Poly-specific neoantigen-targeted cancer vaccines delay patient derived tumor growth

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Abstract

Background: Personalized cancer vaccines based on neoantigens have reached the clinical trial stage in melanoma. Different vaccination protocols showed efficacy in preclinical models without a clear indication of the quality and the number of neoantigens required for an effective cancer vaccine.

Methods: In an effort to develop potent and efficacious neoantigen-based vaccines, we have developed different neoantigen minigene (NAM) vaccine vectors to determine the rules for a successful neoantigen cancer vaccine (NCV) delivered by plasmid DNA and electroporation. Immune responses were analyzed at the level of single neoantigen by flow cytometry and correlated with tumor growth. Adoptive T cell transfer, from HLA-2.1.1 mice, was used to demonstrate the efficacy of the NCV pipeline against human-derived tumors.

Results: In agreement with previous bodies of evidence, immunogenicity was driven by predicted affinity. A strong poly-functional and poly-specific immune response was observed with high affinity neoantigens. However, only a high poly-specific vaccine vector was able to completely protect mice from subsequent tumor challenge. More importantly, this pipeline - from the selection of neoantigens to vaccine design - applied to a new model of patient derived tumor xenograft resulted in therapeutic treatment.

Conclusions: These results suggest a feasible strategy for a neoantigen cancer vaccine that is simple and applicable for clinical developments.

Keywords: Immunotherapy, T cells, Affinity, Cancer vaccine, Neoantigen, Vaccination, Electroporation

Background

Cancer immunotherapy based on immune checkpoint inhibitors (ICI) proved to be significantly successful in the treatment of tumors with poor prognosis [1]. Antibodies targeting the PD1/PDL-1 or CTLA-4 pathways are likely to act by rescuing cytotoxic T cell responses against mutation-derived antigens, known as neoantigens [2]. However, the immune responses induced by ICI are sub-optimal as indicated by the wider immune repertoire detected by priming PBMCs from healthy individuals with cancer-specific neoantigen peptides [3]. Recent evidence in cancer patients has shown that the T cell repertoire of immunogenic neoantigens induced by neoantigen cancer vaccines (NCV) only partially overlaps the specificity reactivated by ICI [4, 5]. Therefore, treatment with ICI does not release all the potential cancer-specific immune responses, leaving room for new therapeutic approaches.

Preclinical studies highlighted the feasibility of targeting mutation-derived neoantigens by a personalized cancer vaccine (reviewed in [2]). The current strategy used to target neoantigen cancer vaccine (NCV) was initially reported in the B16 melanoma model where the possibility of inducing an effective immune response targeting neoantigens by a cancer vaccine was shown [6].

Naked DNA delivered in combination with electroporation (DNA-EP) is considered an efficient delivery system [7] that has moved from preclinical to clinical settings in cancer vaccines as well as in viral vaccine applications (reviewed in [8]). Even though there are 10 ongoing clinical trials registered with www.clinicaltrial.gov [1, 2, 8] using this technology, there have been no
studies that have characterized the DNA-EP delivery of minigenes encoding a string of neoantigens in preclinical tumor models so far. In contrast, many reports in preclinical models support the efficacy of other vaccine methods based on peptides [6] or RNA [9, 10].

Knowing how to predict immunogenicity of neoantigens is still an ongoing debate. The difference between predicted binding affinity to MHC of mutated epitope vs. the natural epitope has been proposed as a relevant factor [11]. This concept was initially explored with peptide vaccines in sarcoma and fibrosarcoma tumor models. The rationale underlying this notion is that the immune response induced by CD8 cells against neoantigens could have been eliminated by immunological tolerance at the central and/or periphery level against the corresponding wild-type (WT) epitope. The author defined this parameter as a differential agretoptic index (DAI). The quality of neoantigens has also been explored from a different perspective. In an attempt to establish correlations between immune responses and different subclasses of neoantigens, it has been proposed that there may be similarities with viral epitopes that can favor better immune responses [12]. Interestingly, this initial evidence was further supported by clinical studies where correlations were established between long term survival pancreatic cancer patients and immune responses against viral-like neoantigens [13]. In general, these sets of evidence underline the need for a better understanding of vaccine induced immune responses against neoantigens. Here, we investigated how the quality and the number of neoantigens affects immunogenicity and anti-tumor activity of neoantigen mini-gene (NAM) vaccines delivered by DNA-EP in murine tumor models and further showed that this approach is effective in patient derived tumors.

**Methods**

**Cell lines and mice**

The B16 melanoma and MC38 colon carcinoma cell lines were purchased from ATCC. Master and working cell banks were generated upon receipt, of which the third and fourth passages were used for all tumor challenge experiments. Cells were mycoplasma free as per internal regular controls. Transfection was carried out with Lipofectamine 2000 according to the manufacturer’s instructions.

