# Spleen-targeted neoantigen DNA vaccine for personalized immunotherapy of hepatocellular carcinoma

Since the first human trial in 2015, personalized neoantigen cancer vaccines (NCVs) have materialized as tangible therapeutic options for multiple malignancy cancers. NCVs, which rely on identifying nonsynonymous genome alterations in cancer cells by next-generation sequencing, have observed the highest efficacy in cancers with established immunogenicity and high tumor mutation burden (TMB). But not all cancers fit this mold and given cancer rates both in the US and worldwide, there is a growing need for personalized immunotherapy. Expanding on the efficacy of NCVs could help address that need.

Hepatocellular carcinoma (HCC) exhibits an immune "cold" microenvironment, a low-to-moderate TMB, and limited tumor-infiltrating lymphocyte (TIL) activity, making immunotherapy treatment especially difficult. With an overall response rate of less than 20% to various immunotherapies and few clinic trails on the horizon, NCV treatment has found limitations in treating HCC. Directly addressing these challenges, Wu and colleagues aimed to increase NCV antitumor activity capabilities against HCC using a spleen-targeted neoantigen DNA vaccine with a red blood cell (RBC) hitchhiking strategy. This detailed study highlights the potential of spleen-targeted DNA nanovaccines in enhancing anti-tumor immunity and improving therapeutic outcomes for HCC.

Compared to other formats of NCVs, including RNA, peptides, and dendritic cells, DNA-based NCVs are relatively easy to generate and offer both molecular flexibility and thermal stability. In addition to these benefits, and perhaps most notably, DNA-encoding neoantigens have

been observed to elicit long-term antigen-specific immune responses. DNA-based NCVs, do however, come with some limitations due to their large size, highly negative charge, and susceptibility to degradation, which overall can result in minimal immunogenicity. Thanks to the development of nanoscaled delivery vehicles, most of these factors can be overcome to effectively protect and transport DNA into cells.

Targeting neoantigens to the spleen can invigorate robust antitumor immunity due to its immuno-centralization and abundance of immune cells such as antigen-presenting cells (APCs) and T cells. To effectively target the spleen and avoid common challenges, the researchers turned to a delivery strategy, recently developed by the Mitragotri group, known as "RBC-hitchhiking". Although the exact mechanism is not yet known, it has been observed that RBCs can capture pathogens on their surface and hand them off to APCs which then form an adaptive immune response. It is this exact and highly-specific interaction that RBC-hitchhiking takes advantage of to effectively target DNA vaccine delivery to spleen to promote an immune response.

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## Methods

### Preparation of DNA nanovaccines

Based on previous findings where murine HCC cell lines were sequenced, the researchers first prepared their DNA nanovaccines encoded for Hepa 1-6 liver cancer cell-specific neoantigens. Using scanning electron microscopy, transmission electron microscopy, and agarose gel electrophoresis, they characterized the size, zeta potential, and encapsulation efficiency of the nanovaccines, finding them to possess both low cytotoxicity and high transfection efficiency in APCs.

### RBC-hitchhiking strategy

Following the preparation and evaluation of the nanovaccines, the researchers turned their attention to the proposed RBC-hitchhiking strategy aimed at improving bioavailability. They first attached the DNA nanovaccines to RBCs *ex vivo*, confirming delivery via intravascular injection with microscopy. Given that nanoparticles have been observed to easily dislodge in the lungs, they next evaluated the *in vivo* biodistribution to assess if RBC-hitchhiking DNA nanovaccines could successfully be delivered to the spleen. Using various nanovaccine-to-RBC ratios, they observed ratios of 50:1 and 100:1 achieved the best enrichment in the spleen, noting that decreased distribution of nanoparticles between the lung and the spleen occurred both above or below these ranges. Finally, they analyzed DiI-labeled RBC-Nanovaccines by flow cytometry and confirmed the internalization of nano-packaged DNA by APCs in the spleen. All together, these findings showed that DNA-nanovaccine hitchhiking on RBCs (RBC-Nanovaccines) can be effective at the appropriate density ratio, evade mechanical dislodgement in the lungs, and promote DNA-based vaccine accumulation in the spleen.

