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# A synDNA vaccine delivering neoAg collections controls heterogenous, multifocal murine lung and ovarian tumors via robust T cell generation

Pratik S. Bhojnagarwala,[1] Alfredo Perales-Puchalt,[2] Neil Cooch,[2] Niranjan Y. Sardesai,[2] and David B. Weiner[1]

[1]Vaccine & Immunotherapy Center, Wistar Institute, Philadelphia, PA 19104, USA; [2]Geneos Therapeutics, Plymouth Meeting, PA 19462, USA

**Neoantigens are tumor-specific antigens that arise due to somatic mutations in the DNA of tumor cells. They represent ideal targets for cancer immunotherapy since there is minimal risk for on-target, off-tumor toxicities. Additionally, these are foreign antigens that should be immunogenic due to lack of central immune tolerance. Tumor neoantigens are predominantly passenger mutations, which do not contribute to tumorigenesis. In cases of multi-focal or metastatic tumors, different foci can have significantly different mutation profiles. This suggests that it is important to target as many neoantigens as possible to better control tumors and target multi-focal tumors within the same patient. Herein, we report a study targeting up to 40 neoantigens using a single DNA plasmid. We observed significant plasticity in the epitope strings arranged in the vaccine with regard to immune induction and tumor control. Different vaccines elicited T cell responses against multiple epitopes on the vaccine string and controlled growth of multifocal, heterogeneous tumors in a therapeutic tumor challenge. Additionally, the multi-epitope antigens induced long-term immunity and rejected a tumor re-challenge several weeks after the final vaccination. These data provide evidence that DNA-encoded long antigen strings can be an important tool for immunotherapeutic vaccination against neoantigens with implications for other *in vivo*-delivered antigen strings.**

## INTRODUCTION

Cancer neoantigens are derived from somatic mutations in tumor cells and are increasingly becoming targets of personalized immunotherapies. They are highly specific to the tumor and hence there is minimal risk of adverse events resulting from the potential for on-target, off-tumor activity that may be associated with targeting some tumor-associated antigens. Neoantigens are foreign antigens and have the potential to be highly immunogenic since they are not subject to central immune tolerance. Studies have shown that in addition to tumor mutation burden (TMB), tumor neoantigen burden is a strong predictor of response to immune checkpoint blockade (ICB) therapy.[1] Neoantigens are generated from non-synonymous mutations, a result of single nucleotide variants, insertions, deletions, frameshifts, or gene fusions. Proteolytic degradation of proteins containing neoantigens creates immunogenic peptides that are uniquely expressed on the tumor cells. These peptides then bind to major histocompatibility complex (MHC) class I or class II molecules and are recognized by T cell receptors (TCRs).[2,3] Neoantigen-targeting T cells can specifically kill those tumor cells, leading to tumor regression. Several recent clinical trials have targeted neoantigens.[4–6] Interestingly, these initial vaccines generated mainly MHC class II-driven $CD4^+$ T cell responses despite the epitopes being selected *in silico* for high binding affinity to MHC class I.[4,5,7] While $CD4^+$ T cells have been shown to recognize neoantigens in tumors, $CD8^+$ T cells are the main driver of neoantigen-based tumor rejection.[8] Targeting fewer neoantigens increases the chances of tumor immune escape, while targeting a high number of neoantigens ensures an adequate pool of immunogenic antigens to drive tumor-targeted responses and could increase the potential for inducing $CD8^+$ T cell responses with more frequency *in vivo*. Studies have also shown that there is a significant difference in the TMB of primary and metastatic tumors from matched patients, indicating that there could be unique neoantigens present in different tumors from the same patient.[9,10] It is important to target all tumors within a patient to provide an optimal clinical response. Vaccine platforms commonly use *in silico* prediction methods to predict neoantigens that bind with high affinity to histocompatibility leukocyte antigens (HLAs) from the patients.[4,5,7] To date, computationally predicted neoantigens have not been consistently immunogenic *in vivo*, indicating a gap in our ability to predict and design neoantigen-targeted therapy.[11–13] Targeting large numbers of neoantigens detected in the patient's tumor bypasses this restriction, but most current studies have only examined limited numbers of epitopes in vaccine formulations.[4,7,14]

The DNA platform represents a useful flexible platform for targeting cancer neoantigens *in vivo*. We have recently described the use of short DNA strings for inducing immunity against neoantigens, which can impact tumors *in vivo*.[15] In the current study, we hypothesize that delivery of an increased epitope payload could improve diversity of immune responses as well as provide improved impact for tumor immunotherapy, without antigen competition. We show that a single vaccine

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**Correspondence:** David B. Weiner, Vaccine & Immunotherapy Center, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA.
**E-mail:** dweiner@wistar.org

 

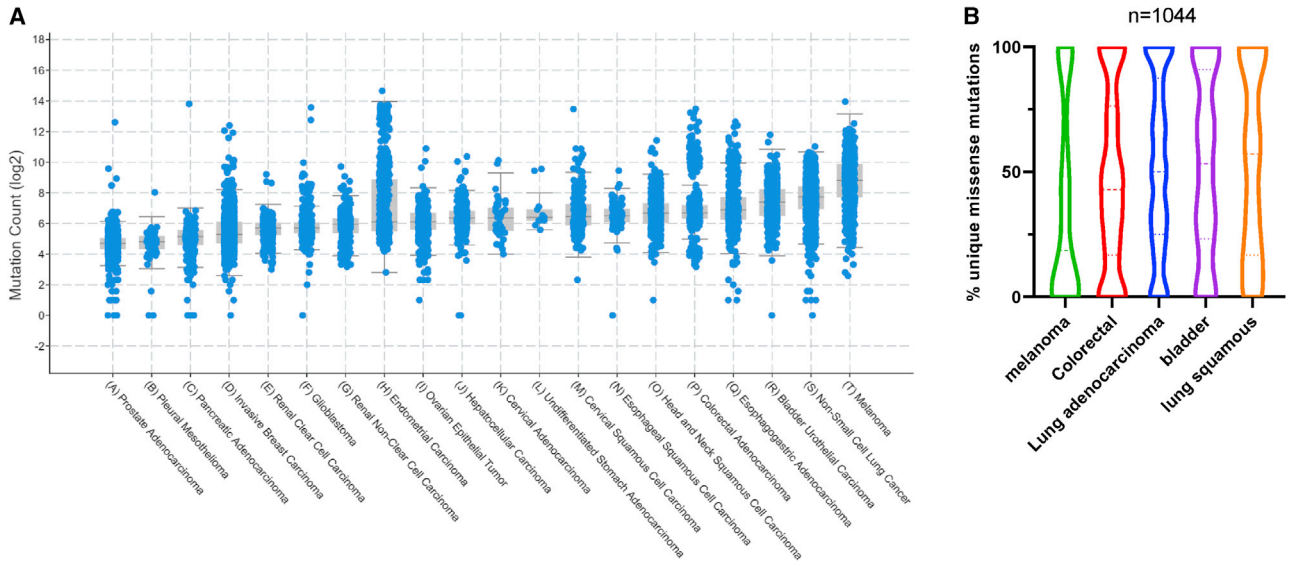


**Figure 1. Human tumors have a high number of mutations**

(A) Plot showing number of mutations per sample across different cancer types. The cancer types have been arranged in increasing order of median number of mutations per sample from left to right. (B) Violin plot showing percentage of unique mutations per patient across different cancer types (see also Figure S1).

encoding 40 neoantigens generates a comparable immune response to vaccines encoding either 10 or 20 neoantigens and that the immune response to individual epitopes is unaffected by the position of the epitope within the vaccine construct. Additionally, we show that a vaccine targeting 40 neoantigens was similarly impactful in targeting TC1 mouse tumor growth and control in a mouse model, supporting that inclusion of more epitopes can be beneficial in a single-vaccine formulation. We also show that these long strings generate long-term immunity, as 100% of the animals were protected when re-challenged with a higher dose of tumor. Finally, we show the efficacy of 40 neoantigen-encoding vaccines to control heterogeneous, multifocal tumors with differing mutation profiles and that the level of tumor control was dependent on the number of immunogenic epitopes encoded in the vaccine. Our data support that encoding multiple neoantigens, in this case as a DNA vaccine, can be a useful tool for targeting heterogeneous multi-focal or metastatic tumors and that the long-term immunity generated appears to have an advantage for minimizing tumor recurrence due to the induction of multi-epitope T cell memory.