6–8 week old C57BL/6 female mice or Rag2<sup>−/−</sup> Il2r<sup>−/−</sup> mice (Envigo) were housed in the Plaisant animal house in accordance with Takis’s ethics committee approval. HHK mice expresses the α1 and α2 domain of human HLA-A0201 fused to the α3 domain of H-2K<sup>β</sup> and were generated in our laboratory (manuscript in preparation).

**Genomic procedure for neoantigen sequencing**

Neoantigen sequences were selected from available data for the MC38 [14] and B16 cells [6, 9]. Sequences of selected neoantigens were confirmed by RNAseq analysis for the MC38, U11 and M285 cells and by NGS target resequencing for B16 cells. For the human derived tumor models, neoantigens were selected according to expression data from RNA sequencing, which was performed as described earlier [15]. In short, total RNA was extracted from tumor cells in culture or from a tumor of 100 mm<sup>3</sup> implanted s.c., ribosome depleted by Ribo Zero Gold and prepared for sequencing using the TruSeq Stranded Total RNA Sample Prep kit (Illumina, Inc., San Diego, CA, USA) following the manufacturer’s instructions. The quality of the libraries obtained was monitored through using a Bioanalyzer, and quantity by qPCR. Sequencing in paired-end mode (2 x 76) was performed on a NextSeq500 (Illumina, Inc., San Diego, CA, USA). The genomic regions encompassing the mutations reported previously for the B16 cell line [6, 9] were amplified by PCR with the primers indicated in Additional file 1: Table S1, controlled on a gel for their specificity and quantity. Subsequently, the PCR products were pooled and purified (QIAquick PCR purification kit, Qiagen, Valencia, CA, USA). 10 ng of the amplicons were further processed using the TruSeq ChiP Library Preparation kit (Illumina, Inc., San Diego, CA, USA) and sequenced on a NextSeq500 (Illumina, Inc., San Diego, CA, USA).

**Bioinformatic procedure for neoantigen selection and prioritization**

The RNA-Seq reads were processed with the cloud pipeline RAP [16] in order to assess quality measures, and map reads to the mouse genome (vv. mm9). Subsequently, we performed variant calling with Freebayes [17] (default parameters). The coverage of each locus of interest was extracted from the resulting VCFs. RPM were calculated by normalizing both read depths with the amount of mapped reads for each library (RPM = (coverage*1e6)/(total_mapped_reads)). For U11 and M285 primary human tumors, we processed RNA-seq reads with the same pipeline, obtaining 45,749,750 and 24,083,207 total mapped reads, that we subsequently analyzed for variants. The mapped reads were, for in vitro and in vivo MC38 samples, respectively 29,610,045 and 34,407,355. The expressed epitopes were calculated from the expressed mutation list with our in-house pipeline Narciso. MHC binding affinity was extracted via Net-MHC4 [18] and the DAI was calculated as a ratio of predicted binding affinity of wild type amino acid sequence and the cognate neoantigen.

**Vaccine and mouse models**

DNA vaccines were generated using codon optimized DNA minigenes encoding 9 or 27 amino acids as it is
listed in Table 1, Table 2, Additional file 1: Tables S2, S4 and S5. In the 27 mer epitope minigenes, the mutated amino acid was in a central position. The peptide sequence was back-translated according to the mouse optimized codon usage and linked to amino acid spacers, i.e. REKR, recognized by the furin protease as previously described [7]. Synthetic genes and expression vectors were generated at Eurofins using pTK1 as a backbone vector, which drives the expression of poly-specific neoantigen expression cassette under the human CMV promoter and enhancer. Control vaccine vectors are the empty pTK1 vectors or pTK1-CEAs, which express the codon optimized sequence for the full length CEA protein as described previously [19]. DNA-EP was carried out as previously described [20]. Peptide vaccination was performed by subcutaneous injection of a mixture of 100 μg peptide and 50 μg CpG-ODN (Sigma) in Incomplete Freund’s Adjuvant (IFA) per mouse. Tumor challenge of mouse models was performed by injecting 3 × 10^5 MC38 cells or 2 × 10^5 B16 cells s.c. in the right flank of the mice.

To test our approach with human primary cancers a new model was established. Immunocompetent mice transgenic for HLA-A0201 (HHD) were vaccinated with neoantigens encoding DNA vaccines and 20 × 10^6 spleenocytes transferred in the peritoneum of immunodeficient Rag2−/−Il2r−/− recipient mice bearing human derived tumors. Screening for the expression of the HLA-2.1 resulted in the selection of the U11 lung cancer tumor model [21] and M285 melanoma tumor model [22], which are low passage human cell lines. For tumor growth 5 × 10^6 cells were injected s.c. and followed over time. All national and institutional guidelines were followed and experiments were approved by governmental authorities (authorization n. 292/2016/PR). All mouse experiments were repeated at least twice with a variable number of animals as described in the figure legends.