The research team next examined the immune response triggered by RBC-Nanovaccines *in vivo*. C57BL/6 mice were immunized twice by intravenous injection with differently engineered vaccines once a week for two weeks. They used a double-injection approach to induce a "priming + boosting" effect to elicit a durable immune response, and four days following the second injection, harvested tissue and blood for immune profiling. Flow cytometry evaluation of MHC I expression on the surface of dendritic cells (DCs) in the spleen showed significantly higher expression on DCs from RBC-Nanovaccines, indicating the neoantigen encoding pDNA was effectively captured and expressed by APCs in the spleen with the help of RBCs. Additionally, the expression of MHC I confirms that maturation of APCs was triggered, generating a favorable environment for a T cell immune response to form.

Next, the researchers assessed the immune response by flow cytometry, observing T cells from the RBC-Nanovaccine group showed significant enhancement in CD45+ CD3+ (effector), CD3+ CD4+ (helper), and CD3+ CD8+ (cytotoxic) populations. The RBC-Nanovaccines group also showed induced secretion of antitumor cytokines of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in peripheral blood.

### Immunization and therapeutic efficacy

After confirming the feasibility of the RBC-hitchhiking strategy with nanovaccines, the team was ready to evaluate the ability of the vaccine to exert an immune-protective effect against tumorigenesis. Seven days after the second RBC-Nanovaccination, immunized mice were subcutaneously challenged with Hepa 1-6 cells to induce HCC tumorigenesis. Tumor progression was monitored via bioluminescence imaging with Revvity's IVIS™ Spectrum and IVISbrite™ D-Luciferin substrate. As expected, mice inoculated with PBS suffered from rapid tumor growth. Mice treated with a single nanovaccine followed by a single inoculation of RBC-Nanovaccine had a moderate delay, but still experienced tumor growth. Remarkably, mice treated with two inoculations of RBC-Nanovaccines showed the highest prevention of tumor growth, with 71.4% (5/7) remaining tumor-free altogether and a prolonged survival rate overall.

Immunological memory is one of the most important factors in promoting tumor rejection, so they next evaluated the memory T cell subtypes in the spleen using flow cytometry. Seven days after the second vaccination, the RBC-Nanovaccinated mice exhibited a 1.5-fold higher percentage of CD8+ memory T cells compared to the control group, indicating T cell memory had formed. The researchers also verified the specificity of the RBC-Nanovaccines against Hepa 1-6 tumors with a B16-F10 melanoma cell injection challenge where they observed all injections to be helpless against the induction of melanoma. This experiment confirmed that the RBC-Nanovaccine created a personalized antitumor response.

**Combination therapy with anti-PD-1 antibody**

Having shown the potent ability of RBC-Nanovaccines to create personalized immunization against HCC, the researchers next assessed antitumor efficacy. Using an established HCC model, C57BL/6 mice were inoculated with Hepa 1-6 cells on negative day seven and then immunized with PBS, nanovaccines, or RBC-Nanovaccines three times on days zero, four, and eight. As shown in Figure 1A, mice injected with PBS and nanovaccine displayed aggressive tumor growth. Although RBC-Nanovaccine mice had significantly delayed and smaller tumor growth, this treatment still failed to prevent tumorigenesis altogether, indicating another pathway may be helping HCC cells evade the immune response. Using immunofluorescence and immunohistochemistry, they discovered PD-L1 to be significantly upregulated in HCC tissue and decided to combine RBC-Nanovaccines with anti-PD-1 antibody from BioLegend (Clone 29F.1A12) to enhance immunotherapy efficacy. Anti-PD-1 antibodies blocked the PD-1 receptor on T cells, preventing interaction with PD-L1 on tumor cells or other cells in the tumor microenvironment, thereby reinvigorating T cell responses against the tumor and enhancing the vaccine-induced immune response. Strikingly, as seen in Figure 1B, this combination increased the tumor regression rate to 75.0% (6/8) and showed a complete cure at day 36 yielding prolonged survival. Paired with this, they also observed the activity of effector memory T cells was significantly increased, suggesting that combination treatment with RBC-Nanovaccines and anti-PD-1 antibody can produce a long-lasting memory immune response to prevent tumor recurrence. Confirming an effective immunological memory, they found 100% cured mice resisted a re-challenge introduced with Hepa 1-6 cells on day 60 as seen in Figure 1C-E.

