



Breast cancer prevention by prophylactic Lalba mRNA-LNP vaccination

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Tumor-suppressive immunity is more evident in early-stage compared to advanced tumors making it the ideal point for cancer interceptive immunotherapies. We previously described that higher peripheral T cell diversity is associated with more pronounced CD8⁺ T cell infiltration in ductal carcinoma in situ of the breast, implying a close interaction between peripheral and intratumor immunity. Here, we developed lipid nanoparticle (LNP)-encapsulated messenger ribonucleic acid (mRNA) vaccines expressing the alpha-lactalbumin (LALBA) protein unique to mammary luminal progenitors (LPs) to test whether enhancing immune response by prophylactic vaccination against the putative cell-of-origin of breast cancer suppresses tumorigenesis. Vaccination of outbred Sprague-Dawley rats with N1-methylpseudouridine-modified or unmodified Lalba mRNA-LNP induced different degrees of LALBA-specific and nonspecific immune responses. The vaccination suppressed carcinogen-induced mammary tumorigenesis and improved tumor-free and overall survival without obvious toxicity in the normal mammary glands and other organs. Single-cell transcriptomic analysis revealed that vaccination decreases the frequency of a proliferative LP population in immunoreactive early epithelial hyperplasia. Overall, we provide proof of principle that prophylactic Lalba mRNA-LNP has the potential to suppress the initiation and progression of early breast neoplastic lesions.

breast cancer | cancer prevention | mRNA-LNP vaccine | progenitors

Breast cancer is the most common cancer in women world-wide and a leading cause of cancer-associated mortality (1). As the incidence continues to rise in densely populated developing countries (1), the global impact of the disease continues to increase and will only be eliminated by focusing on risk prediction and prevention. Cancer prevention has the most profound impact on cancer-associated mortality and morbidity (2). In mammographically highly screened populations, a large fraction of newly diagnosed cases is ductal carcinoma in situ (DCIS), an early stage preinvasive disease (3), providing an opportunity for cancer interceptive strategies aimed at preventing invasive progression. We previously described that DCIS has a more active immune environment with higher fraction of GZMB⁺ CD8⁺ T cells compared to invasive breast tumors (4). We also reported that peripheral blood T cell receptor (TCR) diversity in young (<45) breast cancer patients positively correlated with CD8⁺ T cell infiltration in DCIS, implying a close relationship between peripheral and tumor immunity in preinvasive disease (5). It also implied that boosting peripheral adaptive immunity could be a potential strategy to prevent the progression of preinvasive tumors.

Cancer vaccines represent promising approaches for cancer therapy. In breast cancer, seminal studies have focused on HER2, MUC1, and LALBA as targets (6–9). Among these LALBA has been regarded as a target with minimal potential for off-target effects in vaccination due to its exclusive expression in the lactating breast (10). Recent mammary gland single-cell RNA-seq (scRNA-seq) profiling studies in multiple different species also demonstrated that LALBA expression is restricted to luminal progenitors (LPs) that are thought to serve as normal cell-of-origin of breast cancer (11–15). Moreover, alveolar transformation and upregulation of lactogenic programs is a common feature of mammary tumor initiating cells (16), making LALBA and other milk proteins ideal targets for cancer preventive vaccines. A protein-based LALBA vaccine with a zymosan adjuvant has entered Phase I trials for patients with triple-negative breast cancer (TNBC) of pathologic stage I-III or who had residual disease after standard preoperative systemic therapy (17). Robust immune response determined by frequencies of T cells producing IFN- γ and/or IL-17 in response to recombinant LALBA protein was detected in 12 of 16 patients with high-risk TNBC (18). Meanwhile, mRNA-based neoantigen vaccines in the adjuvant setting have been showing promising results in several cancer types, including melanoma and pancreatic

Significance

Early-stage tumors are the most favorable setting for cancer interception because tumor-suppressive immunity is stronger before cancers become advanced. Here, a prophylactic lipid nanoparticle-encapsulated mRNA vaccine targeting alpha-lactalbumin (LALBA), a protein unique to mammary luminal progenitors (LPs), was developed to test whether strengthening immune response against a putative breast cancer cell-of-origin can suppress tumorigenesis. In outbred Sprague-Dawley rats, vaccination with Lalba mRNA-LNP lowered the incidence of carcinogen-induced mammary tumors and improved both tumor-free and overall survival, without obvious toxicity in normal mammary tissue or other organs. Single-cell transcriptomic analysis further showed that vaccination decreased a proliferative LP population in immunoreactive early epithelial hyperplasia, supporting the potential of prophylactic Lalba mRNA-LNP to block the initiation and progression of early breast neoplastic lesions.

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cancer (19–21). An mRNA vaccine targeting LALBA has also been reported in a preclinical transplantable mammary tumor model within the context of surgical adjuvant therapy (22). However, due to the limited availability of immunocompetent models that recapitulate early breast epithelial lesions including DCIS, the preventive effects of early intervention through vaccination have not been thoroughly investigated.

Rat mammary tumor models have been widely used for breast cancer prevention studies as they reproduce the molecular heterogeneity and hormonal dependency of the human disease (23, 24). We previously described that short-term TGFBR inhibitor treatment of peripubertal rats decreases both estrogen and NMU-induced mammary tumor development in inbred August Copenhagen Irish (ACI) and outbred Sprague-Dawley (SD) rats, respectively (25). scRNA-seq profiling of the mammary glands after treatment revealed that inhibition of TGF- β signaling has a lasting effect on the phenotypes of mammary epithelial progenitors and stem cells (25). Here we utilized the NMU-induced outbred SD rat mammary tumor model that recapitulates preinvasive breast hyperplastic lesions with a robust immune response (25–27) to investigate the effects of prophylactic rat Lalba mRNA-LNP vaccination on mammary tumorigenesis. Targeting LALBA that is specifically expressed in LPs from which tumors thought to originate provides a proof of principle that targeting the putative cell-of-origin of cancer can prevent tumorigenesis.

Results

LALBA Is Dominantly Expressed in Mammary Epithelial LPs/Alveolar-Like Cells. We first explored the expression of *LALBA* mRNA, encoding α -lactalbumin, across tissues. Both bulk and scRNA-seq datasets of human tissue in the Human Protein Atlas revealed that *LALBA* mRNA was almost exclusively expressed in the normal breast tissue (Fig. 1A and SI Appendix, Fig. S1A). Within the normal breast, *LALBA* mRNA was predominantly expressed in a subset of LPs and not in basal (BA) and mature luminal (ML) cells with heterogeneous expression among five subgroups in the human Breast Cell Atlas (14) scRNA-seq data (SI Appendix, Fig. S1B). Expression of *LALBA* mRNA was the highest in the LAMP5 subcluster, which is enriched for proliferation markers, such as *MKI67* and *TOP2A* (14). At the protein level, a subset of human breast epithelial cells was LALBA⁺, and these did not overlap with estrogen receptor- α positive (ER α ⁺) luminal and α -SMA⁺ basal epithelial cells (SI Appendix, Fig. S1C). *LALBA* mRNA is highly expressed in a subset of human basal breast tumors (SI Appendix, Fig. S1D), similar to the ELF5 transcription factor required for mammary alveolar cell development and the expression of milk proteins (28). In the normal rat mammary gland, *Lalba* mRNA was uniquely and ubiquitously expressed in LPs together with other LP markers such as *Elf5*, *Kit*, and *Csn3* mRNAs (Fig. 1B).

Design and In Vitro Evaluation of mRNA-LNP Encoding Rat LALBA. The development of mRNA vaccines was a significant advance as this technology is flexible, and new vaccines can be developed rapidly (29). mRNA modification by pseudouridine and its derivative, N1-methylpseudouridine, increases mRNA stability and reduces innate immune activation compared to unmodified mRNA (30, 31). However, unmodified mRNA is reported to trigger more effective antitumor immunity in some settings because it triggers stronger innate immune activation (32). Thus, we developed both unmodified (U) and N1-methylpseudouridine-modified (M) mRNA-LNP vaccines encoding full-length rat *Lalba* (Lalba-U and Lalba-M). N1-methylpseudouridine-modified firefly luciferase (Luc)-encoding

mRNA-LNP was prepared and utilized as a negative control. Both forms of Lalba mRNA-LNP led to the expression of rat LALBA protein as confirmed by transfection to 293FT cells with the modified mRNA expressing higher levels of rat LALBA protein than the unmodified form (Fig. 1C and SI Appendix, Fig. S2A).