### Immune responses

T-cell peptides-specific poly-functionality responses were determined by using intracellular cytokine staining (ICS) performed by flow cytometric detection. Briefly, PBMCs or splenocytes harvested from immunized mice (or controls) were incubated for 10 min at room temperature in ACK (Ammonium-Chloride-Potassium) Lysing Buffer (Life Technologies) and then washed in RPMI-1640 medium (Gibco-BRL) with 10% fetal bovine serum (FBS). Blood was retro-orbital retrieved in a volume of 100,200 ul and processed, at least 1 × 10^6 PBMCs or splenocytes were cultured in 96-well plates and stimulated for 12–16 h in 10% FBS-supplemented RPMI-1640 medium containing 1 μg/ml of Brefeldin A (Sigma-Aldrich, St Louis, MO, USA), and 10 μg/ml of the single peptides or the indicated pool of peptides in a 1:1 cells/peptide ratio at 37 °C. Following stimulation and surface staining, samples were then fixed and permeabilized by using the Cytofix/Cytperm kit (BD Biosciences, San Jose, CA, USA). We excluded dead cells by using the Violet Dead cell stain kit (Invitrogen, Carlsbad, CA, USA). PBMC or splenocytes were incubated with anti-Fcγ receptor (2.4G2) followed by surface staining with anti-CD3e (142-2C11), anti-CD4 (RM4-5) and anti-TNFα (XMG1.2), anti-IL-2 (JES6-5H4) and anti-IFNγ (MP6-XT22; all from eBioscience, San Diego, CA, USA). The stained samples were acquired through a CytoFLEX flow cytometer (Beckman Coulter), and the data were analyzed using CytExpert software (version 2.1) with the gating strategy reported in Additional file 2: Figure S1. Effector memory T cells were evaluated as CD45^hiCD62L^low using the anti-CD45 (BDCA-3, all from eBioscience, San Diego, CA, USA). We excluded dead cells by using the Violet Dead cell stain kit (Invitrogen, Carlsbad, CA, USA). PBMC or splenocytes were incubated with anti-Fcγ receptor (2.4G2) followed by surface staining with anti-CD3e (142-2C11), anti-CD4 (RM4-5) and anti-TNFα (XMG1.2), anti-IL-2 (JES6-5H4) and anti-IFNγ (MP6-XT22; all from eBioscience, San Diego, CA, USA). The stained samples were acquired through a CytoFLEX flow cytometer (Beckman Coulter), and the data were analyzed using CytExpert software (version 2.1) with the gating strategy reported in Additional file 2: Figure S1. Effector memory T cells were evaluated as CD45^hiCD62L^low using the anti-CD44 (IM7) and anti-CD62L (F13H6) antibodies. The stained samples were acquired through a CytoFLEX flow cytometer (Beckman Coulter), and the data were analyzed using CytExpert software (version 2.1) with the gating strategy reported in Additional file 2: Figure S1. Effector memory T cells were evaluated as CD45^hiCD62L^low using the anti-CD45 (BDCA-3) antibodies. The stained samples were acquired through a CytoFLEX flow cytometer (Beckman Coulter), and the data were analyzed using CytExpert software (version 2.1) with the gating strategy reported in Additional file 2: Figure S1. Effector memory T cells were evaluated as CD45^hiCD62L^low using the anti-CD45 (BDCA-3) antibodies. The stained samples were acquired through a CytoFLEX flow cytometer (Beckman Coulter), and the data were analyzed using CytExpert software (version 2.1) with the gating strategy reported in Additional file 2: Figure S1. Effector memory T cells were evaluated as CD45^hiCD62L^low using the anti-CD45 (BDCA-3) antibodies. The stained samples were acquired through a CytoFLEX flow cytometer (Beckman Coulter), and the data were analyzed using CytExpert software (version 2.1) with the gating strategy reported in Additional file 2: Figure S1. Effector memory T cells were evaluated as CD45^hiCD62L^low using the anti-CD45 (BDCA-3) antibodies. The stained samples were acquired through a CytoFLEX flow cytometer (Beckman Coulter), and the data were analyzed using CytExpert software (version 2.1) with the gating strategy reported in Additional file 2: Figure S1.