## RESULTS

### Multiple tumors from within the same patient can have a high percentage of unique mutations

Neoantigens are derived from non-synonymous mutations. As such, the number of potential neoantigens in a patient tends to increase with the number of mutations within the tumor. We used The Cancer Genome Atlas (TCGA) PanCancer database to define the number of neoantigens present within cancer patients. While there was plenty of heterogeneity as would be expected across cancer types, we identified several patients with more than 1,000 tumor-specific mutations, while one patient had more than 15,000 mutations (Figure 1A). Overall, melanoma patients had the highest number of mutations, followed by non-small cell lung cancer and bladder cancer, which is consistent with mutation numbers reported elsewhere in the literature.[16,17] We wanted to explore the heterogeneity between different nodules of the same tumor or between primary and metastatic tumors. For this purpose, we utilized the American Association for Cancer Research (AACR) Genomics Evidence Neoplasia Information Exchange (GENIE) portal database to identify patients for which two samples (either primary and metastatic or recurrent) had been sequenced. Next, we compared the missense mutations that were present in just one of the two samples to determine the number of unique mutations present. The patient characteristics are listed in Table S1. While there was heterogeneity among patients, across all cancers, each patient had six unique missense mutations on average, which represents roughly 50% of all missense mutations in each patient (Figure 1B; Figure S1). Looking across cancer types, patients with lung adenocarcinoma, lung squamous carcinoma, and bladder cancer had more than 50% unique missense mutations that differed between the two tumor nodules. While these data highlight some of the diversity in the number of potential neoantigens each patient could have, it is likely that the true numbers of potential neoantigens and their diversity are much higher. This is likely, as the studies only captured mutations in a limited number of genes that are known to be either oncogenes or tumor suppressor genes. Neoantigens are generally found in passenger mutations, which do not contribute to tumorigenesis, and hence the real number of unique, targetable neoantigens that are only present in one of the two tumors within a patient is likely to be much higher.

### Size of the vaccine and the position of epitope on the vaccine do not affect the magnitude or type of immune response

We reasoned that including longer strings of neoantigens in a tumor immunotherapy approach could be beneficial to increase the

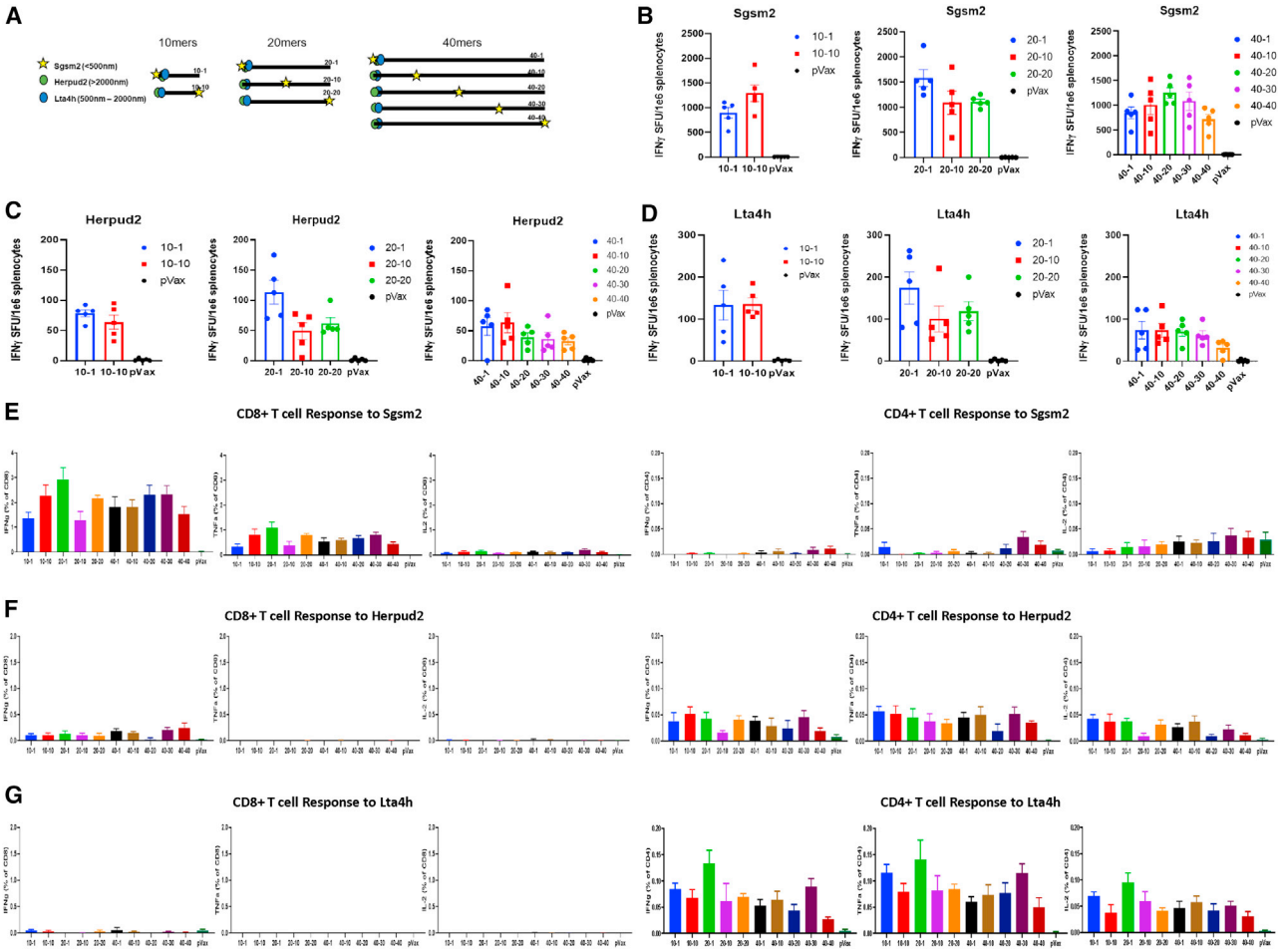


**Figure 2. Immunogenicity is not affected by size of vaccine or location of epitope on vaccine**

(A) Schematic showing different constructs tested and location of Sgsm2 (yellow stars), Herpud2 (green ovals), and Lta4h (blue ovals) on each construct. (B–D) IFN-γ ELISPOT data comparing immune response to (B)Sgsm2, (C) Herpud2, and (D) Lta4h across all constructs. (E–G) Average IFN-γ SFU/1e6 splenocytes for individual epitopes across all constructs. (E) Type of immune response ($CD8^+$ versus $CD4^+$) is not affected by the size of vaccine or position of epitope on vaccine. (F) Type of immune response ($CD8^+$ versus $CD4^+$) is not affected by the size of vaccine or position of epitope on vaccine. (G) Type of immune response ($CD8^+$ versus $CD4^+$) is not affected by the size of vaccine or position of epitope on vaccine. Error bars represent mean + SEM. Single experiment (n = 5 mice/ group).

