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NBTXR3 improves the efficacy of immunoradiotherapy combining nonfucosylated anti-CTLA4 in an anti-PD1 resistant lung cancer model

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The efficacy of immunoradiotherapy consisting of radiation therapy and immune checkpoint blockade relies on effectively promoting the systemic antitumor immune response's activation while simultaneously reducing local factors favoring immune suppression. We previously demonstrated that NBTXR3, a nanoparticle radioenhancer, significantly improved immune responses in a murine anti-PD1-resistant metastatic lung cancer model. We hypothesize that radioactivated-NBTXR3 addition to anti-PD1 and a second-generation anti-CTLA4 could improve treatment effectiveness. To test this hypothesis, we inoculated mice with 344SQR cells in the right and left legs to establish primary and secondary tumors. The primary tumors were intratumorally injected with NBTXR3 nanoparticles on day 7, followed by three fractions of 12 Gy radiation on days 8, 9, and 10. The secondary tumors received two fractions of 1Gy radiation on days 13 and 14. Multiple rounds of anti-PD1, anti-CTLA4 or nonfucosylated anti-CTLA4 were given to the mice. Immune profiling of the tumors revealed that the combination of NBTXR3 with immunoradiotherapy significantly upregulated the activities of a wide range of antitumor immune pathways and reduced the abundance of regulatory suppressor T cells. This combination effectively eradicated the primary and secondary tumors and increased animal survival to 75%. Remarkably, previously

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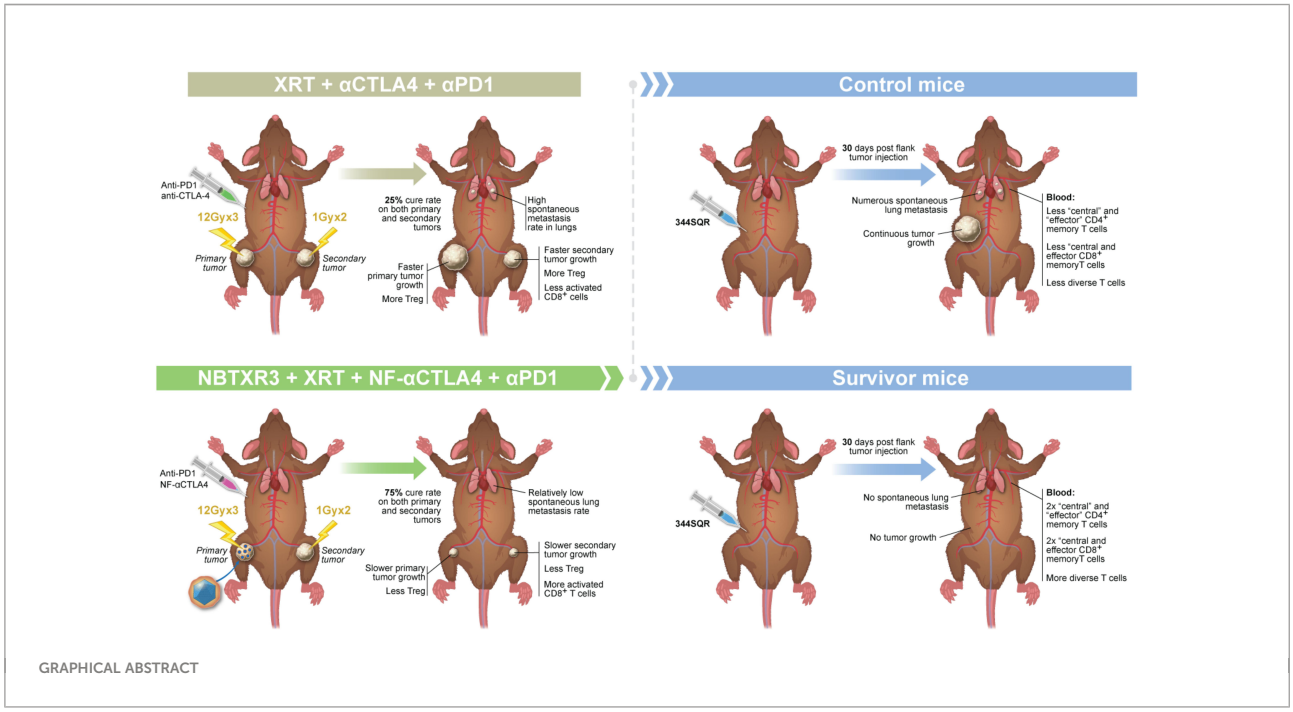
treated with NBTXR3-containing treatment, the survivor mice exhibited a long-lasting antitumor memory immune response. This data provides compelling evidence of the efficacy of NBTXR3 to synergize with the immunoradiotherapy approach when combined with an anti-PD1 and multiple checkpoints such as a second generation anti-CTLA4 and show the potential for clinical uses of antitumor immunomodulatory effects of NBTXR3.