Lalba mRNA-LNP Vaccination Induces Immune Response with No Morphological Changes in the Mammary Gland. To test the effect of Lalba mRNA-LNP vaccination on normal tissues, especially mammary epithelial cells, we intramuscularly injected 10 μ g of the Luc, Lalba-U, and Lalba-M mRNA-LNP or phosphate-buffered saline (PBS) as vehicle control to 23-d-old SD rats three times (Study 1 and 2) or twice (Study 3) with a 2-wk interval between injections (Fig. 1D). Consistent with increased inflammation induced by unmodified mRNA-LNP, we observed scars in all rats in the Lalba-U group at the injection sites, which were not observed in the rats from other groups (SI Appendix, Fig. S2B). The body weight of rats in the Lalba-U group was slightly lower compared to the other groups (SI Appendix, Fig. S2C). We euthanized the animals on day 6 (Study 1), 18 (Study 2), or 12 (Study 3) after the last vaccination and collected peripheral blood and multiple organs to evaluate immune response and potential toxicities. Animals were euthanized following protocols approved by the Dana-Farber Cancer Institute Institutional Animal Care and Use Committee. To evaluate LALBA-specific immune response, we performed IFN- γ ELISPOT assay using blood leukocytes and leukocytes from draining lymph nodes (dLN leukocytes) collected in Study 1. IFN- γ secretion from blood leukocytes was not induced by rat LALBA stimulation, regardless of vaccination group (SI Appendix, Fig. S2D). However, in a subset of samples in the Lalba-M group rat LALBA peptide stimulation was able to induce IFN- γ secretion from dLN leukocytes (SI Appendix, Fig. S2D). To further interrogate T cell responses, we analyzed nonstimulated or rat LALBA peptide-stimulated dLN cells by flow cytometry for selected T cell markers (SI Appendix, Fig. S2E). Among CD4⁺ CD45RC⁺ IRF8⁺ T cells gated as Th1 effector cells (33), a CD25⁺ CD134⁺ early activated population was significantly induced in dLN leukocytes from the Lalba-M group after rat LALBA peptide stimulation compared to the PBS group (Fig. 1E). These populations were slightly but not significantly induced in dLN leukocytes from the Luc and Lalba-U groups, potentially due to anti-CD28 antibody costimulation (Fig. 1E). In contrast, CD8⁺ T cell activation based on increased CD25 expression was not induced by rat LALBA peptide stimulation in dLN leukocytes from any vaccination groups (Fig. 1E). These results suggest that the Lalba-M mRNA-LNP elicits antigen-specific Th1 response. However, the possibility that we could not detect CD8⁺ T cell responses due to the assay conditions used cannot be excluded.

To evaluate whether vaccination induced memory T cells, we performed flow cytometry analyses of blood leukocytes collected in Study 2. We observed a significant increase of memory-like CD45RC⁺ CD4⁺ T cells and CD45RC⁺ CD8⁺ T cells (34) in the Lalba-U group compared to control PBS with a similar trend in the Lalba-M group for CD45RC⁺ CD4⁺ T cells (SI Appendix, Fig. S2F). However, this experiment could not address whether this was due to antigen-specific immune response or nonspecific immune reaction caused by the unmodified mRNA.

Next, we performed ELISA for anti-LALBA IgG, IgA, and IgM to measure antigen-specific humoral immune response using plasma at the endpoint of Study 2. Anti-LALBA IgG was only detectable in samples obtained from the Lalba-M group (Fig. 1F). In contrast, anti-LALBA IgA was induced in both Lalba-U and Lalba-M groups (Fig. 1G). There was no difference in anti-LALBA IgM titers between control groups (i.e., PBS or Luc) and Lalba

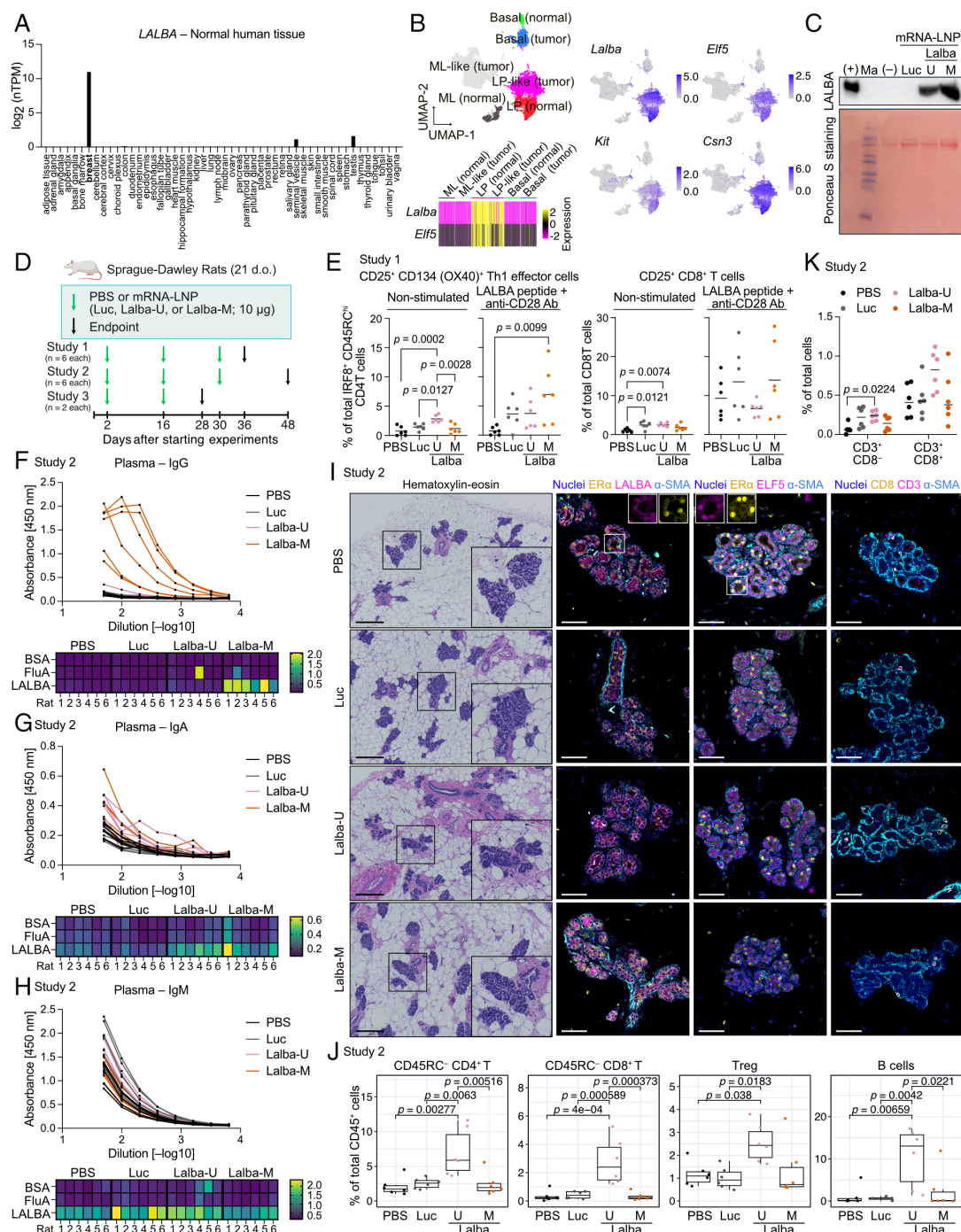


Fig. 1. Characterization of rat LALBA mRNA-LNP vaccine. (A) Plots depicting the expression of LALBA mRNA across human tissues. Consensus RNA data from The Human Protein Atlas was used. nTPM, normalized transcript per million. (B) UMAP of mammary epithelial cell scRNA-seq data colored by cell type or the expression of the indicated LP cell-specific markers. Heatmap indicating expression of LALBA and Elf5 mRNA in randomly selected 100 cells from each cluster (Bottom Left). (C) Immunoblot for LALBA protein in supernatants of 293FT cells transfected with Luc (Luc), and unmodified (U) and modified (M) LALBA mRNA-LNP. Recombinant rat LALBA protein (+) and untransfected cells (-) were used as positive and negative controls, respectively. Molecular weight marker (Ma). Membrane was stained by Ponceau S for loading control. (D) Schematic of mRNA-LNP vaccination experimental design. (E) Flow cytometry analysis of dLN leukocytes collected at the endpoint of Study 1. Cells were either nonstimulated or stimulated with rat LALBA peptide and anti-CD28 antibody. Plots depicting the fraction of the indicated subpopulations. One-way ANOVA with Tukey's test. (F-H) ELISA for anti-LALBA IgG (F), IgA (G), and IgM (H) in the plasma of rats in different vaccination groups. (Top) Line plot showing immunoglobulin binding to rat LALBA protein. (Bottom) Heatmap showing immunoglobulin binding to BSA, Flu A protein, and rat LALBA protein. Plasma was diluted 1:50. (I) Representative images of histology (Scale bar, 200 µm) and immunofluorescence for the indicated markers (Scale bar 50 µm.) (J) Flow cytometry analysis of mammary glands collected at endpoint. Plots depicting the fraction of the indicated subpopulations within CD45⁺ cells. One-way ANOVA with Tukey's test. (K) Plot depicting the quantification of the indicated cell populations in immunofluorescent images from panel I. One-way ANOVA with Tukey's test. Each dot in E, J, and K, and each line in F-H represents one animal.

mRNA-LNP groups (i.e., LALBA-U and LALBA-M) though the ELISA signal was higher in rat LALBA protein-coated plates than bovine serum albumin (BSA) and Influenza A viral Hemagglutinin (Flu A)-coated ones and it was dilution-dependent (Fig. 1H). Induction of anti-LALBA IgG by the LALBA-M mRNA-LNP was also observed in Study 3 (SI Appendix, Fig. S2G). These results indicate different degrees of antigen-specific humoral immune response induced by the LALBA-U and LALBA-M mRNA-LNPs, IgA induction by the LALBA-U mRNA-LNP, while IgG and IgA induction by the LALBA-M mRNA-LNP. Overall, LALBA-M mRNA-LNP was more potent in inducing antigen-specific immune responses than LALBA-U mRNA-LNP.