possibility of induction of $CD8^+$ T cell responses, thus contributing to tumor impact. Such an approach could also limit tumor escape, which is important to provide long-term tumor control. We designed vaccines encoding for 40 neoantigens within a single construct and studied the position of epitopes as well as epitope string size on the resulting immune responses. To test whether the immunogenicity of a particular epitope is affected by the number of neoantigens encoded or the location of the epitope on the vaccine string, we designed two vaccine constructs containing 10 neoantigens (10-mer), three constructs containing 20 neoantigens (20-mer), and five constructs containing 40 neoantigens (40-mer) (Figure 2A). For the first set of experiments, we tested immunogenicity of three different epitopes, i.e., Sgsm2, Herpud2, and Lta4h, which we had previously found to be highly immunogenic in C57BL/6 mice. The epitopes also segregated based on binding affinity to MHC class I, where Sgsm2 had the highest affinity (<500 nM), Lta4h had medium affinity (500–2,000 nM), and Herpud2 had the lowest affinity (>2,000 nM).[15] We found that vaccine length did not impact immunogenicity of the individual epitopes. Interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) results for Sgsm2 showed no difference in the number of spots across different vaccine constructs (Figure 2B). Similarly, for Herpud2 and the Lta4h, there were no significant differences in the number of spots regardless of the size of the vaccine and number of epitopes on the vaccine construct (Figures 2C and 2D). The consistency in the presence of responses and the similar number of IFN-γ-secreting cells for these three epitopes elicited by the vaccines of different lengths reinforce the concept that the number of epitopes included in the vaccine does not significantly impact the ability of the immunogenic epitopes to respond. These results suggest that the presence or magnitude of the

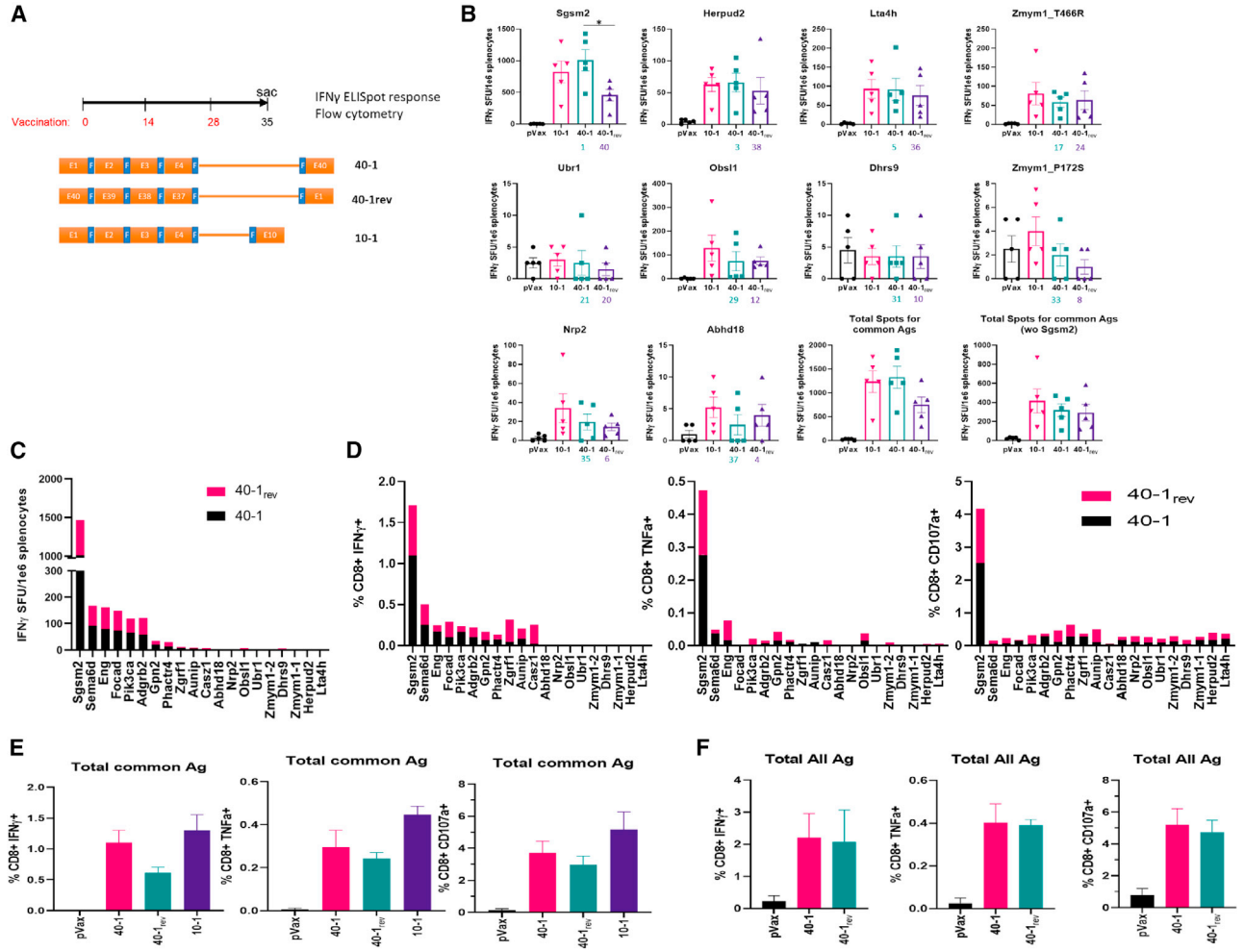


**Figure 3. Changing position of epitopes does not affect immunogenicity**

(A) Schematic showing design of the three vaccines used for this experiment. (B) IFN-γ ELISPOT results from all common antigens across the three constructs. The numbers below the graphs show position of epitope on the 40-1 (green) or 40-$1_{rev}$ (purple) constructs. (C) Comparison of IFN-γ ELISPOTs for all epitopes generated by 40-$1_{rev}$ (pink bars) versus the 40-1 (black bars) constructs. (D) Comparison of IFN-γ, TNF-α, and CD107a production from $CD8^+$ T cells across 40-$1_{rev}$ (pink bars) versus the 40-1 (black bars) constructs. (E) Total IFN-γ, TNF-α, and CD107a production from $CD8^+$ T cells in response to all common antigens from the three vaccines. (F) Total IFN-γ, TNF-α, and CD107a production from $CD8^+$ T cells in response to all antigens in the 40-1 and 40-$1_{rev}$ constructs. Single experiment (n = 5 mice/group). Error bars represent mean + SEM. Ordinary one-say ANOVA. *p < 0.05. See also Figures S2 and S3.

immune response remains unaffected by the location of individual epitopes in a vaccine construct.