### Table 1

| # | Symbol | WT Peptide | H2-Db | H2-Kb | Neoantigen | DAI | Imm.Resp. |
|---|--------|------------|-------|-------|------------|-----|-----------|
| 1 | Wbp7   | SNHFMCAR   | 39,828| 499   | SNHFMCAL  | 7213| > 5       |
| 2 | Hace1  | QINAFLQGF  | 44,767| 1128  | QYAFILQGF | 37,598| > 5       |
| 3 | Hdgfrp2| KGYPHWPAR  | 43,395| 4131  | KGYPHWPA  | 12,706| > 5       |
| 4 | Kpn6   | CTLQFAEAW  | 34,408| 1319  | CTLQFEEA  | 8030| > 5       |
| 5 | Aurkai1| RTRFLRKRV  | 45,504| 6329  | RTRFLRKL  | 42,713| 7062      |
| 6 | Sreb2  | HSFVDSVGF  | 33,887| 3985  | HSFVSVG   | 38,481| 135       |
| 7 | Mttp   | TGYVERSPL  | 41,635| 11,661| TGYVERS   | 9548| 131       |
| 8 | Nle1   | MALSTDYAL  | 1982  | 1278  | MALSTY    | 407 | 46        |
| 9 | Zbtb24 | SLLEHMSLH  | 43,119| 18,788| SLLEHMSSL | 7649| 265       |
| 10| Hmmpf  | GYWKLRGL   | 42,720| 3065  | GYWKLRGL  | 38,966| 438       |

Predicted binding are expressed as nM values according to NetMHC-4. Immunogenic neoantigens (+) and intermediate response (+/) are indicated. Bold residues in the peptide sequence are mutated.
and gated on CD3+CD8+IFNγ+ T cells. Gating strategy is depicted in Additional file 2: Figure S1.

IFN-γ ELISPOT

The assay was performed according to the manufacturer’s instructions (U-Cytech, Utrecht, Netherlands). Briefly, standard 96-well plates (Millipore) were coated with anti-mouse IFNγ antibody diluted 1:200 in sterile PBS (final conc. 10 μg/ml). Splenocytes were plated at 4 × 10^5 and 2 × 10^5 cells/well, in duplicate, with MC38 neoantigens, Reps1, both WT and mutated at decreasing concentration from 1 pM to 100 μM. After overnight stimulation at 37 °C, plates were washed and incubated with biotinylated anti-mouse IFNγ antibody, washed and incubated for 2 h at room temperature with streptavidin-AP conjugated antibody. After extensive washing, 50 μl/well of the substrate (NBT/BCIP-1 step solution, Pierce) was added to measure spot development. The washing plates were thoroughly washed with distilled water to stop the reaction. Plates were allowed to air-dry completely, and spots were counted using an automated ELISPOT reader (Aelvis ELISpot reader, A.EL.VIS Gmbh, Germany).

Statistical analysis

Log-rank test, ANOVA and two-tailed Student's t-tests were utilized where indicated. All analyses were performed in JMP version 5.0.1 (SAS Institute, Cary, NC).

Results

High affinity drives immunogenicity of NCV delivered by DNA-EP

To develop a pipeline process for NCV based on DNA-EP, we first asked whether published neoantigens previously delivered in the form of a peptide or an RNA were effective through using our technology platform. Starting from data in the literature [9, 23], we generated NAM expressing neoantigens from the B16 melanoma cell line (Fig. 1). The B1 vector expresses 10 neoantigens while the B2 expresses only two neoantigens, M30 and M48, with the last one also expressed in B1. We were able to detect immune responses against the pool of neoantigens in the peripheral blood (Fig. 1b) and at the level of a single neoantigen in the splenocytes by flow cytometry (FC) against two out of the eleven neoantigens (Additional file 1: Table S2). The immune response against the M48 neoantigen was similar in mice vaccinated with B1 or B2 vaccine vectors suggesting that the presence of additional neoantigens in The B1 vaccine vector does not affect immunogenicity (data not shown). Vaccinated mice were not protected from tumor challenge (Fig. 1c) while mutations were confirmed to be present by genomic sequencing (see M&M). We noticed that the predicted binding values for ten out of eleven neoantigens were over 500 nM (Additional file 1: Table S2) and a recent pan-cancer analysis suggests that immunogenicity of neoantigens is driven by lower predicted binding values [24]. Therefore, we looked at the MC38 tumor model for which high affinity neoantigens have been described [14].