We next investigated changes in tissue morphology and immune phenotypes in vaccinated rats. No noticeable histologic differences were observed in the mammary gland, liver, lung, heart, kidney, and brain among groups collected in Study 2, or in cytokine expression profiles in plasma collected in Study 1 and 2 (Fig. 1I and SI Appendix, Fig. S2H and I). To evaluate potential changes in LALBA⁺ mammary epithelial cells, we performed immunofluorescence for LALBA. We found that a major fraction of intralobular luminal cells was positive for LALBA (Fig. 1I), but we could not conclusively identify LALBA⁺ LP cells by immunofluorescence due to LALBA being a highly abundant secreted protein. Thus, we utilized staining for the ELF5 transcriptional factor to detect

LP cells (Fig. 1I), since based on our scRNA-seq data *Elf5* mRNA was specifically expressed in LALBA⁺ LPs (Fig. 1B). Immunofluorescence for ER α , ELF5, and α -SMA demonstrated that vaccination did not affect the cellular composition and architecture of the mammary epithelium (Fig. 1I). To evaluate immune cell composition in the mammary tissues, we performed flow cytometry analysis using dissociated tissues. We detected a significant increase in T and B lymphocytes in the mammary tissues of rats from the Lalba-U group (Fig. 1J and SI Appendix, Fig. S1J). However, this was not corroborated by immunofluorescence of the mammary glands for CD3⁺ and CD8⁺ T cells (Fig. 1I and K). These data suggest that the observed increase of T and B cells by flow cytometry in mammary tissues of rats in the Lalba-U group is not likely to be a LALBA-specific immune response. Overall, neither Lalba-U nor Lalba-M mRNA-LNP induced noticeable changes in normal mammary gland morphology and cellular composition in virgin rats at the time points analyzed.

Lalba mRNA-LNP Vaccination Suppresses NMU-Induced Mammary Tumorigenesis. We next evaluated the effect of Lalba mRNA-LNP vaccination on NMU-induced mammary tumorigenesis. 23-d-old rats were injected with Luc, Lalba-U, or Lalba-M mRNA-LNPs twice intramuscularly with a 2-wk interval. 10 d after the last vaccination we injected two doses of NMU via intraperitoneal injection on two consecutive days (Fig. 2A). Animal body weight was not affected by vaccination (SI Appendix, Fig. S3A). To evaluate tumor-free survival, we applied the Fleming-Harrington weighted long-rank test, a statistical method designed for cancer immunotherapy studies where delayed treatment effects often cause nonproportional hazards and reduced power for standard log-rank tests (35, 36). Tumor-free survival was significantly improved by Lalba-M mRNA-LNP vaccination compared to the Luc group, while no significant difference was detected between the Luc and Lalba-U groups (Fig. 2B). We also evaluated the total numbers of palpable tumors over time using a generalized linear mixed model. While the effect of Lalba-U vaccination on tumor number was moderate, Lalba-M mRNA-LNP vaccination reduced the number of tumors per rat compared to the Luc group (Fig. 2C). When any of the rats reached the ethical endpoint, all rats were euthanized to enable comparison of tumors at the same time point. The total number of collected tumors was not always the same as the number of palpable tumors since some adjacent tumors merged into one large tumor and nonpalpable tumors were also detected in several animals. The weight of individual mammary tumors was significantly lower in both Lalba-U and Lalba-M groups compared to the Luc group (Fig. 2D). In addition to macroscopic tumors, microscopic DCIS-like lesions were observed in the mammary glands of 2 out of 6 rats in the Luc, 1 out of 6 rats in the Lalba-U, and none of the six rats in the Lalba-M group (SI Appendix, Fig. S3B). Vaccination did not alter tissue morphology and CD8⁺ T cell infiltration in normal mammary glands based on histologic and immunofluorescence analyses (SI Appendix, Fig. S3B and C), consistent with results in the cohort with no NMU injection. Microscopic DCIS in one rat from the Lalba-U group exhibited higher CD8⁺ T cell infiltration compared to the one from the Luc group (SI Appendix, Fig. S3C). We also observed high focal accumulation of CD8⁺ T cells in one mammary gland from one rat in the Lalba-M group (SI Appendix, Fig. S3C).

We then characterized tumor epithelial cells to test whether vaccination influenced the cellular composition of tumors. Histological analysis revealed that 2 out of 15 in the Luc and 4 out of 13 tumors in the Lalba-M group displayed fibroadenoma-like morphology, which has been observed with low frequency among NMU-induced tumors (37) (SI Appendix, Fig. S3D). We therefore omitted

fibroadenoma-like tumors from the downstream analysis as they are not epithelial origin. To evaluate the effect of vaccination on LP-like cells within tumors, we performed immunofluorescence for ER α , LALBA, and α -SMA or ER α , ELF5, and α -SMA (Fig. 2E and F). We observed variable frequencies of LALBA⁺ and ELF5⁺ cell populations across tumors (Fig. 2E and F). Lower fraction of ELF5⁺ cells showed a slight nonsignificant correlation with higher tumor weight in the Luc group ($R^2 = 0.258$, P -value = 0.0766), consistent with the fact that NMU-induced mammary tumors are mostly ER α ⁺ (Fig. 2G). Considering that Lalba mRNA-LNP vaccination reduced tumor weight, the change in the relative fraction of ELF5⁺ LP-like cells was evaluated by linear regression analysis between cell population ratio and tumor weight. There were no differences in slope (P -value = 0.968) and intercept (P -value = 0.355) of the regression line between the Luc and Lalba-M groups (Fig. 2G), suggesting that the Lalba-M mRNA-LNP did not decrease the frequency of ELF5⁺ LP-like cells in residual macroscopic tumors. However, there were significant differences in intercept (P -value = 0.0022) of the regression line between the Luc and Lalba-U groups (Fig. 2G), implying the reduction of LP-like cells in residual tumors of the Lalba-U group. The relative fraction of ER α ⁺ and α -SMA⁺ cells in tumors from Luc and Lalba-U groups was heterogeneous and exhibited no clear correlation with tumor weight (Fig. 2G). In contrast, higher tumor weight significantly positively correlated with higher ER α ⁺ and lower α -SMA⁺ cell ratio in the Lalba-M group (ER α vs. tumor weight, $R^2 = 0.696$, P -value = 0.0052; α -SMA vs. tumor weight, $R^2 = 0.480$, P -value = 0.0386) (Fig. 2G). These results imply a more dominant ER-driven luminal tumor growth in the Lalba-M compared to the Luc or Lalba-U group.

We next explored the effect of vaccination on immune phenotypes. We could not robustly detect anti-LALBA IgG, IgA, and IgM in the plasma of vaccinated rats at endpoint (SI Appendix, Fig. S3E). This might reflect, at least in part, the formation of antigen/antibody complexes (LALBA is a secreted protein) that mask detection in the ELISAs or deposition of the antibodies in the incipient tumor tissues. We then performed flow cytometry analyses of dissociated tumors to evaluate the tumor immune microenvironment (Fig. 2H and I and SI Appendix, Fig. S3F). We found a significant decrease in CD163⁺ macrophages in both Lalba-U and Lalba-M groups and Tregs in the Lalba-U group compared to the Luc group (Fig. 2H). These cells showed a significant positive correlation with tumor weight, consistent with their presumed immunosuppressive roles (Fig. 2I). The difference in CD8⁺ T cell fraction between Lalba and Luc groups was not significant in part due to the higher relative fraction of CD8⁺ T cells in smaller tumors in the Luc group (Fig. 2H and SI Appendix, Fig. S3G). These results suggest that tumors in the Lalba mRNA-LNP groups have a tumor-suppressive immune microenvironment, consistent with the lower tumor weight compared to the Luc mRNA-LNP group. Overall, both Lalba-U and Lalba-M mRNA-LNPs suppressed tumor progression, with Lalba-M mRNA-LNP showing a greater suppression of tumor incidence.

Lalba-M mRNA-LNP Vaccination Prolongs Both Tumor-Free and Overall Survivals. We further evaluated the cancer-preventive efficacy of the Lalba mRNA-LNP vaccination in a longer-term experiment. We focused on the Lalba-M mRNA-LNP due to its positive impact on antigen-specific immune responses in our previous studies. Given the variability in vaccination responses attributed to the outbred nature of SD rats, we conducted three independent experiments with varying numbers of rats and NMU administration (Fig. 3A). Notably, the vaccination did not affect the body weight of the rats (SI Appendix, Fig. S4A). Meta-analyses