We next explored whether vaccine size or positioning of a particular epitope across different epitopes impacted the type of immune response generated. To address these questions, we performed flow cytometry analyses to tease out whether the immune response is $CD8^+$ or $CD4^+$ T cell mediated. We found that Sgsm2 drove a strong $CD8^+$ T cell response across all constructs, as evidenced by the high levels of IFN-γ and tumor necrosis factor (TNF)-α produced by $CD8^+$ T cells. In contrast, there was no $CD4^+$-mediated immune response, as levels of IFN-γ, TNF-α, and interleukin (IL)-2 were similar to background levels (Figure 2E). While Herpud2 did drive some IFN-γ responses from $CD8^+$ T cells, the $CD4^+$ immune response was much stronger, as these cells produced IFN-γ, TNF-α, and IL-2 in response to Herpud2, further indicating a polyfunctional response (Figure 2F). Lta4h also drove polyfunctional, but predominantly $CD4^+$, responses, secreting IFN-γ, TNF-α, and IL-2. $CD8^+$ T cells did not respond to Lta4h (Figure 2G). This observation was true across all of the vaccine constructs tested where the magnitude of immune response and the type of immune response were similar. These data suggest that the immune response driven by any given epitope is dependent solely on its intrinsic immunogenicity. The size of the vaccine construct or the position of a particular epitope within the vaccine did not significantly affect the magnitude or the type of immune induction (MHC class I- versus MHC class II-driven responses) we observed.

**Table 1. Immunogenicity comparison between 40-1 and 40-$1_{rev}$**

| Epitope | 40-1 | 40-$1_{rev}$ |
|---|---|---|
| Sgsm2 | Yes | Yes |
| Lta4h | Yes | Yes |
| Adgrb2 | Yes | Yes |
| Obsl1 | Yes | Yes |
| Herpud2 | Yes | Yes |
| Zmym1 (T466R) | Yes | Yes |
| Nrp2 | No | No |
| Gpn2 | No | No |
| Sema6d | No | No |
| Zgrf1 | No | No |
| Dhrs9 | No | No |
| Phactr4 | No | No |
| Ubr1 | No | No |
| Abhd18 | No | No |
| Zmym1 (P172S) | No | No |
| Casz1 | No | No |
| Eng | No | No |
| Pik3ca | No | No |
| Aunip | No | No |
| Focad | No | No |

### Changing the position of epitopes does not affect their immunogenicity

Based on these results, we next assessed whether these conclusions held true for multiple epitopes. We also examined whether some low-immunogenicity epitopes became more immunogenic when moved to a different location on the 40-mer string. For this, we designed a new 40-$1_{rev}$ construct, which had the same epitopes as the 40-1 construct in reverse order. For instance, epitope no. 1 in the 40-1 construct was in the 40th position in the 40-$1_{rev}$ construct, and so on (Figure 3A). We vaccinated C57BL/6 mice with 25 μg of the 10-1 construct and 50 μg of the 40-1 and 40-$1_{rev}$ constructs three times at 2-week intervals. This was done to ensure that all groups received equimolar amounts of DNA vaccine. The immune response was measured via IFN-γ ELISPOT and flow cytometry. We found a 100% concordance between the 40-1 and the 40-$1_{rev}$ groups where, of the 20 representative epitopes tested, all epitopes that were immunogenic in the 40-1 group were also immunogenic in the 40-$1_{rev}$ group and vice versa (Table 1). Overall, we observed that the magnitude of the immune response was comparable for 9 of 10 antigens that were common across all three constructs (Figure 3B). Sgsm2 remained the most immunogenic epitope in all constructs, although there was a significant reduction in the number of IFN-γ spots in the 40-$1_{rev}$ group compared to the 40-1 group. The total immune response measured by the sum of number of spots generated by all of the epitopes was also lower in the 40-$1_{rev}$ construct, although this difference was driven by the reduced immune response from Sgsm2 (Figure 3B). The other 10 epitopes for which we measured immune response to in the 40-1 and 40-$1_{rev}$ constructs did not elicit any immune response from both constructs (Figure 3C). This illustrates that moving epitopes either earlier or later on the 40-mer string does not alter their immunogenicity significantly. We also controlled whether the immune response generated against these vaccines was specific to neoantigens by co-culturing splenocytes with the wild-type peptides. All three vaccine constructs generated immune responses specific to the mutated peptides only (Figure S2). Flow cytometry analysis showed that $CD8^+$ T cells from 40-1 and 40-$1_{rev}$ groups produced comparable amounts of IFN-γ, TNF-α, and CD107a for all antigens except for Sgsm2 (Figure 3D). The total immune response from all common epitopes across the three constructs also remains the same (Figure 3E). Similarly, the total $CD8^+$ immune response to all antigens tested was comparable in the 40-1 and 40-$1_{rev}$ constructs (Figure 3F). The $CD4^+$ immune response to individual antigens was slightly more variable, although it showed a similar trend where the amount of IFN-γ, TNF-α, and CD107a production was comparable between the 40-1 and 40-$1_{rev}$ constructs (Figure S3A). Similarly, the total immune response to common antigens (Figure S3B) as well as all antigens in 40-mer constructs (Figure S3C) was comparable across all constructs. These data indicate that the immunogenicity of epitopes is independent of the size of the vaccine construct as well as the position of the epitope on the vaccine string.

### Long neoantigen vaccine strings can control tumor growth and significantly enhance animal survival

Next, we tested the ability of the epitope string vaccines to control tumor growth *in vivo*. 100,000 TC1 cells were implanted subcutaneously into the right flank of C57BL/6 mice. 3 days following tumor implantation all mice were given either empty vector pVax, 10-1, 40-1, or 40-$1_{rev}$ at the indicated doses. All mice received vaccines 1 week apart for a total of four doses. Tumors in all five mice in the pVax group grew out, and all of them had to be eventually sacrificed because of tumor burden (median survival of 50 days). In contrast, all five mice receiving either 10-1 or 40-1 cleared tumors, and there was no evidence of tumors up to 124 days after tumor challenge (Figure 4B; Figure S3). In the 40-$1_{rev}$ group, four of five mice completely cleared their tumors while one of them delayed tumor growth but eventually lost tumor control. In this case, the tumor grew significantly slower, and the mouse survived 50% longer (24 days more) than those in the pVax group (Figure 4B). The tumor clearance in all vaccine-treated groups resulted in significantly enhanced survival of the mice (Figure 4C).

To examine whether the vaccine strings generated induction of memory T cell immunity, we re-challenged the surviving mice in all groups with 200,000 TC1 cells on the contralateral flank. The mice were followed for an additional 39 days, and all of them were able to suppress growth of tumors (Figure 4C; Figure S4). These data indicate that long string DNA-encoded immunogens can generate antigen-specific $CD8^+$ memory T cells that are able to protect from tumor recurrence over the long term. The data also indicate that memory T cell generation is similar across all groups, again indicating that all vaccines

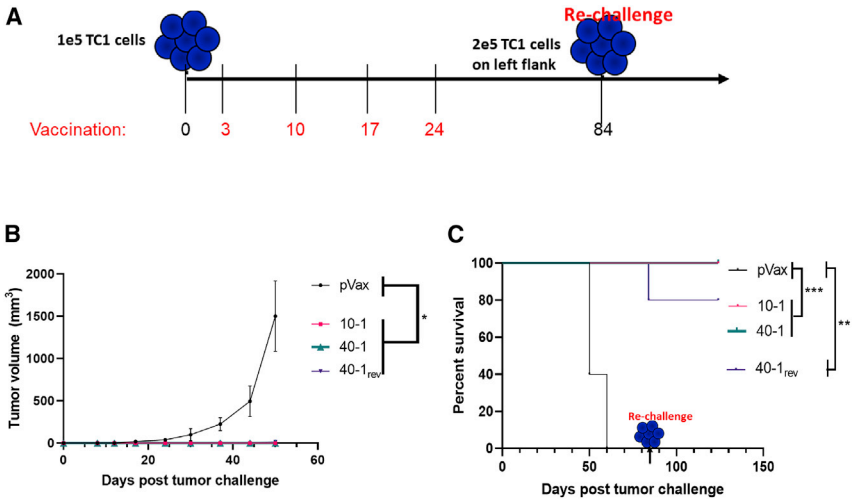


**Figure 4. All vaccines provide significant long-term protection against tumors regardless of vaccine size and epitope position**

(A) Schematic explaining tumor challenge experiments in (B) and (C). (B) Mean tumor sizes of mice bearing TC1 tumors followed by vaccination with either pVax (50 μg), 10-1 (25 μg)+pVax (25 μg), 40-1 (50 μg), or 40-$1_{rev}$ (50 μg). (C) Survival of mice with TC1 tumors treated with either pVax (50 μg), 10-1 (25 μg)+pVax (25 μg), 40-1 (50 μg), or 40-$1_{rev}$ (50 μg). Single experiment (n = 5mice/group). Error bars represent mean + SEM. Two-way ANOVA, log-rank (Mantel-Cox) test. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figures S4 and S5.

perform equally *in vivo* regardless of the vaccine size or the positioning of an epitope within the construct.

