but malignant cells exploit this mechanism to shut down spontaneous tumor-specific T cells responses. Indeed, a recent report by Xiong and colleagues showed that neo-epitope specific CD8 T cells express a high level of co-inhibitory molecules, including PD-1.[47] When CD8 T cells are properly activated, by means of vaccination, for example, they also upregulate PD-1 and are therefore more susceptible to immune suppression. Accordingly, integration of anti-PD-1 blockade therapy resulted in complete response in both RNA- and peptide-based vaccination clinical studies for, respectively, one[11] and two[12] patients that experienced recurrence after vaccination. Altogether these observations show that specific immunotherapy can synergize with PD-1 checkpoint therapy most likely by supporting adequate effector functions of the increased frequencies of tumor-specific T cells.

Immunization with multiple epitopes in one formulation may result in reduced responses to individual epitopes. The occurrence of immune-dominant neoantigens has been reported in several studies.[48-50] Nevertheless, a study identifying neoepitopes in patients with chronic leukemia reports how immune-dominance plays a role primarily in the induction of spontaneous responses, while vaccination against multiple epitopes diversifies the tumor-specific T cell repertoire and amplifies the heterogeneity of tumor-specific T cell responses.[50] In addition, tumor immunoediting could lead to antigen loss and the outgrowth of resistant tumor variants that do not possess one or more of the targeted neoantigens. The inclusion of multiple epitopes in a vaccine may be important to avoid the outgrowth of such resistant clones. Therefore, the beneficial effects of a more diversified T cell response are likely to outweigh a potential reduction of individual T cell specificities due to immuno-dominance.

Interestingly, we observed that primarily the Adpgk neoepitope appears to induce effector T cells which are able to recognize and eliminate antigen-loaded cells (see Figure2(d)). In contrast, DNA-induced Reps1-specific T cells were not able to kill target cells as opposed to the responses induced by peptide vaccination. Differences in induction of T cell responses depending on the method of immunization have also been reported in RNA vaccination studies.[9] Why these differences between peptide and gene immunization occur is as yet not clear; however, considering the notion that some responses to tumor neoantigens can still be irrelevant for tumor eradication,[51] these observations support the rationale of including multiple potential neoantigens in therapeutic cancer vaccines.

Personalized therapy against tumor neoantigens represents an exciting prospect for clinical translation. A personalized therapeutic cancer vaccine requires a flexible, cost-effective vaccine platform. Here, we show a proof of concept of a DNA vector as a versatile vaccine platform for the inclusion of multiple tumor neoantigens. Moreover, we show that this DNA vaccine synergizes with anti-PD-1 treatment in tumor control. Our data report the potency of stimulating tumor-specific responses via DNA vaccination in a string-of-bead design to achieve effective immunotherapy and underline the importance of combining different immunotherapy strategies in order to achieve effective clinical responses.

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Authors' contributions

ET, KO, MC, SvD, WH, JvB designed and performed experiments. ET, JvB, RA, GZ, and FO interpreted the experiments. ET and TA wrote the manuscript. All authors contributed to and approved the final manuscript.

Disclosure of potential conflicts of interest

KO, WH, JvB, and GZ are employees of Immunetune BV.

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