To explore the impact that the quality of neoantigens has on immunogenicity, we looked at two classes of neoantigens delivered in the context of NAMs by DNA-EP vaccination, [7] neoantigens with predicted high or low affinity according to a threshold of 50 nM [24]. To this end, we generated two vaccine vectors, M1 and M2, encoding twenty neoantigens from MC38 colon cancer cells [14]. Neoantigen expression in our in vitro and in vivo samples was confirmed by the RNAseq analysis (Additional file 1: Table S3). Nine neoantigens encoded in the M1 vaccine (Fig. 2a) have a predicted affinity at least five-fold higher than that of the corresponding WT peptide (ratio WT/mut > 5), indicated as DAI (Table 1). Mice were vaccinated with three biweekly vaccinations and immune responses were analyzed by the FC analysis.

| #   | Symbol | Seq | H2-Db | H2-Kb | Seq | H2-Db | H2-Kb | DAI | Imm. Resp. |
|-----|--------|-----|-------|-------|-----|-------|-------|-----|------------|
| 11  | Tmem135| FALMNKRKAL | 12 | 1426 | FALMNKRKAL | 12 | 681 | < 5 | + |
| 12  | Aatf   | MAPIDHTAM | 297 | 5239 | MAPIDHTAM | 90 | 6675 | > 5 | - |
| 13  | Spire1 | SAIRSYQDV | 814 | 271 | SAIRSYQDV | 28 | 43 | > 5 | + |
| 14  | Zbtb40 | KSFHYCRL | 7827 | 4 | KSFHYCRL | 1318 | 4 | < 5 | - |
| 15  | Slic12a4| LSAARYALL | 26,865 | 16 | LSAARYALL | 27,697 | 14 | < 5 | - |
| 16  | Nfe2l2 | ASYSQVAHI | 4627 | 66 | ASYSQVAHI | 1309 | 23 | < 5 | - |
| 17  | Herc6  | CGYEHTAVL | 7636 | 140 | CGYEHTAVL | 9017 | 91 | < 5 | - |
| 18  | Copb2  | MSYFQTLGL | 6858 | 72 | MSYFQTLGL | 2039 | 51 | < 5 | - |
| 19  | Reps1  | AQLPNDVL | 89 | 9261 | AQLPNDVL | 16 | 8591 | > 5 | + |
| 20  | Adpgk  | ASMTNRELML | 6 | 2267 | ASMTNRELML | 4 | 1288 | < 5 | + |

Predicted binding affinity by NetMHC-4 software. Immunogenic neoantigens (+) are indicated. Mutated residues are in bold.

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in the peripheral blood on day seven after the last treatment. Significant immune responses through
CD8+IFN-γ+, CD8+TNFα+, and poly-functional CD8+IFN-γ+TNFα+, were
observed by FC against the pool of neoantigen peptides in
the peripheral blood (Fig. 2b). Mice were then sacrificed on
day forty-two and immune responses analyzed at the level
of a single neoantigen in restimulated splenocytes (Fig. 2c).
CD8+IFN-γ+ T cells were detected against two neoantigens,
Wbp7 and Hace1. Of note, two neoantigens out of the five
with predicted high affinity (< 50 nM) but none of the five
neoantigens with predicted low affinity (> 50 nM) were
immunogenic. To exclude that the result could be influ-
enced by the position of neoantigens and the length of
DNA construct, we included a CD8 epitope reference from
the Carcino-Embryonic Antigen (CEA) at the end of the
open reading frame. Frequency of CEA specific CD8 im-
mune responses induced by the minigene construct was
similar to the full-length CEA protein (Additional file 2:
Figure S2) [19]. These data validate the architecture with
ten neoantigens and moreover suggest that high affinity
is the driving force behind immunogenicity.

To further support the immunogenicity of high affinity
neoantigens, we generated the M2 vaccine (Fig. 3a). Table 2
shows that seven of the neoantigens encoded by the M2
vaccine are characterized by high affinity (Tmem135, Aatf,
Spire1, Reps1, Adpgk Zbtb40, Slc12a4, Nfe2l2), and three
with a value higher than 50 nM (Aatf, Herc6, Copb2). Table
2 reported also on the DAI to compare the previous vector
and to verify whether the neoantigens with high affinity
and DAI are more immunogenic.

Splenocytes from vaccinated mice showed a CD8+
IFN-γ+ specific T cell response against four out of seven
high affinity neoantigens: Tmem135, Spire1, Reps1 and
Adpgk (Fig. 3b). The cumulative data obtained with B1, B2,
M1 and M2 vaccine vectors delivered by NAMs via
DNA-EP demonstrate that higher frequency of immuno-
genic neoantigens are observed in the presence of predicted
high affinity (6/12) with respect to predicted lower affinity
(2/19) (p < 0.05 one tailed Mann-Whitney test). Among the
immunogenic neoantigens there is a trend favoring those
with positive DAI (4/6 have a DAI > 5). The limited number
of immunogenic neoantigens tested prevents us to come to
any conclusions on the impact of DAI on immunogenicity
of neoantigens delivered by DNA-EP. To prove the specifi-
city of DNA-EP delivered neoantigens, we compared the
immune responses of a neoantigen with that of the cognate
epitope. The IFN-γ ELISPOT analysis for the Reps1 neoan-
tigen showed a clear specificity for the neoantigen com-
pared to the WT peptide (Fig. 3c). The difference was more
evident in restimulated splenocytes with a decreasing
concentration of peptides. Similar results were observed in the peripheral blood as measured by the FC (Additional file 2: Figure S3).