### Long neoantigen vaccine strings control heterogeneous multifocal tumors

To test our hypothesis that targeting multiple neoantigens will allow for better control of multifocal or metastatic tumors with differing mutation profiles, we injected ID8 tumors on the right flank of C57BL6 mice. At the same time, we injected TC1 tumors on the contralateral flank and vaccinated mice with either the 40-1 or 40-$1_{rev}$ vaccine construct and followed tumor size over time. As controls, we also vaccinated the mice with either a ID8vax 12-mer or TC1vax 12-mer, which we have previously described (Figure 5A). The number of immunogenic epitopes against each tumor are listed in Table 2. Mice vaccinated with TC1vax (two immunogenic TC1 epitopes) demonstrated partial control compared to mice vaccinated with pVax or ID8vax, and four of five mice eventually grew tumors. Alternatively, 100% of the mice receiving either 40-1 or 40-$1_{rev}$ vaccines (three immunogenic TC1 epitopes) prevented growth of TC1 tumors (Figure 5B; Figure S6A). Importantly, mice receiving ID8vax, 40-1, or 40-$1_{rev}$ vaccines (two immunogenic ID8 epitopes) all shrank established ID8 tumors compared to the pVax or TC1vax groups to equal measures, demonstrating the need for including as many epitopes as possible (Figures 5C and 5D; Figure S6B). These data provide strong evidence supporting the hypothesis that targeting multiple neoantigens will allow for better control of multifocal tumors with different mutation profiles.

## DISCUSSION

In this study, we identified that there are a significant number of missense mutations that are unique to a single tumor nodule in case of multi-focal or metastatic tumors, in patients through analysis of TCGA database. Chung et al.[18] have also reported differences in the TMB of primary versus metastatic tumors in prostate cancer. Barroso-Sousa et al.[19] have reported a higher TMB in metastatic breast cancer tissue compared to primary tumors. Although neither of these studies compared matched samples from the same patient, they highlight the diversity in mutational burden between primary and metastatic tumors. Also, early neoantigen studies with shorter peptide strings induced mostly $CD4^+$ T cell responses,[4,5] suggesting that increasing the epitope number could provide more opportunities for induction of $CD8^+$ T cell neoantigen-targeting immunity. In order to improve clinical responses, it is important to target multiple nodules and hence as many neoantigens as possible. With this in mind, we designed long strings of synthetic neoantigens assembled in a DNA vaccine vector. These long strings target up to 40 epitopes as neoantigens. Antigen interference is a concern with the delivery of multiple epitopes at the same time. Several studies have shown that delivery of multiple epitopes at the same time dampens the immune response from individual epitopes compared to when the same epitope was delivered as a single antigen in murine models.[20,21] To test whether antigenic interference occurred in these DNA vaccines coding for 40 neoantigens, we compared immune responses against three highly immunogenic epitopes in a range of construct sizes and changed the position of individual epitopes within constructs. We observed that the magnitude of immune response against all three epitopes was similar in 10-mer, 20-mer, and 40-mer regardless of the position of the epitope on the neoantigen string. Similarly, the type of immune response (MHC class I versus MHC class II mediated) remained unchanged across all constructs, indicating that antigen processing and presentation remain unaffected by larger construct sizes.

Next, we examined the immunogenicity of 20 different neoantigens that display varying degrees of immunogenicity. We also tested whether low-immunogenicity epitopes became more immunogenic by moving them to a different location on the 40-mer vaccine, and vice versa. While we did observe some limited variability in the immune responses across constructs, overall, we found that immune responses were comparable for all of the 10 test antigens across all three constructs. Also, crucially, across the total 20 antigens tested, all antigens that yielded a response in one construct did so in all constructs, and those that were non-immunogenic in one construct were non-immunogenic across all of the constructs. This 100% concordance indicates that antigen processing and presentation and induction of

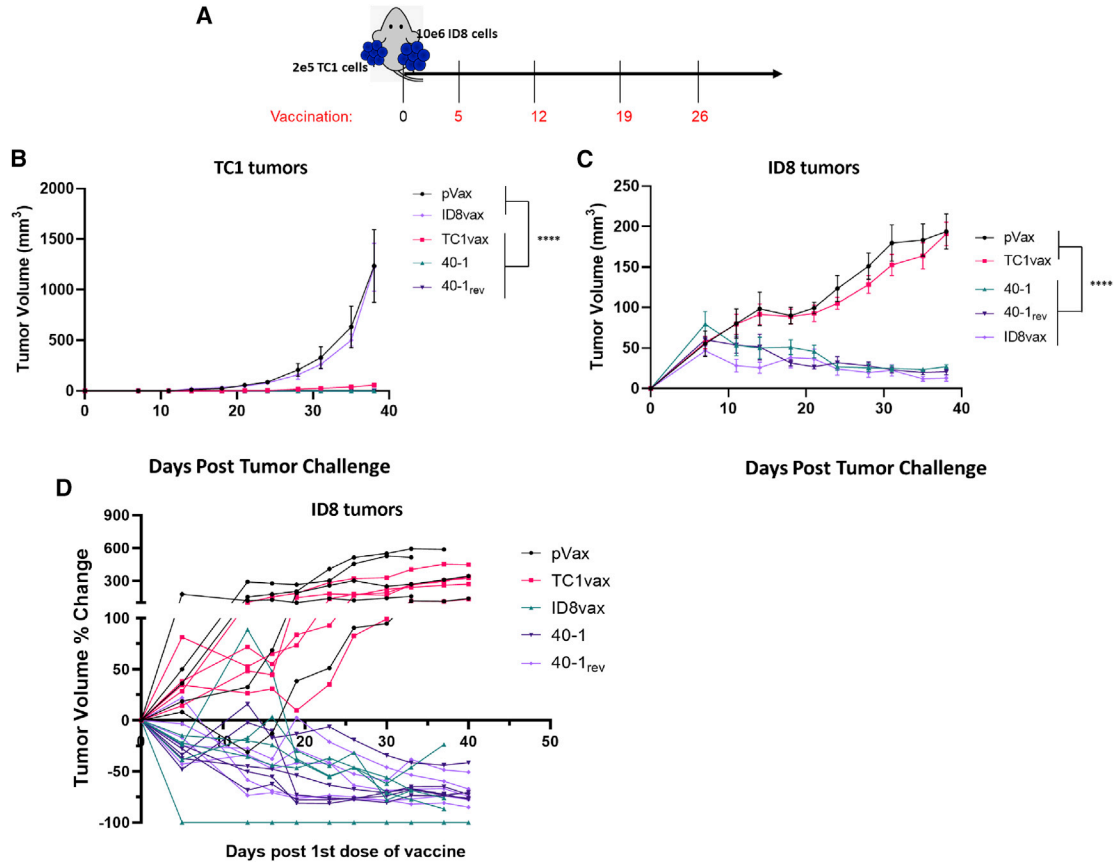


**Figure 5. The vaccines control multifocal tumors with completely different mutation profiles**

(A) Schematic to explain tumor challenge experiments in (B)–(D). (B and C) Mean tumor sizes of TC1 (B) and ID8 (C) tumors in mice vaccinated with pVax (50 μg), TC1vax (25 μg)+pVax (25 μg), ID8vax (25 μg)+pVax (25 μg), 40-1 (50 μg), or 40-$1_{rev}$ (50 μg). (D) % of ID8 tumor size change after vaccination with pVax (50 μg), TC1vax (25 μg)+pVax (25 μg), ID8vax (25 μg)+pVax (25 μg), 40-1 (50 μg), or 40-$1_{rev}$ (50 μg). Error bars represent mean + SEM. Two-way ANOVA. ****$p < 0.0001$. See also Figure S6.