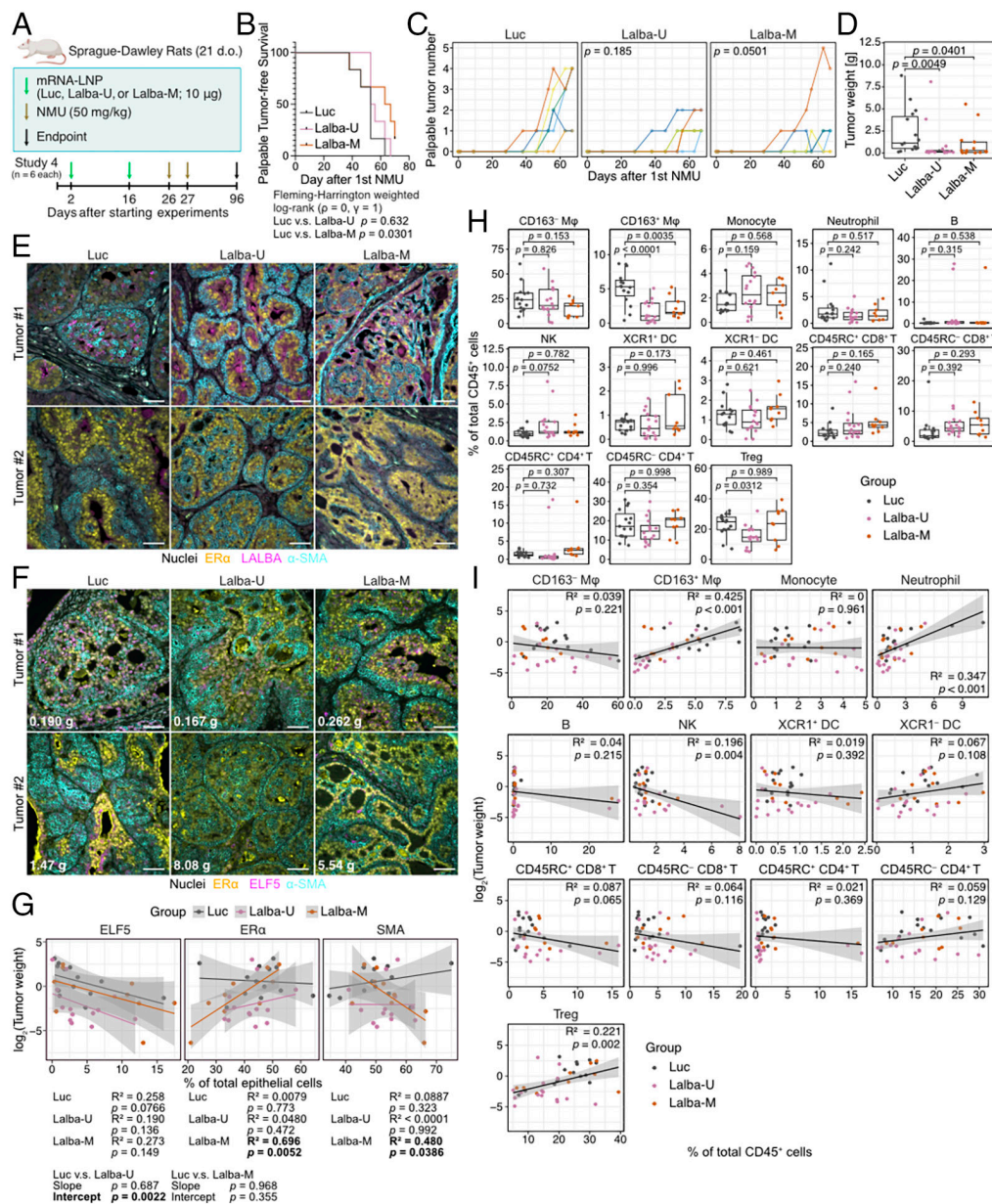


Fig. 2. Prevention of NMU-induced mammary tumors by rat Lalba mRNA-LNP. (A) Schematic outline of experimental design with two vaccinations and two NMU injections. (B) Kaplan-Meier plot depicting palpable tumor-free survival of rats. (C) Graph depicting palpable tumor numbers per rat in different groups. Generalized linear mixed model with Dunnett's test. (D) Box plot depicting tumor weight at endpoint. Kruskal-Wallis test with Dunnett's test. (E) Representative images of immunofluorescence for ER α , LALBA, and α -SMA in mammary tumors. (Scale bar, 50 μ m.) (F) Representative images of immunofluorescence for ER α , ELF5, and α -SMA in mammary tumors. (Scale bar, 50 μ m.) (G) Correlation plot between log2-transformed tumor weight and the percentage of ELF5 $^+$, ER $^+$, or α -SMA $^+$ cells over epithelial cells. Linear regression analysis. (H and I) Flow cytometry analysis of NMU-induced mammary tumors. (H) Graph depicting the fraction of the indicated cell types in CD45 $^+$ cells. One-way ANOVA with Dunnett's test. (I) Correlation plot between log2-transformed tumor weight and the fraction of the indicated subpopulations within CD45 $^+$ cells. Linear regression analysis. Each dot in D and each line in G-I represents one animal. Each dot in G-I represents one tumor.

of these experiments revealed that the Lalba-M mRNA-LNP vaccination significantly extended both tumor-free and overall survival (Fig. 3 B and C). At the endpoint of each experiment, we collected tumors and mammary glands to inspect microscopic lesions. In Study 5, while the incidence of palpable tumors was the same between the Lalba-M and Luc groups (10 out of 12 rats in each group), micro-DCIS was present in all remaining rats in the Luc group but absent in the Lalba-M group (Fig. 3D and SI Appendix, Fig. S4B). In Study 7, neither palpable tumors nor micro-DCIS were detected in 9 out of 12 rats in the Lalba-M group, compared to 6 out of 12 in the Luc group (Fig. 3D and SI Appendix, Fig. S4C).

Subsequently, we assessed the impact of Lalba-M mRNA-LNP on tumor growth (Fig. 3E and SI Appendix, Fig. S4D). Considering the variable tumor latency in the NMU-induced tumorigenesis model, tumors in Studies 5 and 6 were categorized into three subgroups based on latency (T1, T2, and T3) to compare growth rates (Fig. 3E). Among each time interval, growth rates of tumors initiated in the mid-term (T2) in the Lalba-M group were significantly lower than those in the Luc group (Fig. 3F). This finding

suggests that the Lalba-M mRNA-LNP vaccination exerts optimal tumor-suppressive effects on tumors initiated during this period.

We next characterized the immune microenvironment of tumors developing post-Lalba-M mRNA-LNP vaccination. Our previous studies showed that NMU-induced mammary tumors exhibit a heterogeneous immune microenvironment, with varying latency periods, and that tumor growth rate and size are correlated with differences in the tumor immune microenvironment (4, 25). Therefore, we investigated potential growth rate-dependent differences in the immune microenvironment through flow cytometry analyses of palpable tumors. We analyzed the tumor from each rat that first reached humane endpoint, typically also the first tumor detected. Among analyzed cell population, the fractions of XCR1 $^+$ DCs and CD45RC $^+$ CD4 $^+$ memory-like T cells were significantly inversely correlated with tumor growth rate in both Studies 5 and 6 (Fig. 3G). In tumors initiated in the mid-term (T2), CD45RC $^+$ CD4 $^+$ memory-like T cells were significantly more abundant in tumors from the Lalba-M group compared to those from the Luc group (Fig. 3H and SI Appendix, Fig. S4E). These results suggest a substantial role for CD4 $^+$ T cells in the immunosuppression of