KEYWORDS

immunoradiotherapy, NBTXR3 nanoparticle, immume checkpoint blockade, abscopal effect, lung cancer



GRAPHICAL ABSTRACT

Introduction

Checkpoint inhibitors (CPIs) have been a revolution in cancer therapy (1), boasting unprecedented responses in many cancers deemed previously intractable (2). The first CPI was directed against cytotoxic T lymphocyte antigen 4 (CTLA4) (3, 4). CTLA4 is a competitive antagonist with the T cell co-receptor CD28, which delivers the second signal essential for full T cell activation. By binding to the same ligands as CD28, CTLA4 denies the T cell this activating signal, replacing it with an inhibitory one (5). Anti-CTLA4 (αCTLA4) binds to the extracellular region of CTLA4, preventing it from binding the B7 ligands and thereby blocking this inhibitory signal (6).

Since its approval in 2011, αCTLA4 has become a mainstay of cancer immunotherapy (7). Both as a monotherapy and in combination with αPD1, αCTLA4 has been widely used in many types of solid tumors with significant treatment benefits (8). Preclinical results demonstrated that a nonfucosylated (NF) version of αCTLA4 (NF-αCTLA4) may achieve better treatment outcomes than traditional αCTLA4 by increasing Treg depletion at the tumor site (9). The lack of a fucosyl group on the fragment crystallizable (Fc) region of the NF-αCTLA4 antibody results in this region being bound with higher affinity by the Fcγ receptor CD16, which is predominantly expressed on natural killer (NK) cells. The binding of antibodies by CD16 triggers antibody-dependent cellular cytotoxicity (ADCC), resulting in the death of any cells expressing CTLA4. As CTLA4 is predominately expressed by T regulatory cells (Tregs), this results in the preferential depletion of this immunosuppressive cell population (10, 11). Thus, NF-αCTLA4 improves upon existing αCTLA4 treatments by effectively reducing the tumor-resident Treg population (12).

More recently, immunoradiotherapy that combines stereotactic body radiation therapy (SBRT) with CPIs (both αCTLA4 and αPD1) has proven effective in treating metastatic cancers by inducing systemic and specific antitumor immune responses (13–15). To maximize the efficacy, it is crucial to promote the priming of effector cells and reduce concomitant

immune suppression. NBTXR3, a hafnium oxide nanoparticle, was initially introduced as a radiation-enhancer for treating localized tumors (16). Lately, it has been discovered that NBTXR3-mediated radiotherapy can also serve as an immune enhancer that promotes antitumor activities, extending its treatment benefits to distant lesions (17–19). In preclinical models, NBTXR3 was found to facilitate the infiltration of $CD8^+$ T cells into abscopal tumors and elevate the expression of genes that favor tumor killing. Phase I clinical data evaluating NBTXR3/RT/αPD1 in patients with advanced cancers show that intratumoral injection of NBTXR3 is feasible and well-tolerated with promising signs of efficacy (20). These findings are of great significance, as only a minority of cancer patients (<20%) respond to αPD1 treatment (21). Given the excellent treatment potential of NBTXR3 and NF-αCTLA4, we hypothesize that a novel immunoradiotherapy integrating NBTXR3, radiotherapy, NF-αCTLA4, and αPD1 would further improve the treatment outcome of αPD1-resistant lung cancer.

Materials and methods

Materials

NBTXR3 nanoparticles were kindly provided by Nanobiotix and were stored at room temperature in darkness before use. Bristol-Myers Squibb kindly provided the mouse αCTLA4, NF-αCTLA4, and αPD1 antibodies. Antibodies for flow cytometry, including αCD45:PE-Cy7 (Cat. #147704), αCD4:APC-Cy7 (Cat. #100414), αCD8:PerCP-Cy5.5 (Cat. #126610), αCD3:BV510 (Cat. #100234), αCD49b:APC (Cat. #108910), αCD19:PE/Dazzle (Cat. #115554), αFoxP3:PE (Cat. #126404), αGranzyme B:Pacific Blue (Cat. #372218), αCD45:Pacific Blue (Cat. #103126), αCD4:APC/Fire750 (Cat. #100460), αCD44:APC (Cat. #103012), αCD62L:PE-Cy7 (Cat. #104418), and αCD27:AF700 (Cat. #124240) were purchased from BioLegend.

Cell line and culture conditions

The cell line used throughout this study was αPD1-resistant lung cancer cell line 344SQR (22). It was cultured by methods described in previous reports (17, 19).