immune responses were driven by the intrinsic property of the epitope to yield an immune response during their processing and presentation. Importantly, relative positioning along the synthetic DNA multi-epitope construct or the presence of other neoepitopes had a relatively modest impact on immune responses. We did not see any antigenic interference with this vaccine approach, as the immune responses induced from vaccines strings coding for 40 epitopes behaved similarly to those from a construct coding for only 10 epitopes. This again indicates that the immune response generated by these long vaccines is dependent on the intrinsic immunogenicity of individual epitopes encoded within the vaccine construct. Coding for a higher number of epitopes does not significantly affect the immune response driven from individual epitopes.

Our observations were further confirmed in an *in vivo* tumor challenge study where all three constructs were able to completely control tumor growth. Strikingly, we observed that 100% of mice remained tumor-free after re-challenge with a higher dose of tumor 50 days after the last vaccination. This suggests that these long epitope DNA vaccines generate a memory T cell response that is prevalent long term and can be reactivated to fight off tumors, which is important in cases of recurrence. This would be of utility in a clinical setting where the vaccine platform is able to generate long-term immunity and minimize the chances of tumor recurrence. The long-term immunity would also potentially reduce the number of doses a patient would have to take, which further provides possible clinical advantages.

The number of neoantigens in a tumor vary vastly from patient to patient and depend on the tumor type. This number is generally thought to be between 33 and 163 expressed, nonsynonymous mutations in human tumors.[22] Based on the tested number of 40 neoantigens per construct, all of the expressed neoantigens could be targeted by co-delivering four constructs. Additionally, since the presence of non-immunogenic epitopes does not affect immunogenicity, this similarly eliminates the requirement for experimentally validating each neoantigen before treatments. This reduces the cost of therapy as well as the time required to treatment, which are critical for cancer therapy. In clinical settings, DNA vaccines have been shown to generate $CD8^+$ and $CD4^+$ T cell responses against multiple antigens

**Table 2. No. of relevant immunogenic epitopes in each vaccine**

| Construct | No. of immunogenic TC1 epitopes | No. of immunogenic ID8 epitopes |
|---|---|---|
| pVax | 0 | 0 |
| TC1vax | 2 | 0 |
| ID8vax | 0 | 2 |
| 40-1 | 3 | 2 |
| $40\text{-}1_{rev}$ | 3 | 2 |

in humans, and multiple doses of DNA vaccines have been well tolerated in patients,[23–25] supporting further study in this context. To our knowledge, the delivery of 40 neoepitopes as a single polynucleotide string has not been previously reported and could be important in immunotherapy approaches for driving diverse T cell expansion.

Our neoepitope insert design incorporates a furin cleavage site that ensures that each individual neoantigen is chopped up into 33-mer peptides matching the sequence of the mutation in the tumor. These would then require further processing by the proteasome into 8- or 9-mer before being presented on MHC class I. Not including the furin cleavage site could result in generation of random junctional epitopes by combination of amino acids from two sequential epitopes. Intracellular protein production followed by proteasomal degradation is essential for antigen presentation of MHC class I molecules.[26] DNA vaccination followed by electroporation allows for expression of antigens in their native form. This leads to antigen processing such that it increases the chances of presentation on MHC class I and skews the immune response toward a more $CD8^+$ T cell response. Additionally, our group has recently demonstrated that DNA vaccine plus electroporation causes transient local injection site inflammation, leading to an increased infiltration of $CD11b^+F4/80^+$ M1 macrophages and $CD8\alpha^+$ conventional dendritic cells (cDCs) into the injection site. The increased infiltration of antigen-presenting cells (APCs) is associated with an increased $CD8^+$ T cell response.[27]

In conclusion, we have shown the feasibility of targeting up to 40 neoantigens using a simple combination system. Multiple neoantigens were immunogenic in this approach, and the magnitude or type of immune response was independent of the size of the construct and the position of individual epitope within the vaccine. Neoantigen DNA vaccines also generated long-term immune responses and were able to protect animals from tumor growth for as many as 89 days after the final vaccination. Finally, long neoantigen vaccine strings controlled the growth of heterogeneous, multifocal tumors, even with completely different mutation profiles. Additionally, the level of tumor control was dependent on the number of immunogenic epitopes included in the vaccine. Eliminating even a single immunogenic epitope significantly reduced the efficacy of the vaccine in controlling tumors. These data highlight the need to vaccinate against as many epitopes as possible. The data also suggest that such long neoantigen string vaccines could be studied to simultaneously target primary and metastatic disease and also be useful for preventing tumor recurrence. Further translational development of longer neoantigen vaccines is likely of importance.

## MATERIALS AND METHODS

### Animals and cell lines

6- to 8-week-old female C57BL/6 mice were purchased from The Jackson Laboratory. All animal experiments were approved by the Institutional Animal Care and Use Committee at The Wistar Institute. The TC1 cell line was provided by Y. Paterson (University of Pennsylvania) in 2011. TC1 tumors were generated by injecting 100,000 TC1 cells into the right flank. For the re-challenge experiment to test long-term protection, 200,000 TC1 cells were injected into the left flank. All cell lines were maintained at low passage (<10 passages) and thawed directly from a master stock generated upon receipt of the cells for all experiments. Cells were routinely tested for mycoplasma contamination prior to freezing them for storage, most recently in 2019. The cell lines were not genetically authenticated but were examined for morphologic authenticity in cell culture.

Mice were vaccinated by injecting indicated amounts of DNA resuspended in 50 μL of water into the tibialis anterior muscle followed by electroporation with the CELLECTRA-3P device (Inovio Pharmaceuticals). For each vaccination, mice were delivered two 0.1 amp electric constant current square-wave pulses.

### Vaccine design

All plasmid DNA vaccine constructs were designed using previously described methods and were based on neoantigens previously identified and tested.[15] 10-mer constructs were designed selecting the 10 most immunogenic epitopes previously identified from TC1, ID8, and LLC tumors. 20-mer constructs contained all 10 epitopes from the 10-mer construct with an additional 10 epitopes randomly interspersed in between. This was designed to model a clinical scenario where the immunogenicity of different epitopes within a single patient is unlikely to be known before vaccine design. A similar strategy was chosen to design 40-mer epitopes where all 20 epitopes from the 20-mer construct (including the 10 most immunogenic epitopes encoded by the 10-mer construct) were combined with 20 others that were previously identified to be weakly or not immunogenic. Importantly, all vaccines contained a mixture of epitopes derived from TC1, ID8, and LLC tumors. The $40\text{-}1_{rev}$ vaccine was designed as a reverse to the 40-1 vaccine, where the position of all 40 epitopes relative to the N terminus was correspondingly switched to be relative to the C terminus of the encoded polypeptide string. For example, epitope no. 1 (i.e., N-terminal epitope) from the 40-1 vaccine was epitope no. 40 (i.e., C-terminal epitope) in the $40\text{-}1_{rev}$ vaccine, epitope no. 2 from the 40-1 vaccine was epitope no. 39, and so on for all 40 epitopes. The naming protocol for each vaccine that we used throughout the study was X-Y, where X represents the total number of epitopes in the vaccine and Y represents the position of epitope Sgsm2 V656A, which was the most immunogenic epitope of all epitopes studied.[15] The positions of Herpud2 and Lta4h in each of the vaccine constructs are listed in Table S2. The empty vector, pVax, devoid of any encoded neoantigens was used as a negative control in the immunization and challenge studies.