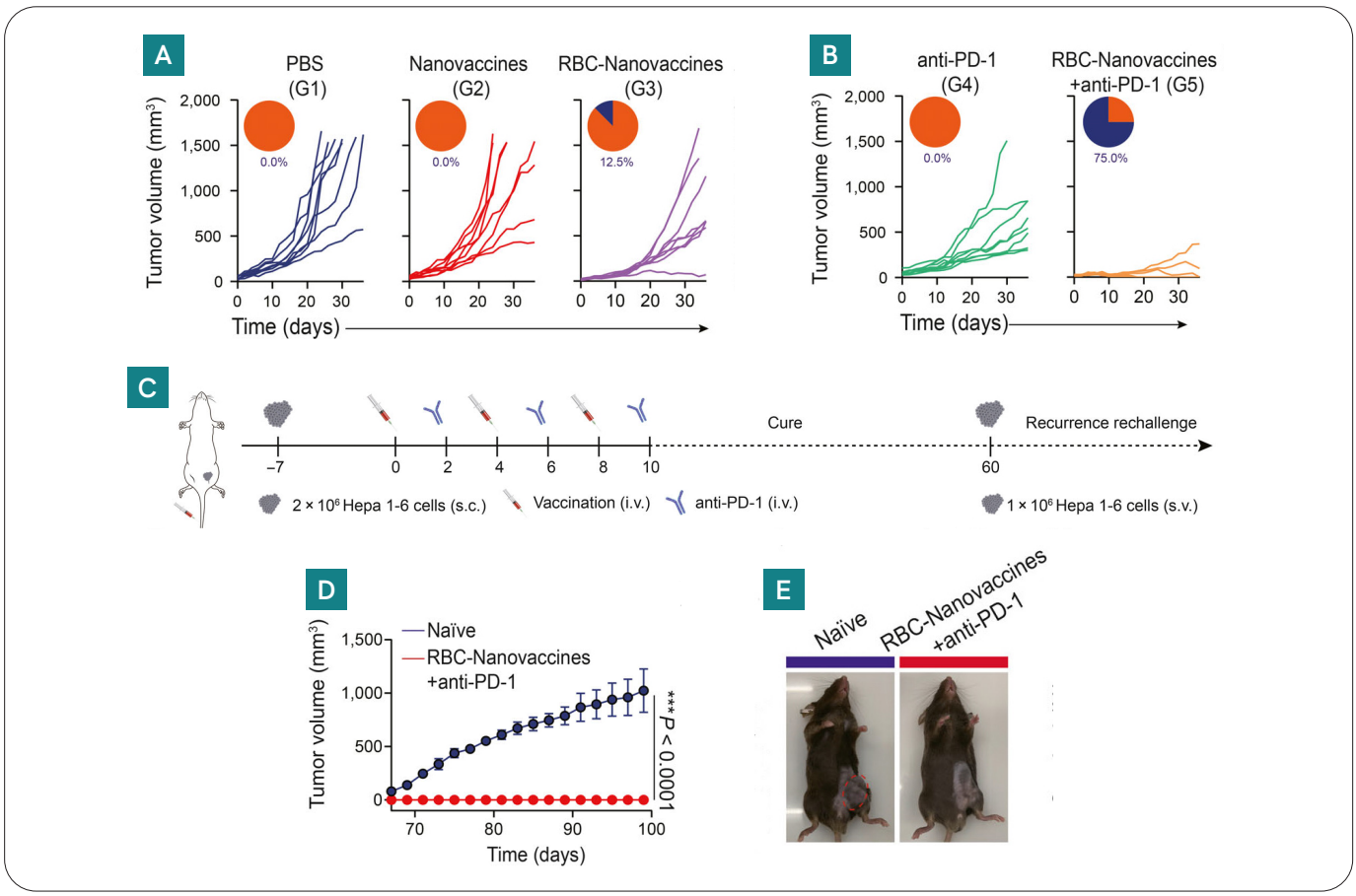


Figure 1: Combination therapy with anti-PD-1 increases tumor regression rate and creates immunological memory. A) Individual Hepa 1-6 tumor growth with fraction of completely tumor regression (CR) after inoculation with PBS, nanovaccines, and RBC-Nanovaccines three times (n = 8 animals per group). B) Individual Hepa 1-6 tumor growth with fraction of CR after receiving different treatments as indicated (n = 8 animals per group). C) Schematic illustration of the schedule for the tumor subcutaneous re-challenge. D) Individual Hepa 1-6 tumor growth with average tumor growth curves after subcutaneous re-challenge of Hepa 1-6 cells (n = 4 animals per group). E) Representative images of Hepa 1-6 tumor-bearing mice in each group at the 100th day after subcutaneous rechallenge.

Inspired by their findings, the team moved to test this treatment against an orthotopic HCC mouse model, which more closely mimics the microenvironment where tumor cells grow in the liver. Luc-Hepa 1-6 cells were surgically inoculated into the liver lobes of C57BL/6 mice on day 0, and RBC-Nanovaccines were injected three times (D10, 14, and 18) while tumor growth was monitored by bioluminescence imaging with Revvity's IVIS Spectrum every eight days. As clearly shown in Figure 2A-C, RBC-Nanovaccines in combination with anti-PD-1 antibody successfully eliminated orthotopic Hepa 1-6 tumors and inhibited tumor metastasis. Furthermore, they also confirmed this treatment is most effective when administered by intravenous injection rather than subcutaneous inoculation, as it resulted in complete tumor regression and prolonged survival over 100 days. These findings highlight the efficacy of RBC-Nanovaccines in orthotopic tumor models and displays that enhanced immunotherapy can be achieved.