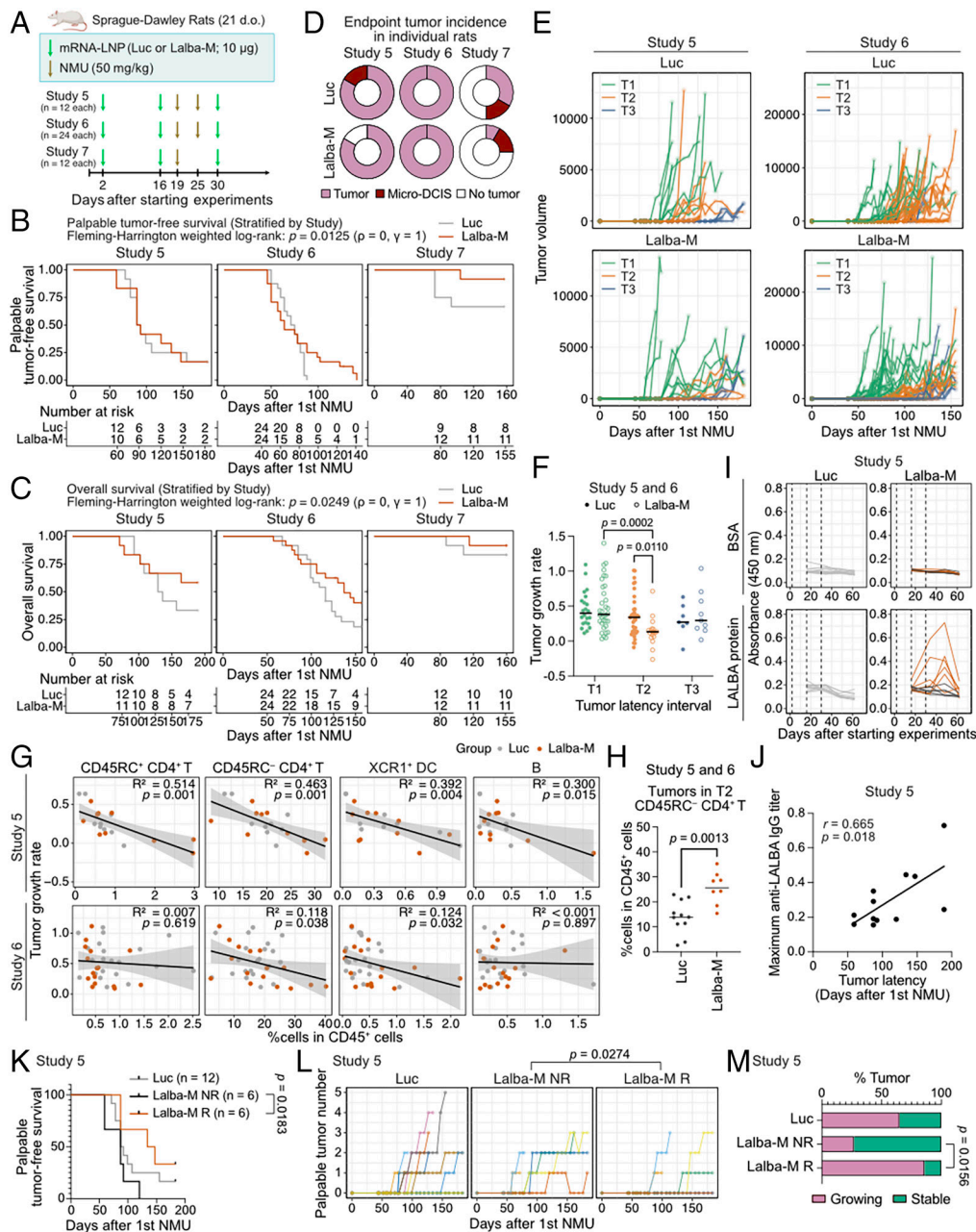


Fig. 3. Analysis of mammary tumor-preventive and tumor-suppressive effects by rat Lalba mRNA-LNP. (A) Schematic of experimental design. (B and C) Kaplan-Meier plot depicting palpable tumor-free (B) and overall (C) survival of rats in each study. (D) Pie chart depicting the ratio of palpable tumor-bearing, micro-DCIS-bearing, and tumor-free rats in the indicated groups and studies. (E) Plot depicting individual tumor volumes in the indicated vaccination groups and studies. Colors represent the timing of palpable tumor initiation (tumor latency). Study 5; T1, day 59 to 98; T2, day 99 to 137; T3, day 138 to 176. Study 6; T1, day 46 to 76; T2, day 77 to 107; T3, day 108 to 137. (F) Plot depicting tumor growth rates of individual tumors. One-way ANOVA with Tukey's test. (G) Flow cytometry of cells in the first palpable NMU-induced mammary tumors in each rat collected at endpoint. Plot depicting the fraction of the indicated subpopulations within CD45⁺ cells and average tumor growth rate. Linear regression analysis. (H) Plot depicting the percentage of CD45RC⁺ CD4⁺ T cells within the CD45⁺ population in tumors classified as T2. Welch's *t* test. (I) ELISA for anti-LALBA IgG in the plasma of rats at different time points. Graph showing immunoglobulin binding to BSA or rat LALBA protein. Orange and black lines in the Lalba-M group represent IgG-responder and IgG-nonresponder, respectively. (J) Plot depicting the correlation between tumor latency and maximum anti-LALBA IgG titer in individual animals at any of the time points. Pearson's *R*. (K) Kaplan-Meier plot depicting rat tumor-free survival. NR, nonresponder. R, Responder. Log-rank test. (L) Graph depicting palpable tumor numbers per rat in the indicated groups. Generalized linear mixed model. (M) Graph depicting the ratio of growing and stable tumors in each group. Chi-square test. Each dot in J and each line in I and L represents one animal. Each dot in F-H and each line in E represents one tumor.

NMU-induced tumors and align with the antigen-specific Th1 responses induced by Lalba-M mRNA-LNP vaccination.

Lalba-M mRNA-LNP Vaccination Effectively Inhibits the Progression of Immunoreactive Tumor Subsets. We further correlated the peripheral immune responses elicited by the Lalba-M mRNA-LNP vaccination with tumor outcomes. To achieve this, we collected PBMCs and plasma at various time points to monitor both antigen-specific and nonspecific peripheral immune responses. Plasma cytokine profiling in vaccinated rats indicated that the vaccination was unlikely to trigger autoimmune-related cytokines (SI Appendix, Fig. S4F). We then evaluated the antigen-specific adaptive immune response using the IFN- γ ELISPOT assay. However, we were unable to detect an antigen-specific adaptive immune response, possibly due to the low frequency of antigen-specific T cells in the blood (SI Appendix, Fig. S5A).

Subsequently, we assessed the antigen-specific humoral immune response. Despite administering three vaccinations, IgG binding

affinity to the rat LALBA protein was detected in the plasma of only 6 out of 12 rats at a minimum of one of the assessed time points (Fig. 3I). This response was observed exclusively in IgG, not in IgM or IgA, and was only noted in Study 5, not in Study 6 (SI Appendix, Fig. S5B and C), likely due to interindividual variability in immune response within this outbred rat strain. Notably, in Study 5, a high anti-LALBA IgG titer in plasma was significantly associated with prolonged tumor latency (Fig. 3J). Consequently, we divided the twelve animals in the Lalba-M group in Study 5 into IgG-responders (Lalba-M R; *n* = 6) and IgG-nonresponders (Lalba-M NR; *n* = 6) to assess their tumor outcomes. The palpable tumor-free survival was significantly extended in the Lalba-M R group compared to the NR group (Fig. 3K). Tumor incidence was also significantly reduced in the Lalba-M R compared to the NR group (Fig. 3L). The composition of normal mammary epithelial cells was consistent across groups (SI Appendix, Fig. S5D). We assessed tumor growth rates in each group, categorizing tumors as either growing or stable (Materials and Methods

and *SI Appendix, Fig. S5E*). Notably, two out of six rats in the Lalba-M R group were tumor-free; among those with tumors, a significantly lower proportion of tumors were classified as stable in the Lalba-M R group compared to the NR group (*Fig. 3M and SI Appendix, Fig. S5E*). These findings indicate that a robust immune response to Lalba-M mRNA-LNP is associated with a reduced number of tumors, although residual tumors exhibit growth rates comparable to those in control groups. Overall, Lalba-M mRNA-LNP vaccination may effectively target immunoreactive subsets of NMU-induced tumors.

Lalba-M mRNA-LNP Vaccination Limits the Proliferation of Basal-Like Tumor Epithelial Cells. We next evaluated the effect of Lalba-M mRNA-LNP vaccination on the cellular compositions and phenotypes of NMU-induced mammary tumors by scRNA-seq on growing tumors with comparable sizes from each group (*Fig. 4A and B and SI Appendix, Table S1*). Although there was considerable variability, no consistent differences were observed in the relative fractions of major cell types based on vaccination status (*Fig. 4C*). Subclustering analysis of tumor epithelial cells revealed that *Lalba* mRNA expression was not reduced in LP-like cells in tumors from the Lalba-M groups (*SI Appendix, Fig. S6A and B*). The number of differentially expressed genes (DEGs) was highest in the basal-like cancer cell population when comparing the Lalba-M NR and Lalba-M R groups, followed by CD4⁺ T cells (*Fig. 4D*). Louvain clustering analysis of basal-like cancer cells revealed enrichment of distinct clusters in each sample among the Luc, Lalba-M NR, and the Lalba-M R groups (*Fig. 4E and F*). Cells within each cluster displayed varying expression levels of cytokine and chemokine ligands and receptors, contraction-associated markers, extracellular matrix components, and differentiation markers (*SI Appendix, Fig. S6C and D and Dataset S1*). While most basal-like cells in all Lalba-M R samples were part of cluster 1 or 3, different clusters were enriched in each sample of the Luc and Lalba-M NR groups (*Fig. 4E and F*). To explore the functional implications of gene expression differences, we conducted gene set enrichment analysis comparing the Lalba-M NR and R groups. Basal-like cancer cells from the Lalba-M NR group significantly enriched gene signatures associated with cell proliferation, such as MYC_TARGETS and G2M_CHECKPOINT while no pathways were enriched in the Lalba-M R group (*Fig. 4G and Dataset S1*). The same level of enrichment was not observed when comparing LP-like or ML-like cancer cells except the enrichment of MYC_TARGETS_V1 in ML-like cells from the Lalba-M NR group (*Fig. 4G and Dataset S1*). In contrast, inflammation and metabolism-related signaling pathways were enriched in the LP-like and ML-like cancer cell comparisons, while epithelial_mesenchymal_TRANSITION, MTORC1_SIGNALING, and MYOGENESIS were commonly enriched in all three cell populations (*Fig. 4G and Dataset S1*).

To comprehensively evaluate all growing tumors from each vaccination group, we performed flow cytometry analysis for epithelial markers. We found a significantly higher relative ratio of proliferative Ki67⁺ cells in CD29⁺EpCAM⁺ basal-like cancer cells within growing tumors in the Lalba-M NR compared to the Lalba-M R group (*Fig. 4H*). Conversely, the difference in CD24⁺EpCAM⁺ luminal (LP-like and ML-like) cancer cells in growing tumors was not significant (*Fig. 4H*). A strong positive correlation was observed in the relative fraction of Ki67⁺ cells within CD29⁺EpCAM⁺ and CD24⁺EpCAM⁺ cells when analyzing all tumors with varying growth statuses across each vaccination group (*Fig. 4I*). The Lalba-M R group exhibited the highest R value, although the differences in R values among the vaccination groups were not statistically significant (Luc vs. Lalba-M R, $P = 0.1237$; Lalba-M NR vs. Lalba-M R, $P = 0.1176$) (*Fig. 4I*). These results

imply that tumor epithelial cells are more homogenous in the Lalba-M R group and that Lalba mRNA-LNP vaccination may preferentially affect basal-like cancer cell populations.

We also examined immune cell populations focusing on CD4⁺ T cells and myeloid cells due to their higher numbers of DEGs compared to other cell types. CD4⁺ T cells were categorized into Treg, effector or memory, IL17⁺, naïve, and proliferative subpopulation (*SI Appendix, Fig. S6E and F*), which showed no consistent differences in cellular composition among the vaccination groups (*SI Appendix, Fig. S6G*). DEG analysis of each CD4⁺ T cell subpopulation between the Lalba-M NR and Lalba-M R groups revealed that Treg had the most DEGs (*SI Appendix, Fig. S6H*). Milo neighborhood analysis of Treg subpopulation revealed that the abundance of *Tnfrsf4*-high (CD134) and *Tnfrsf18*-high (GITR) Tregs was lower in the Lalba-M R group than in the Lalba-M NR group (*SI Appendix, Fig. S6J and K*). Among myeloid cells there were no consistent differences in cell populations among vaccination groups (*SI Appendix, Fig. S6L–N*). Overall, these results implied modest differences in the tumor immune microenvironments of growing tumors between the Lalba-M R and NR groups.