### Tumor challenge experiment

100,000 TC1 cells (in PBS) were injected on the right flank subcutaneously. 4 days after tumor implantation, mice were treated with either 10-1+pVax (25 μg each), 40-1 (50 μg), or 40-$1_{rev}$ (50 μg) or just pVax empty vector control (50 μg). The mice were vaccinated weekly for a total of four vaccinations. Tumor size was monitored via caliper measurements. Mice were euthanized when the length of tumor reached 20 mm or tumor volume reached greater than 2,000 $mm^3$. Tumor volume was calculated using the formula V = [(length × width2) × 3.14]/2, where width is considered the side with smaller measurement. For the re-challenge experiment, 200,000 TC1 cells were implanted subcutaneously into the left flank 61 days after the final vaccination. The mice received no further treatment, and the mice were euthanized 39 days after re-challenge. To evaluate efficacy in heterogeneous, multifocal tumors, we injected 10e6 ID8 tumors in 50% Matrigel/50% PBS on the right flank of C57BL/6 mice. At the same time, we injected 2e5 TC1 tumors on the contralateral flank. The mice were vaccinated with 10-1+pVax (25 μg each), 40-1 (50 μg), or 40-$1_{rev}$ (50 μg) or just pVax empty vector control (50 μg). As controls, we also vaccinated the mice with either TC1vax 12-mer or ID8vax 12-mer vaccine as previously described.[15]

### Flow cytometry

Directly conjugated antibodies against murine CD3e (17A2), CD4 (RM4-5), CD8b (YTS156.7.7), IFN-γ (XMG1.2), TNF-α (MP6-XT22), and IL-2 (JES6-5H4) were purchased from BioLegend. A Live/Dead Violet viability kit (Invitrogen) was used to exclude dead cells from analysis. To determine intracellular cytokine production, 2 million splenocytes from vaccinated mice were cultured with peptides (5 μg/mL) derived from corresponding mutated neoantigen, protein transport inhibitor (eBioscience), and CD107a antibody (1D4B, BioLegend) for 5 h. Surface staining followed by intracellular cytokine staining was done to determine cytokine production. Cells were permeabilized using the eBioscience FoxP3 staining kit as per the manufacturer's instructions. Data were acquired on a BD FACSymphony (BD Biosciences) and analyzed using FlowJo. The neoantigen peptides consisted of 15-mer peptides overlapping by 9 aa. The peptides were designed to cover the entire 33-mer used for vaccination. Mice were vaccinated three times at 2-week intervals and euthanized 1 week after the last vaccination. Spleens were harvested, and splenocyte suspensions were obtained using a Stomacher 80 Biomaster (Thomas Scientific), followed by red blood cell lysis (Thermo Fisher Scientific).

### ELISPOTs

Mice were vaccinated three times at 2-week intervals. 1 week following the final vaccination, splenocytes were harvested and co-incubated with each neoantigen-derived peptide pool comprising 15-mer overlapping by 9 aa (5 μg/mL). Splenocytes were cultured at 37°C. 24 h later, we performed the murine IFN-γ ELISPOT according to the manufacturer's instructions (Mabtech, no. 3321-4APT-10). Spots were read using an ImmunoSpot CTL reader, and spot-forming units (SFU) were calculated by subtracting media-alone wells from stimulated wells. Concanavalin A was used as a positive control. The threshold for immunogenicity was set at 30 SFU per million splenocytes.

### Statistical analysis

The difference between the means of experimental groups was calculated using a Tukey's multiple comparison test. Comparisons between two or more groups with multiple subjects was done using ordinary one-way ANOVA. Comparisons between tumor size at each time point were done using two-way ANOVA with Fisher's least significant difference (LSD) test. Error bars represent standard error of the mean. For mouse survival analysis, significance was determined using a log-rank (Mantel-Cox) test. All statistical analyses were done using GraphPad Prism 8.0. $p < 0.05$ was considered statistically significant. Error bars represent SEM unless otherwise stated.

### Evaluation of the number of mutations per tumor type

Mutation data originally published by Chang et al.[28] were obtained from cBioportal (https://www.cbioportal.org/comparison/clinical?sessionId=5e76cceae4b0ff7ef5fdb3c2; accessed July 10, 2020) from TCGA PanCancer Atlas studies. The dataset was reduced to the top 20 cancer types based on the number of samples in the study, and the number of mutations per sample was obtained.

### Calculation of unique mutations per patient

We queried data from the AACR Project GENIE database[29] to obtain this information. We selected five cancers that had the highest number of mutations per sample (non-small cell lung cancer was split into lung adenocarcinoma and lung squamous cell carcinoma). To obtain data about unique mutations, we chose patients where two samples had been submitted per patient. Next, we eliminated patients where sequencing was done on fewer than 200 genes. Then we looked at the total number of missense mutations in each patient. Unique missense mutations were defined as those mutations that were present in only one of the two samples sequenced. The percentage of unique mutations was calculated as (total unique mutations/total missense mutations) × 100. Patients with no missense mutations or missense mutations in only one of two samples sequenced were not included in final analysis. The data of all patients analyzed can be found at https://genie.cbioportal.org/study/summary?id=5f1f517ee4b070725d7d7314; accessed July 27, 2020.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.04.005.

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## AUTHOR CONTRIBUTIONS

P.S.B., A.P-P., N.Y.S., and D.B.W. conceptualized the study and designed the experiments. P.S.B. and A.P-P. performed experiments, acquired, and analyzed the data. P.S.B. and N.C. provided administrative support for the project. All authors contributed to writing and revision of the manuscript.

## DECLARATION OF INTERESTS

D.B.W. discloses the following paid associations with commercial partners: GeneOne (consultant), Geneos (Advisory Board, research funding, stock), Astrazeneca (Advisory Board, speaker), Inovio (BOD, SRA, stock), Pfizer (speaker), Merck (speaker), Sanofi (Advisory Board), and BBI (Advisory Board). A.P-P, N.C., and N.Y.S., are employees of Geneos Therapeutics. The remaining authors declare no competing interests.