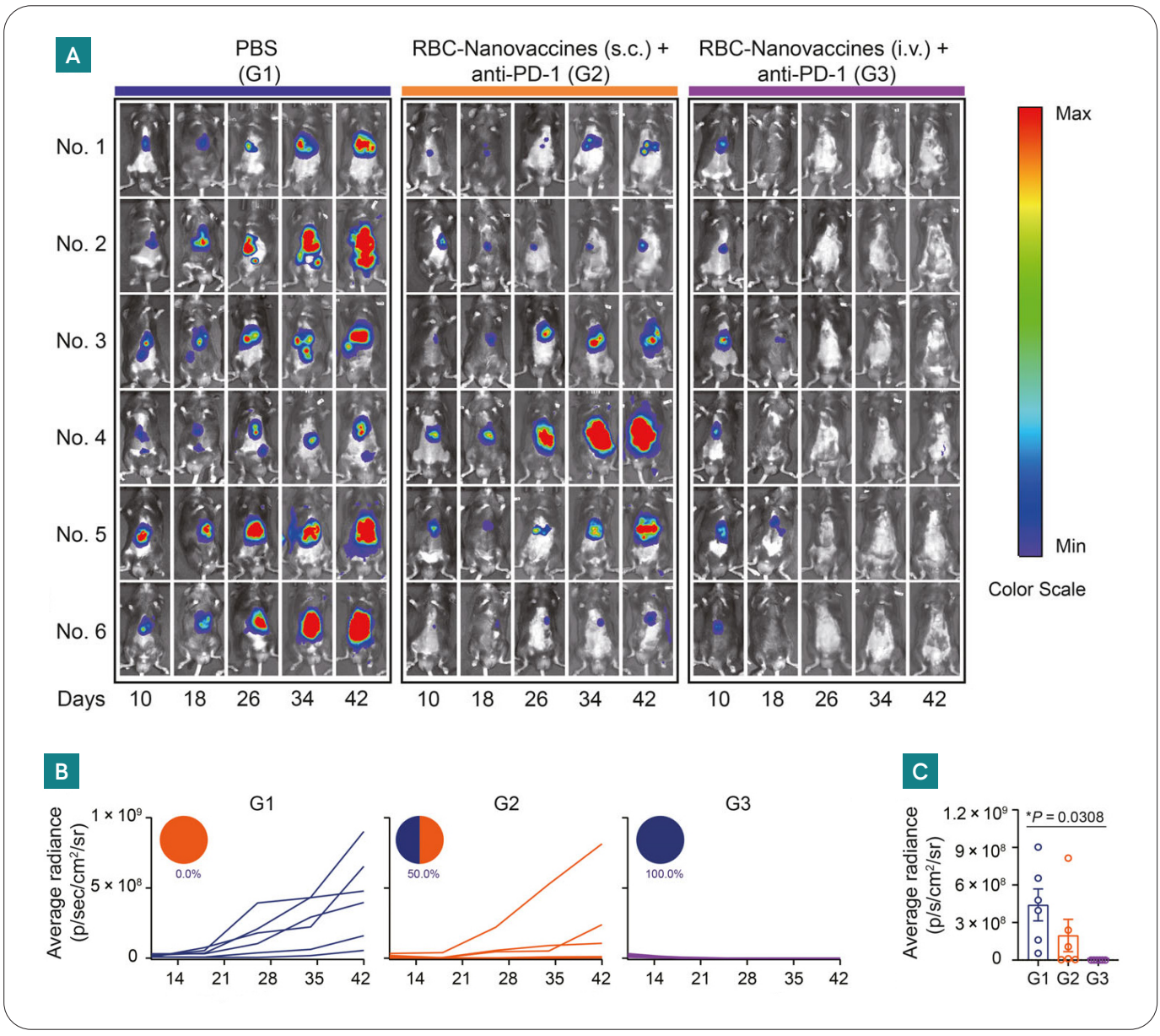


Figure 2: RBC-hitchhiking DNA nanovaccines combined with anti-PD-1 antibody eliminates orthotopic Hepa 1-6 tumors and inhibits tumor metastasis. A) Bioluminescence imaging of mice in each group on days 10, 18, 26, 34, and 42 as indicated. B) Individual orthotopic Hepa 1-6 tumor growth (measured by average radiance) after subcutaneous or intravenous inoculation with RBC-Nanovaccines three times (n = 6 animals per group). C) The average radiation statistics for each group at 42 days.

## Conclusion

The study concluded that a spleen-targeted DNA vaccine, delivered via RBC-hitchhiked nanoparticles, effectively induced robust neoantigen-specific T cell responses in HCC. This approach not only inhibited tumor growth, but also showed potential for complete tumor regression when combined with anti-PD-1 therapy, offering a promising strategy for personalized immunotherapy in HCC. This novel treatment opens the door to the expansion of personalized immunotherapy for other immune “cold” microenvironments.

## Reference

1. Wu, M., Luo, Z., Cai, Z., Mao, Q., Li, Z., Li, H., Zhang, C., Zhang, Y., Zhong, A., Wu, L., & Liu, X. (2023). Spleen-targeted neoantigen DNA vaccin for personalized immunotherapy of hepatocellular carcinoma. EMBO molecular medicine, 15(10), e16836. https://doi.org/10.15252/emmm.202216836