Lalba-M mRNA-LNP Vaccination May Suppress Immunoreactive Early Lesions. We next investigated the impact of Lalba-M mRNA-LNP vaccination on the earliest stages of mammary tumor development. The Luc and Lalba-M mRNA-LNP, along with a PBS vehicle, were administered to 23-d-old SD rats three times at 2-wk intervals, coinciding with two NMU injections (*Fig. 5A*). We then collected all animals at the same time when the first palpable tumor was detected in one of the rats at day 78 (*Fig. 5A and SI Appendix, Table S1*). At the endpoint, we were unable to consistently detect anti-LALBA IgG, IgA, and IgM in the plasma of vaccinated rats (*SI Appendix, Fig. S7A*). Following the collection of mammary tissues, histological analysis of hematoxylin-eosin-stained mammary gland sections was performed. Mammary tissues were classified as hyperplastic (score 2), partially hyperplastic (score 1), or normal mammary gland (score 0) (*Fig. 5B and C and SI Appendix, Table S1*). The hyperplasia scores were significantly lower in the Lalba-M group compared to the control groups (PBS and Luc) (*Fig. 5B and C*). Notably, one rat in the Lalba-M group exhibited micro-DCIS (*Fig. 5C*).

To investigate the impact of Lalba-M mRNA-LNP vaccination on hyperplastic early lesions, we performed scRNA-seq on cells from mammary tissues collected at the study endpoint (*Fig. 5D and SI Appendix, Fig. S7B and C*). Due to the substantial presence of endothelial cells, pericytes, and fibroblasts, likely resulting from the inclusion of subcutaneous tissues surrounding the mammary glands, these cell populations were excluded from further analysis (*SI Appendix, Fig. S7B*). Clustering analysis of epithelial cells identified distinct histology-specific clusters within each mammary epithelial cell type, including ML, LP, Basal, and secretory basal cells (SBC) (*Fig. 5D–F*). Notably, samples with score 2 hyperplasia and DCIS were the most distinct. The expression levels of *Lalba* and *Elf5* in LP cells were not reduced following Lalba-M mRNA-LNP vaccination, indicating that the vaccination does not diminish normal LALBA⁺ LP cells (*Fig. 5G*). Score 2 hyperplasia was characterized by the presence of the hyperplasia-specific LP cell subcluster. These populations were present in the PBS and Luc mRNA-LNP groups but absent in any samples from Lalba-M mRNA-LNP-vaccinated animals (*Fig. 5E and Dataset S1*). This hyperplastic LP cluster showed enrichment for multiple GSEA hallmark gene signatures related to cell proliferation and contained a larger population of cells with proliferation markers compared to the normal LP cluster (*Fig. 5H and I and SI Appendix, Fig. S7D*). A direct comparison of transcriptomic differences in

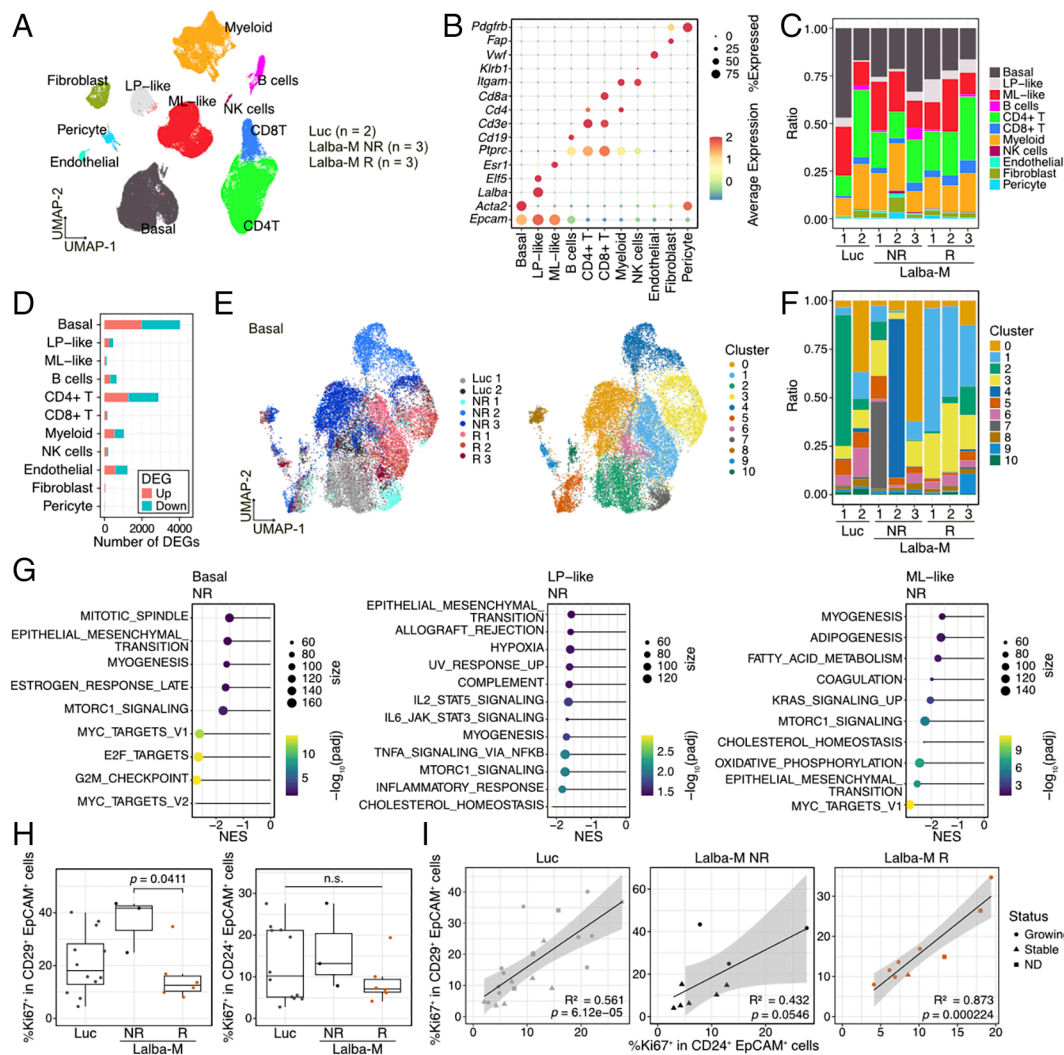


Fig. 4. Single-cell RNA-seq of mammary tumors. (A) UMAP plot of all cell types from all tumors combined. (B) Dot plot depicting the expression of known cell type-specific genes in each cluster. (C) Stacked bar plots depicting the relative cell fractions in each tumor. (D) Stacked bar plots depicting the number of up or downregulated DEGs in each cluster. (E) UMAP plots of basal epithelial cells colored by tumor sample (Left) and Louvain clustering (Right). (F) Stacked bar plot depicting the relative cell fractions in each tumor. (G) Gene set enrichment analysis of basal-like, LP-like, and ML-like cells comparing Lalba-M responders and nonresponders. Only significantly enriched signatures (adj. P -value ≤ 0.05) are indicated. (H) Flow cytometry analysis of growing mammary tumors collected at endpoint. Box plot depicting Ki67⁺ cells within CD29⁺ EpCAM⁺ (Left) or CD24⁺ EpCAM⁺ (Right) subpopulations. One-way ANOVA with Tukey's test. (I) Scatter plots depicting the correlation between the fraction of Ki67⁺ cells within CD24⁺ EpCAM⁺ and CD29⁺ EpCAM⁺ cell populations. All tumors from all groups were included in the analysis. Linear regression analysis. Each dot in H and I represents one tumor.

LP cells between the Luc and Lalba-M groups also revealed enrichment of G2M_checkpoint and Mitotic_spindle in the Luc group (SI Appendix, Fig. S7E and Dataset S1). These findings suggest a reduced frequency of proliferative LP cells in the Lalba-M vaccination group than in controls.

Last, we assessed immune phenotypes of mammary tissues to explore potential mechanisms underlying the preferential reduction of hyperplastic lesions in the Lalba-M group. Flow cytometry and scRNA-seq analysis of mammary tissues revealed numerous differences in immune cell abundance in mammary tissues of different groups as exemplified by significantly lower ratio of CD45RC⁺ CD4⁺ T cells in the Lalba-M group compared to the Luc group by flow cytometry (SI Appendix, Fig. S7F–L). However, there was no consistent difference between the Lalba-M group and the PBS groups (SI Appendix, Fig. S7F–L). These results suggested that Lalba-M mRNA-LNP-specific alteration of immune cell population was minimal in mammary tissues at this time point. We then explored the association between hyperplasia and T cell infiltration in mammary gland focusing on the abundance of LP cells and Ki67⁺ mammary epithelial cells. Immunofluorescence analysis revealed that hyperplasia score 1 or 2 mammary glands exhibited higher LP cell ratio and CD8⁺ cell infiltration than score 0 (Fig. 5J and K and SI Appendix, Fig. S7M). Of note, there was a positive correlation between CD8⁺ T cell infiltration and the abundance of ELF5⁺ or Ki67⁺ epithelial cells in the mammary gland while CD3⁺ T cells were less correlative (Fig. 5L). Considering this correlation, we performed cell-to-cell communication analysis of these

two cell types in scRNA-seq data (i.e., LP cells and T/NK/ILC cells) using the NichNet algorithm to find enriched pathways in hyperplasia (score 2) over normal (score 0) mammary tissues (SI Appendix, Fig. S7N and O). The top 10-enriched pathways from LP cells to T/NK/ILC cells in score 2 samples included signaling pathways known to enhance T cell activation, as exemplified by *Il33* (SI Appendix, Fig. S7N and O) (38). Overall, these results highlight the immunoreactive features of LP-rich hyperplastic early lesions, and that Lalba mRNA-LNP vaccination may predominantly suppress immunoreactive early mammary epithelial lesions.