We then asked whether the CD8+ T cells induced by DNA-EP against the MC38 specific neoantigens could recognize cancer cells. To do this, mice were vaccinated with the M2 vaccine vector as described in Fig. 4a and the FC analysis was performed on day 7 after the last vaccination. A strong immune response against the M2 peptide pools was observed via poly-functional CD8+IFN-γ+TNF-α+ and CD8+IFN-γ+TNF-α+IL2+ T cells (Fig. 4b). In order to verify whether the M2 neoantigens were naturally processed and presented, splenocytes from vaccinated mice were incubated overnight with MC38 cells. The comparison between the response induced in control mice to animals vaccinated with M2 vector showed a statistically significant increase in CD8+ IFN-γ+ T cells upon incubation with MC38 cells, suggesting that M2 neoantigens are present on the cell surface and are specifically recognized by M2 vaccinated mice. (Fig. 4c). Moreover, the frequency of CD8+ IFN-γ+ T cells further increased when splenocytes were incubated with MC38 cells transfected with the M2 vaccine as compared to untransfected MC38 cells or MC38 cells transfected with a control plasmid. We cannot exclude that mice vaccinated with the M2 vaccine can develop immune responses against additional cryptic epitopes, which could be present when cells are transfected with the M2 plasmid. However, higher immune responses detected with the M2 transfected MC38 cells support the concept that the expression level of a neoantigen is an important aspect of tumor recognition. As expected the percentage of neoantigen-specific CD8+IFN-γ+ T cells decreased at day 30 but was still measured in the order of single digits (Fig. 4d).

Poly-functional and poly-specific immune responses protect mice from tumor challenge
To verify whether the use of a poly-functional and poly-specific neoantigen vaccine delivered by DNA-EP platform has an impact on tumor growth, we explored the MC38 tumor model in a prophylactic setting. In fact, the MC38 tumor model is fast-growing and our vaccination protocol with three biweekly DNA-EP is too long to mount a therapeutic immune response (data not shown). For this reason, we focused on tumor prevention rather than on the therapeutic setting. Therefore, on day fifty-nine vaccinated mice were challenged with MC38 cells resulting in a statistically significant delay in tumor growth.
growth compared to control mice (Fig. 5a). The analysis of memory T cells at day 59 in an independent experiment revealed that most of the M2 specific T cells were effector memory (CD83⁺CD8⁺IFNγ⁺CD44⁺CD62L⁻LO), thus suggesting that a boost in the immune response could further improve tumor protection (Additional file 2: Figure S4). We then asked whether not only an immune response boost but also the degree of poly-specificity would affect tumor growth. To evaluate this aspect, we generated a third vaccine vector, M3 expressing only two immunogenic neoantigens, Dpagt1 and Reps, expressed by the M2 vector and previously identified in MC38 cells by mass spectrometry [14]. We choose these two neoantigens to allow comparison with previous vaccinations, which were carried using peptides. Similar immune responses were observed with Adpgk and Reps1 neoantigens delivered as peptides or as DNA-EP (Additional file 2: Figure S5). Mice were vaccinated as described in Fig. 5b with the M2 or M3 vaccines. To maximize the impact of the vaccine treatment, we performed an immunological boost on day fifty-eight, that is one week before the tumor challenge. Figure 5c shows the immune response at the time of the boost using the peptides as a stimulus for the two neoantigens, Adpgk and Reps1, shared between M2 and M3 vaccine vectors. M3 vaccine induced a slightly higher immune responses, which can be explained by the expression of a lower number of neoantigens. Although the immune responses were not statistically different through CD8⁺IFN-γ⁺ or CD8⁺TNFα⁺ T cells, complete protection from tumor challenge was observed only in mice vaccinated with the M2 vaccine vector (Fig. 5d). These results support the concept that high levels of poly-specificity induced with M2 vaccine via the four immunogenic neoantigens is key in safeguarding mice from tumor take. The efficacy of adjuvant immunotherapy with ICI was recently demonstrated in the clinic for anti PD-1 pembrolizumab [25] and it was previously shown to be effective for the anti CTLA-4 ipilimumab [26]. For comparison purposes we verified whether anti PD1 and anti CTLA-4 could prevent MC38 tumor growth by starting the treatment before tumor challenge. We observed a complete protection from tumor challenge with the anti PD1 and in four out of five animals treated with the anti CTLA-4 antibody (Additional file 2: Figure S6) which is in line with the protection degree of the NCV delivered by DNA-EP for adjuvant personalized treatment.