## REFERENCES


1. Snyder, A., Makarov, V., Merghoub, T., Yuan, J., Zaretsky, J.M., Desrichard, A., Walsh, L.A., Postow, M.A., Wong, P., Ho, T.S., et al. (2014). Genetic basis for clinical response to CTLA-4 blockade in melanoma. N. Engl. J. Med. *371*, 2189–2199.
2. Schumacher, T.N., Scheper, W., and Kvistborg, P. (2019). Cancer neoantigens. Annu. Rev. Immunol. *37*, 173–200.
3. Schumacher, T.N., and Schreiber, R.D. (2015). Neoantigens in cancer immunotherapy. Science *348*, 69–74.
4. Ott, P.A., Hu, Z., Keskin, D.B., Shukla, S.A., Sun, J., Bozym, D.J., Zhang, W., Luoma, A., Giobbie-Hurder, A., Peter, L., et al. (2017). An immunogenic personal neoantigen vaccine for patients with melanoma. Nature *547*, 217–221.
5. Sahin, U., Derhovanessian, E., Miller, M., Kloke, B.-P., Simon, P., Löwer, M., Bukur, V., Tadmor, A.D., Luxemburger, U., Schrörs, B., et al. (2017). Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. Nature *547*, 222–226.
6. Carreno, B.M., Magrini, V., Becker-Hapak, M., Kaabinejadian, S., Hundal, J., Petti, A.A., Ly, A., Lie, W.R., Hildebrand, W.H., Mardis, E.R., and Linette, G.P. (2015). A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. Science *348*, 803–808.
7. Kreiter, S., Vormehr, M., van de Roemer, N., Diken, M., Löwer, M., Diekmann, J., Boegel, S., Schrörs, B., Vascotto, F., Castle, J.C., et al. (2015). Mutant MHC class II epitopes drive therapeutic immune responses to cancer. Nature *520*, 692–696.
8. Durgeau, A., Virk, Y., Corgnac, S., and Mami-Chouaib, F. (2018). Recent advances in targeting CD8 T-cell immunity for more effective cancer immunotherapy. Front. Immunol. *9*, 14.
9. Schnidrig, D., Turajlic, S., and Litchfield, K. (2019). Tumour mutational burden: Primary versus metastatic tissue creates systematic bias. IOTECH *4*, 8–14.
10. Stein, M.K., Pandey, M., Xiu, J., Tae, H., Swensen, J., Mittal, S., Brenner, A.J., Korn, W.M., Heimberger, A.B., and Martin, M.G. (2019). Tumor mutational burden is site specific in non–small-cell lung cancer and is highest in lung adenocarcinoma brain metastases. JCO Precis. Oncol. Published online July 26, 2019. https://doi.org/10.1200/PO.18.00376.
11. González, S., Volkova, N., Beer, P., and Gerstung, M. (2018). Immuno-oncology from the perspective of somatic evolution. Semin. Cancer Biol. *52*, 75–85.
12. Hacohen, N., and Sarkizova, S. (2017). How T cells spot tumour cells. Nature *551*, 444–446.
13. Topalian, S.L., Taube, J.M., Anders, R.A., and Pardoll, D.M. (2016). Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. Nat. Rev. Cancer *16*, 275–287.
14. Johanns, T.M., Miller, C.A., Liu, C.J., Perrin, R.J., Bender, D., Kobayashi, D.K., Campian, J.L., Chicoine, M.R., Dacey, R.G., Huang, J., et al. (2019). Detection of neoantigen-specific T cells following a personalized vaccine in a patient with glioblastoma. OncoImmunology *8*, e1561106.
15. Duperret, E.K., Perales-Puchalt, A., Stoltz, R., G H, H., Mandloi, N., Barlow, J., Chaudhuri, A., Sardesai, N.Y., and Weiner, D.B. (2019). A synthetic DNA, multi-neoantigen vaccine drives predominately MHC class I $CD8^+$ T-cell responses, impacting tumor challenge. Cancer Immunol. Res. *7*, 174–182.
16. Lawrence, M.S., Stojanov, P., Polak, P., Kryukov, G.V., Cibulskis, K., Sivachenko, A., Carter, S.L., Stewart, C., Mermel, C.H., Roberts, S.A., et al. (2013). Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature *499*, 214–218.
17. Chalmers, Z.R., Connelly, C.F., Fabrizio, D., Gay, L., Ali, S.M., Ennis, R., Schrock, A., Campbell, B., Shlien, A., Chmielecki, J., et al. (2017). Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. Genome Med. *9*, 34.
18. Chung, J.H., Dewal, N., Sokol, E., Mathew, P., Whitehead, R., Millis, S.Z., Frampton, G.M., Bratslavsky, G., Pal, S.K., Lee, R.J., et al. (2019). Prospective comprehensive genomic profiling of primary and metastatic prostate tumors. JCO Precis. Oncol. *3*, PO.18.00283.
19. Barroso-Sousa, R., Jain, E., Cohen, O., Kim, D., Buendia-Buendia, J., Winer, E., Lin, N., Tolaney, S.M., and Wagle, N. (2020). Prevalence and mutational determinants of high tumor mutation burden in breast cancer. Ann. Oncol. *31*, 387–394.
20. Sedegah, M., Charoenvit, Y., Minh, L., Belmonte, M., Majam, V.F., Abot, S., Ganeshan, H., Kumar, S., Bacon, D.J., Stowers, A., et al. (2004). Reduced immunogenicity of DNA vaccine plasmids in mixtures. Gene Ther. *11*, 448–456.
21. Wang, K.Y., Guo, Y.J., Zhang, Y.L., Lv, K., and Sun, S.H. (2007). Combined DNA vaccination against three animal viruses elicits decreased immunogenicity of a single plasmid in mice. Vaccine *25*, 4429–4436.
22. Vogelstein, B., Papadopoulos, N., Velculescu, V.E., Zhou, S., Diaz, L.A., Jr., and Kinzler, K.W. (2013). Cancer genome landscapes. Science *339*, 1546–1558.
23. Trimble, C.L., Morrow, M.P., Kraynyak, K.A., Shen, X., Dallas, M., Yan, J., Edwards, L., Parker, R.L., Denny, L., Giffear, M., et al. (2015). Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: A randomised, double-blind, placebo-controlled phase 2b trial. Lancet *386*, 2078–2088.
24. Tebas, P., Roberts, C.C., Muthumani, K., Reuschel, E.L., Kudchodkar, S.B., Zaidi, F.I., White, S., Khan, A.S., Racine, T., Choi, H., et al. (2017). Safety and immunogenicity of an anti-Zika virus DNA vaccine—preliminary report. N. Engl. J. Med. Published online October 4, 2017. https://doi.org/10.1056/NEJMoa1708120.
25. Aggarwal, C., Cohen, R.B., Morrow, M.P., Kraynyak, K., Bauml, J., Weinstein, G.S., Boyer, J., Yan, J., Mangrolia, D., Oyola, S., et al. (2017). Immunogenicity results using human papillomavirus (HPV) specific DNA vaccine, INO-3112 (HPV16/HPV18 plasmids + IL-12) in $HPV^+$ head and neck squamous cell carcinoma (HNSCCa). J. Clin. Oncol. *35* (15, Suppl), 6073.
26. Rock, K.L., Reits, E., and Neefjes, J. (2016). Present yourself! By MHC class I and MHC class II molecules. Trends Immunol. *37*, 724–737.
27. Xu, Z., Chokkalingam, N., Tello-Ruiz, E., Wise, M.C., Bah, M.A., Walker, S., Tursi, N.J., Fisher, P.D., Schultheis, K., Broderick, K.E., et al. (2020). A DNA-launched nanoparticle vaccine elicits $CD8^+$ T-cell immunity to promote *in vivo* tumor control. Cancer Immunol. Res. *8*, 1354–1364.
28. Weinstein, J.N., Collisson, E.A., Mills, G.B., Shaw, K.R., Ozenberger, B.A., Ellrott, K., Shmulevich, I., Sander, C., and Stuart, J.M.; Cancer Genome Atlas Research Network (2013). The Cancer Genome Atlas Pan-Cancer analysis project. Nat. Genet. *45*, 1113–1120.
29. Consortium, A.P.G.; AACR Project GENIE Consortium (2017). AACR project GENIE: powering precision medicine through an international consortium. Cancer Discov. *7*, 818–831.