Discussion

As the incidence of breast cancer is growing worldwide, the need for prophylactic intervention is becoming an even greater issue. Cancer prevention studies have been challenging due to the higher level of safety requirements for interventions prior to disease onset. Vaccines are some of the safest medicines ever produced, and vaccine-mediated targeting of progenitors driving tumor initiation and progression is an attractive approach for breast cancer prevention. Here, we provide proof-of-concept in a rat model of breast cancer that prophylactic mRNA-LNP vaccine targeting LALBA, a LP marker, can prevent the development of full-blown mammary tumors in animals that mounted a measurable immune response to vaccination with no discernible side effects.

We employed two different formats of Lalba mRNA-LNP, one containing N1-methylpseudouridine instead of uridine and one

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without nucleoside modification. While nucleoside-modified mRNA is typically favored for infectious disease vaccines due to its more favorable safety profile and superior ability to induce humoral immune responses (39), some data suggest that unmodified mRNA-LNP may offer advantages as a cancer therapeutic vaccine due to its ability to induce a robust type I interferon immune response (40). In our study, we identified notable differences in the effects of the two Lba mRNA-LNP formulations on tumor progression and the immune microenvironment. The Lba-U mRNA-LNP elicited a more pronounced antigen-nonspecific immune reaction compared to the Lba-M mRNA-LNP. Conversely, the Lba-M mRNA-LNP induced a robust antigen-specific immune response, characterized by a Th1 response and LALBA-specific IgG antibody production. Vaccination with Lba-M mRNA-LNP significantly extended tumor-free survival, unlike Lba-U mRNA-LNP. However, in cases where tumors did develop, those in both Lba-U and Lba-M group were smaller than those in the Luc group. Tumors that emerged after a specific interval postvaccination in the Lba-M group exhibited reduced growth rates compared to the control Luc group, correlating with a higher proportion of non-Treg CD4⁺ T cells. Th1 effector cells may enhance antigen-specific or bystander CD8⁺ T cell activation, thereby facilitating antitumor immune responses during both tumor growth and immunotherapy. These findings underscore the selective impact of Lba-M mRNA-LNP on tumors with an active immune microenvironment. Further investigation is required to determine whether the vaccination enhances the activation and migration of antigen-specific Th1 effector cells to the tumor site.

Enhancing the identification of actionable points in vaccination and its implications for tumor subtypes remains crucial. The absence of hyperplastic and immunoreactive early lesions, which are heavily infiltrated by CD8⁺ T cells, in rats of the Lba-M group suggests that vaccination-primed immunity may target these lesions or even earlier stages of tumor initiation. However, technical challenges associated with the comprehensive analysis of small, microscopic lesions throughout mammary gland make it difficult to definitively confirm this possibility. NMU-induced mammary tumors are estrogen receptor-positive (ER⁺) and estrogen-dependent based on prior studies testing the effects of oophorectomy and treatment with antihormonal therapies (41). Nonetheless, our prior RNA-seq profiling of a large cohort revealed the presence of both hormone-receptor-positive luminal and more basal tumors with low ER levels (26). Our scRNA-seq analysis of growing tumors in Lba-M mRNA-LNP-vaccinated animals identified basal-like cells as the most affected by vaccination, exhibiting a marked reduction in proliferation compared to more luminal cancer cells. Conversely, in hyperplastic lesions, LP-like cells showed the most pronounced vaccine effect, followed by basal-like cells. These findings suggest several possibilities: 1) basal-like cells are derived from LP-like cells, 2) LP-like cells may regulate basal-like cell proliferation, or 3) basal-like cells may be primarily affected by the bystander immune effects induced by the vaccination. Dissecting these relationships would necessitate the engineering of barcoded rats, allowing for the tracking of tumor evolution at the single-cell level.

We employed LALBA as a model antigen and mRNA-LNP as a platform, recognizing the significant potential of prophylactic vaccines encoding alternative antigens. This interest is underscored by the demonstrated efficacy of several cancer vaccines in patients with DCIS. A phase I clinical trial of MUC1 peptide vaccine (NCT06218303) is currently enrolling postmenopausal patients with newly diagnosed, hormone receptor-positive DCIS aiming to elicit an immune response against the tumor-specific form of MUC1 and has advanced to a phase II clinical trial for recurrent colorectal adenoma (42). The NeuVax HER2-targeting vaccine, which combines the HER2-derived MHC class I peptide E75

(nelipepimut-S, NPS) with GM-CSF, has shown good tolerability and a numerical increase in NPS-specific cytotoxic T lymphocytes compared to GM-CSF alone (43). While our study demonstrated a short-term immune response, a significant advantage of the mRNA-LNP platform is its capacity to enable combination strategies with immune-activating factors such as IFN- β to induce long-term immunity or with modulators of Treg function. The use of LNPs to induce more humoral- or cellular-focused immune response can be another alternative strategy to improve the protective efficacy of Lba mRNA-LNP vaccines (44). Future studies are warranted to identify optimal combinations that mount an effective and sustained immune response with minimal side effects.

Breast cancer vaccine-based strategies may have the greatest impact in preventing the development of invasive tumors in patients diagnosed with DCIS. In this context, it is essential to design experimental models that accurately reflect this clinical scenario. The NMU mammary tumor model is limited by its rapid progression from early lesions to palpable tumors, a transition that occurs much faster than in human disease, thereby restricting the timeframe available for vaccination. This presents challenges in designing a model to evaluate postinitiation vaccination exclusively. Our three-vaccination experiment, where the third vaccination was administered post-NMU treatment, may offer a viable experimental model to simulate the DCIS interception setting. Of note, multiple carcinogenic stimuli due to the injection of two doses of NMU may hinder the cancer preventive effects of the vaccination in our study. Optimization of the number of NMU doses and timing relative to vaccination would be required to improve the cancer preventive effects of the Lba mRNA-LNP vaccination.

Neither Lba-U nor Lba-M mRNA-LNP affected the normal mammary epithelial cells, including LP cells with high LALBA expression. However, from a translational perspective, further research is necessary to assess its impact on the mammary gland during pregnancy and lactation, when LALBA expression is significantly elevated, and to characterize potential immunosuppressive effects, such as Treg accumulation in mammary tissues observed with unmodified Lba mRNA-LNP vaccination. The outbred nature of SD rats presents a limitation in experimentally demonstrating long-term protective immunity, such as through a tumor rechallenge experiment. Additional data on safety and efficacy in long-term settings are crucial for clinical translation. These studies could be conducted using inbred rat models such as Buffalo/N rats.

In conclusion, our findings suggest that prophylactic vaccination targeting genes highly and specifically expressed in epithelial cells and early initiated lesions is a viable cancer prevention strategy for women at high risk of cancer. However, further studies are needed to optimize the targets and timing of vaccination.

Materials and Methods

Ethics Statement. All animal studies were conducted in compliance with the relevant ethical guidelines and approved by the Dana-Farber Cancer Institute Animal Care and Use Committee protocol #15-005.

Animal Model. Female virgin SD rats were obtained from Envigo and housed in an AAALAC-accredited, SPF rodent-only barrier facility at Dana-Farber Cancer Institute (Boston, MA). Rats were housed in individually ventilated, solid-bottom, polysulfone 135 sq. in. microisolator cages within the Optimice[®] rack system, which includes an integrated automatic watering system. Environmental conditions were maintained at a temperature of 20 °C and relative humidity of 35 to 55%, with a 12-h light/dark cycle controlled by an automated system.

mRNA-LNP Production. To produce the Lba and Luc mRNAs, the sequences were codon-optimized, synthesized, and cloned into an mRNA production plasmid (45). N1-methylpseudouridine-5'-triphosphate (m1 Ψ -5'-triphosphate)

(TriLink #N-1081) instead of UTP was used to generate modified nucleoside-containing mRNAs, and the mRNAs were transcribed to have 101 nucleotide-long poly(A) tails. Cotranscriptional capping of the in vitro transcribed mRNAs was performed using the trinucleotide cap1 analog, CleanCap (TriLink #N-7413). Cellulose (Sigma-Aldrich #11363-250G) was used for mRNA purification (45). All mRNAs were evaluated by agarose gel electrophoresis and were stored frozen at -20°C . mRNAs were encapsulated in LNP using a self-assembly process in which mRNA in an aqueous acidic buffer was rapidly mixed with an ethanolic lipid mixture of an ionizable cationic lipid, distearoylphosphatidylcholine, cholesterol, and a polyethylene glycol lipid as previously described (46). The ionizable cationic lipids and LNP composition are described in patent application WO 2017/004143A1. All LNP were sized by Malvern Zetasizer and RNA content was quantitated using the Ribogreen assay. All LNPs were measured to have sizes below 80 nm, PDI < 0.14 and encapsulation efficiency of $> 95\%$.

In Vivo Experiment and Tissue Collection. For all experiments, 21-d-old female virgin SD rats were purchased from Envigo. mRNA-LNP was diluted with PBS to be $200\text{ }\mu\text{g/mL}$ and $50\text{ }\mu\text{L}$ ($10\text{ }\mu\text{g}$) was intramuscularly injected to the right hindlimb of rats at indicated time points. N-nitroso N-methylurea (NMU) was reconstituted in either pH 6.0 or pH-non-adjusted distilled water and injected to rats intraperitoneally at 50 mg/kg . Tumor growth was monitored by palpation once or twice a week.