NCV generated for human cancer models
To move closer to the clinical setting and test whether our pipeline was effective in dealing with human tumors, we developed an innovative tumor model based on patient derived tumors and adoptive T cell transfer. Adoptive T cell therapy is effective in melanoma patients and
recent evidence suggests that T cells recognize neoantigens [27]. Screening of tumor cells derived from primary tumors for the expression of HLA-A0201 resulted in the selection of the M285 melanoma model [22] and the U11 lung cancer model [21]. As reported for mouse cell lines, neoantigens were selected according to predicted binding to HLA-A0201 and their expression measured by RNAseq (Additional file1: Tables S4 and S5). Selected neoantigens were used to generate the NAM vaccine vectors TK-U11 and TK-M285 (Fig. 6a). We then vaccinated HLA-2.1 transgenic mice (HHK) and transferred splenocytes in Rag2−/−Il2r−/− mice bearing the corresponding human tumors. Neoantigen-specific immune responses were measured in the splenocytes at the time of splenocyte transfer in TK-U11 and TK-M285 vaccinated mice (Fig. 6b). A significant tumor regression was observed in the U11 tumor model while a significant tumor delay was observed in M285 tumor bearing mice (Fig. 6c). These results demonstrate that adoptive transfer of a neoantigen-specific immune response is able to reduce tumor growth of human derived tumors.

**Discussion**

In this study, we showed that NCV delivered by DNA-EP are able to provide antitumor effects in murine models and can be used to treat human xenograft tumor models. Our first observation was that well established neoantigens such as M30 [6, 9, 23] did not turn out to be immunogenic when administered through the DNA-EP delivery system. In contrast, the M48 neoantigen was immunogenic using two different NAM vaccine vectors (B1 and B2). Moreover, the two immunogenic neoantigens M21 and M48, which have been previously reported as CD4 epitopes [9], showed a CD8-specificity in our experiments (Additional file 1: Table S2). More importantly, induction of a B16-specific effector T cell response did not correlate with tumor protection. In agreement with this notion, another preclinical study on ovarian cancer showed that immune responses against neoantigens with low affinity did not result in tumor protection [28]. This disappointing result in the context of DNA-EP prompted us to look at the quality of neoantigens in other tumor models.
The analysis of immune responses induced with the twenty predicted neoantigens in MC38 cancer cells and expressed by M1 and M2 vaccines, suggests that DNA-EP induced immunogenicity is driven by high affinity neoantigens. One potential concern regarding neoantigens is safety because of the potential auto-immunity against healthy tissue expressing the cognate self-antigens. In line with previous evidence [14], we showed that the immune response against one of these neoantigens, Reps1, is highly specific compared to the wt epitope. However, further experiments are required to define cross reactivity and potential toxicity. Overall, we used quite a large set of mouse neoantigens (n = 31) and reported individual immunological values via the FC analysis. We have to acknowledge that while we confirmed in our vaccination platform the immunogenicity of neoantigens with high affinity such as Reps1 and Adpgk [14], this was not the case for other neoantigens. In contrast, a neoantigen such as Aatf, which is presented on MC38 cells, was not immunogenic when administered as a peptide [14] as well as in our NAM vaccine. Discrepancies with other vaccination methods highlight the fact that neoantigen pipelines, from
prediction to delivery methods, need to be experimentally validated. Using the DNA-EP vaccination method, we identified new immunogenic CD8+ neoantigens (Wbp7, Hace1, Tmem135 and Spire1) that had been selected on the basis of predicted high affinity to MHC-I. The minigene DNA-EP technology allowed to accommodate a sufficient number of neoantigens to obtain significant poly-specific immune responses. Our evidence suggests that the quality, as well as the number of neoantigens, are key parameters for a productive immune response.

We observed a strong poly-functional immune response particularly with the M2 vaccine. The Adpgk neoantigen from MC38 cells showed an immune response dominated by IFN-γ upon delivery by a very efficient system based on peptide embedded in liposome disk [23]. In contrast, we observed a clear poly-functional response mostly due to CD8+IFN-γ+TNF-α+ T cells. However, further experiments with more neoantigens compared side by side using different vaccine platforms are required before drawing any conclusions. Interestingly, poly-functionality was observed...
also in a clinical study, where a personalized vaccine delivered as an RNA for melanoma patients showed poly-functional CD8+IFN-γ+TNF-α+ immune responses [5]. Poly-functionality was not limited to a vaccine-induced immune response but was also reported for natural immune responses against neoantigens in ovarian cancer patients [29].