Peripheral blood was collected from the tail vein of rats into Vacutainer™ Plastic Blood Collection Tubes with Lithium Heparin (BD Biosciences) using the Vacutainer Winged Safety Push Button Blood Collection Sets without Luer Adapter (BD Biosciences). Collected blood was centrifuged at $1,500\times g$ for 4 min, and plasma was collected and stored at -80°C .

For the tissue collection, rats were euthanized at the humane endpoint using a CO_2 chamber, followed by blood puncture from the left ventricle. Collected blood was centrifuged at $1,500\times g$ for 4 min, and plasma was stored at -80°C . Red blood cells were then lysed with TheraPEAK ACL Lysing Buffer (Lonza Bioscience) at room temperature for 5 min and centrifuged at $1,500\times g$ for 4 min. After this process was performed twice in total, cells were fixed with 4% PFA-PBS for 10 min, washed with PBS, and stored in the freezing media (90% FBS containing 10% DMSO) at -80°C . Right inguinal dLNs were collected for the ELISPOT assay and mashed on the $100\text{ }\mu\text{m}$ cell strainer. Tissues were harvested as previously described (25). Briefly, a small piece of each organ, tumor, and the inguinal mammary gland was dissected and fixed with 10% neutral buffered formalin solution (Millipore Sigma) overnight, followed by storage in 70% ethanol at 4°C . FFPE sectioning and H&E staining was performed by the Rodent Pathology Core at Harvard Medical School (HMS) or iHisto. Mammary tumors and axillary and inguinal mammary glands were minced using scalpel and incubated in the tissue dissociation media [DMEM/F12 medium containing 2 mg/mL collagenase type IV (Worthington), 2 mg/mL hyaluronidase from bovine testes (Millipore Sigma), and 2 mg/mL BSA (Millipore Sigma)] for 1 to 2 h at 37°C , followed by storage in the freezing media (90% FBS containing 10% DMSO) in liquid nitrogen.

Growth Rate Calculation. Tumor growth rate was calculated based on the linear regression of the tumor diameter changes throughout individual tumor progression after each tumor became palpable using the following formula.

$$\text{Imer(Tumor diameter} \sim 0 + \text{Day} + (0 + \text{Day} - 1) \text{Sample})).$$

Individual tumors were then subjected to categorization of "growing tumors" and "stable tumors" based on their tumor growth ratios, i.e., higher 50% as growing tumors and lower 50% as stable tumors, respectively.

Hyperplasia Score. Mammary tissues were inspected by a pathologist in the Rodent Pathology Core at HMS. Mammary glands with fully hyperplasia, partially hyperplasia, and no hyperplasia were considered as score 2, 1, 0, respectively.

Single-Cell RNA-Seq Data analysis. Rat mammary epithelial cell analysis in Fig. 1B was performed as previously described (27). Analysis of human single-cell RNA-seq data in the human breast cell atlas (14) was conducted as we previously described (27). scRNA-seq data of mammary tumors (Fig. 4 and SI Appendix, Fig. S6) and normal mammary glands (Fig. 5 and SI Appendix, Fig. S7) were analyzed as follows. Sequenced read processing and alignment were performed as previously described (27). Briefly, reads were aligned to the rat reference genome Rnor_6 using Cell Ranger v6.1.2 ($10\times$ Genomics). Downstream analysis was performed in the RStudio (R v4.2.2) and the Seurat package (v5.1.0). Data filtration

was performed according to following criteria: 1) $500 \leq \text{nCount_RNA} < 50,000$, 2) $\text{nFeature_RNA} \geq 300$, 3) $\text{percent.mt} \leq 20$, and 4) $\text{percent.rb} \leq 40$. Filtered data were then normalized using "SCTransform." Clustering was conducted by "FindNeighbors" using the first 30 dimensions and "FindClusters" at the resolution of 0.1. Cluster names were manually annotated based on the representative gene expression in each cluster found by "FindMarkers" function in the Seurat package. Azimuth Cell Types 2021 in Enrichr (<https://maayanlab.cloud/Enrichr/>) was also used to support the cell type annotation. Subclustering analysis was conducted after the subset of cell types of interest, followed by FindNeighbors using the first 30 dimensions and FindClusters at the resolution depending on each cell type. For reclustering of CD4^+ T cells in the mammary tumor cohort and epithelial cells in the mammary gland cohort, clusters which did not express CD4 and EpCAM was removed from the downstream analysis, respectively. Subcluster names were also manually annotated based on the representative gene expression in each cluster. Feature plot and stacked bar plot were visualized using scCustomize package (v2.1.2) and ggplot2 package (v3.5.1), respectively.

After data were normalized with "NormalizeData" function in the Seurat package, FindMarkers package was used to compare gene expression of two group comparisons. Only genes with adjusted P -value equal to or less than 0.05 were considered as DEGs. GSEA was conducted by fgsea package (v1.24.0) using mouse hallmark gene sets (*ms.all.v2023.2.Mm.symbols.gmt*) provided by MSigDB (47, 48). Only signatures with adjusted P -value equal to or less than 0.05 were considered as significantly enriched signature. Data were visualized using ggplot2 package (v3.5.1). Gene expression was visualized using "DoHeatmap" function using the Seurat package. Reactome pathway analysis was performed using the Reactome 2022 database by Enrichr (<https://maayanlab.cloud/Enrichr/>).

Cell-cell communication analysis. MultiNicheNet (multinichenetr v2.0.0) was used for the cell-cell communication analysis. Only epithelial cells and immune cells were subset from the whole cell population and reclustered to perform the analysis. "alias_to_symbol_SCE" function was used to convert aliases to mouse official gene symbols. MultiNicheNet core analysis was performed following the tutorial. The top 10 predictions of pathways were selected by assigning senders as LP and receivers as T/NK/ILC cells and were visualized using "make_sample_lr_prod_activity_plots_Omnipath" function.

Public Dataset Acquisition. Expression of *LALBA* mRNA and *ELF5* mRNA in breast tumors was evaluated using METABRIC dataset acquired from the cBioportal for Cancer Genomics (<https://www.cbioportal.org/>). Expression of *LALBA* mRNA in whole tissues was evaluated using consensus RNA expression dataset and single cell type dataset from the Human Protein Atlas (<https://www.proteinatlas.org/>).

Statistical Analysis. All statistical analyses were performed using either Prism 10 or R (v4.2.2). Overall, P -value ≤ 0.05 is considered as statistical significance. Parametric multiple group comparison was performed by one-way ANOVA with Tukey's test or Dunnett's test if multiple groups were compared with a single control group. Nonparametric multiple group comparison was performed by the Kruskal-Wallis test with Dunnett's test. Survival analysis was stratified by independent studies and performed using R with Fleming-Harrington weighted log-rank test with the parameter of $\rho = 0$ and $\gamma = 1$, a test that down-weights early events, or Prism 10 with Long-rank (Mantel-Cox) test. Hyperplasia score and the ratio between growing tumors and stable tumors were assessed by the Chi-square test. Fisher exact test was used when either of group compared has less than five samples. Linear regression analysis was performed using "lm" function in R or Prism 10. Difference of regression lines was assessed by Prism 10 by the "Test whether slopes and intercepts are significantly different" function. Palpable tumor number comparison was conducted with "glmer" function in lme4 package (v1.1.35.5). The presence or absence of the interaction effect in the model was selected based on the Akaike Information Criterion (AIC) value.

For Study 4 (Fig. 2), the following formula was used.

$$\text{glmer(Tumor number} \sim \text{Treatment} + \text{Time} + (0 + \text{Time}|\text{Subject}), \\ \text{family} = \text{"poisson"}).$$

For Study 5 (Fig. 3), the following formula was used.

$$\text{glmer(Tumor number} \sim \text{Treatment} * \text{Time} + (0 + \text{Time}|\text{Subject}), \\ \text{family} = \text{"poisson"}).$$

Data, Materials, and Software Availability. Single-cell RNA-seq data were deposited to NCBI Gene Expression Omnibus under accession number [GSE300205](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE300205) (49). A supporting data values file with all reported data values is available as part of the [SI Appendix](#). This study does not report original code. All data were analyzed and processed using published software packages, the details of which are provided and cited in *Materials and Methods* section.

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Competing interest statement: M.B.B. is an employee of Acuitas Therapeutics. P.Y. is a current employee of Glaxo Smith Klein, PA. P.Y. contributed to this work prior to their employment at Glaxo Smith Klein. T.M.B. is a shareholder of Merck and Co., Novo Nordisk A/S, AbbVie Inc., and Johnson and Johnson. K.P. serves on the Scientific Advisory Board of Ideaya Biosciences, is an adviser to CurieBio, holds equity in Antares Therapeutics and stock options in Ideaya Biosciences, receives funding from Novartis, and received honoraria from Astra-Zeneca, and Novartis, and payment from Eli Lilly for their purchase of Scorpion Biosciences in the past 24 mo; and is an inventor on patents on S100A7 antibody and BET inhibitor resistance. N.P. served on the mRNA strategic advisory board of Sanofi Pasteur in 2022, Pfizer in 2023–2024, and AldexChem in 2024–2025, is a member of the Scientific Advisory Board of BioNet, and has consulted for Vaccine Company Inc, Optimeos, Piezo Therapeutics, and Pasture Bio.

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