Our preliminary data with the humanized “immunovatar” model indicates that transferring splenocytes from HHK vaccinated mice blocks the growth of the melanoma M285 xenografted mice and induces tumor regressions in lung cancer U11 transplanted mice. The current model answers the question whether the NCV can induce an immunogenic immune response specific for the patient in the surrogate model of HLA-A0201 transgenic mice and define the potential efficacy as a means of T cell adoptive transfer. Further improvements to this model will be the use of in vitro primed human T cells against neoantigens and their transfer into patient derived xenografts to prove their efficacy.

Conclusion

Our study suggests that a vaccine endowed with high poly-specificity and linked to poly-functionality is the most efficient in preventing tumor growth. We did not aim to establish a threshold of poly-specificity or a specific combination of neoantigens but rather to show, in a direct comparison, the superiority of a vaccine encoding more neoantigens. This observation supports the concept that a NCV has the potential to broaden the repertoire of immune responses against cancer, a feature that could be particularly relevant in the treatment of tumors with high heterogeneity [30]. In the described setting for MC38 checkpoint inhibition is very effective. The two different treatment strategies employ differing immunologic mechanisms, and as both are comparable in terms of activity it is reasonable to conclude that NCV approach is a potential alternative to currently established therapies. A second significant aspect is the possibility of inducing a long-lasting response to prevent tumor relapse. We observed a significant tumor delay (Fig. 5) after more than one month after the last vaccination when the response was diminishing. In contrast, mice boosted one week in advance (Fig. 6) were completely protected from the tumor challenge, suggesting that in order to maintain high levels of circulating tumor-specific T cells the protocol requires a further boost.

The possibility of extending the NCV approach to tumors other than melanoma is expected based on the high neoantigen load observed for instance in lung cancer [31]. Here, we can show that a vaccine could be designed using RNAseq data and HLA-A0201 binding prediction, although we are confident that improvements in predictive algorithms or the introduction of in vitro binding or functional assays can further increase the identification of immunogenic neoantigens. This is of particular relevance in the context of tumors for which biopsy material is limited but sufficient for NGS approaches [32]. Finally, it is interesting to note that DNA-EP does not induce any neutralizing immune response as it is the case for viral vaccines. Indeed, we demonstrated the feasibility and the clinical efficacy of a repetitive DNA-EP vaccination in a veterinary trial [33]. The relevance of an adjuvant setting for the development of NCV is in line with the human clinical trials exploiting this approach [4, 5, 34].

Many clinical trials registered on https://clinicaltrials.gov/ with DNA-EP do not indicate any selection criteria for neoantigens, however it would be interesting to explore the immunological and clinical outcomes as we trust that poly-specificity and poly-functionality of high affinity neoantigens will be highly relevant for the success of this approach.

Additional files

Additional file 1: Tables S1. Primers used for resequencing of B16 neoantigens. Table S2. B16 selected neoantigens. Table S3. Summary results of RNAseq of in vitro and in vivo MC38 cells. Table S4. M285 selected peptides. Binding affinity prediction of M285 neoantigens according to NetMHC-4 and RNAseq values. Table S5. U11 selected peptides. Binding affinity prediction of U11 neoantigens according to NetMHC-4 and RNAseq values. (DOCX 116 kb)

Additional file 2: Figure S1. Representative gating strategy for the identification of neoantigen specific immune responses. Figure S2. Comparison of CEA specific immune responses induced by the M1 vaccine vector vs. the full length CEA protein delivered by DNA-EP. Figure S3. Comparison in peripheral blood by IFN-γ+ICS analysis for the Rep1 neoantigen and cognate WT peptide. Figure S4. M2 specific memory T cells. Figure S5. M3 specific T cells. Figure S6. Immuno modulators prevent MC38 tumor growth. (PPTX 1226 kb)

Abbreviations

ACK: Ammonium-Chloride- Potassium; CEA: Carcino-Embryonic Antigen; DAI: Differential agnetic index; EP: Electroporation; FBS: Fetal bovine serum; FC: Flow cytometry; HHK: Immunocompetent mice transgenic for HLA-A0201; ICI: Immune checkpoint inhibitors; RPM: Reads per milion; WT: Wild-type

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Availability of data and materials

Not applicable.

Authors’ contributions

LA designed the experiments and contributed to the writing; ES, LL, SB, RA, AC, LL produced the immunological data and developed the tumor models; FDN, FG, generated NGS data; MP performed the bioinformatics analysis with the supervision of MF; GC contributed with ideas and in writing; FP conceived the experiments and wrote the manuscript. All authors read and approved the final manuscript.
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