Tumor establishment and treatment

The animals used in all the experiments in this study were 8-12-week-old 129/SvEv female mice purchased from Taconic Biosciences. The 344SQR cells (5×10^4 in 100 μL PBS) were injected into the right leg on day 0 to create primary tumors and into the left leg on day 4 to create secondary tumors. Tumor size was monitored with digital calipers at least twice a week, and tumor volumes were calculated using the formula: tumor volume = $0.5 \times \text{width}^2 \times \text{length}$. Mice were divided into six treatment groups, with eight mice in each group: 1) control (no treatment), 2) NBTXR3+XRT, 3) XRT+αCTLA4+αPD1, 4) XRT+NF-αCTLA4+αPD1, 5) NBTXRT3+XRT+αCTLA4+αPD1, and 6) NBTXRT3+XRT+NF-αCTLA4+αPD1. NBTXR3 nanoparticles in 5% glucose with 25% of the tumor volume were intratumorally injected into the primary tumors on day 7. CPIs, including αCTLA4 (50 μg), NF-αCTLA4 (50 μg), and αPD1 (200 μg), were intraperitoneally injected into mice on days 7, 11, and 14. For experiments that evaluate the efficacy of intratumoral injection of NF-αCTLA4, the primary tumors were intratumorally delivered with 50 μg NF-αCTLA4 on day 7 (IT1) or on days 7 and 11 (IT2). Anti-PD1 treatment continued on days 21, 28, 35, and 42. The primary tumors were irradiated with three fractions of 12 Gy, each with a PXi X-Rad SmART irradiator on days 8, 9, and 10 (total dose of 36 Gy). The secondary tumors were irradiated with two fractions of 1 Gy each, also with a PXi X-Rad SmART irradiator on days 13 and 14 (total dose of 2 Gy). The dose was delivered with two opposing beams from anteroposterior and posteroanterior positions and a 15-mm circular collimator. The dosimetry and treatment planning was performed using the Advanced Treatment Planning software supplied by the vendor. Precision XRay Corporation commissioned all collimators at the time of installation. Routine output checks were done with an ion chamber to ensure that the outputs had not changed and that the treatment plans were accurate. Mice were euthanized when primary or secondary tumors reached 14 mm in any dimension. All animal procedures followed the guidelines of the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center.

Tumor rechallenge

Mice from the XRT+NF-αCTLA4+αPD1, NBTXR3+XRT+αCTLA4+αPD1, and NBTXR3+XRT+NF-αCTLA4+αPD1 groups that had survived more than 156 days past the initial tumor challenge were rechallenged with 5×10^4 344SQR cells in 100 μL PBS in their right flank. Five mice of similar age were also implanted with the same number of 344SQR cells and served as control. No further treatment was given. As before, mice were euthanized when the tumor reached 14 mm in cross-section. The blood samples were collected 20 days before tumor rechallenge and 7 and 21 days post tumor rechallenge for immune profiling. The lungs were also harvested at the end of the experiment to count the number of lung metastases.

Tumor processing

Primary and secondary tumors were harvested on day 16 for flow cytometric immune profiling and on day 18 for NanoString

analysis. The tumor tissues were minced and digested with 250 μg/mL of Liberase (Roche, Cat. #05401127001) and 20 μg/mL DNAse (Sigma-Aldrich, Cat. #4716728001) at 37°C for 30 min. The digestion process was stopped with 1 mL fetal bovine serum (FBS), and the samples were filtered and washed with PBS (2% FBS) buffer. The cells were either stained with antibodies for flow cytometry analysis (FACS) or frozen in TRIzol for RNA extraction.

Flow cytometric analysis

The above-processed cells on day 16 were stained with αCD45:PE-Cy7, αCD4:APC-Cy7, αCD8:PerCP-Cy5.5, αCD3:BV510, αCD49b:APC, αCD19:PE/Dazzle, αFoxP3:PE, and αGranzyme B:Pacific Blue. The blood samples from the tumor rechallenge study were collected on day 21 post tumor rechallenge and were stained with αCD45:Pacific Blue, αCD4:APC/Fire 750, αCD8:PerCP-Cy5.5, αCD44:APC, αCD62L:PE-Cy7, αCD3:BV510, αCD19:PE/Dazzle, and αCD27:AF700. Samples were analyzed using a Gallios Flow Cytometer (Beckman Coulter) with the Kaluza software Version 2.1.

Counting numbers of lung metastases

The lungs collected either on day 16 or at the end of the tumor rechallenge experiment were stored in Bouin's fixative solution (Polysciences Inc., Cat. #16045-1) for 3 days, after which the number of lung metastases was counted.

Analysis of immune-related genes in tumor immune microenvironment *via* Nanostring

Total RNA extracted from both primary and secondary tumors harvested on day 18 were analyzed with an nCounter PanCancer Immune Profiling Panel and an nCounter MAX Analysis System (both from NanoString Technologies, Seattle, WA, USA) by following the manufacturer's instructions; the expression of immune-related genes was analyzed with the PanCancer Immune Profiling Advanced Analysis Module (also from NanoString Technologies).

TCR repertoire analysis

Blood was collected from 4 mice in the control group and the NBTXR3+XRT+NF-αCTLA4+αPD1 group 21 days post tumor rechallenge. Total RNA was subsequently extracted from the blood. TCR analysis was performed using a method described in the previous study (17). The raw TCR sequencing data was processed using MiXCR (version 3.0.13) with default parameters (23). In brief, the raw reads were aligned to the *Mus musculus* reference T cell receptor genes based on the ImMunoGeneTics database (IMGT) (24). Then, the aligned reads were assembled to construct the CDR3 (complementarity-determining region 3). Finally, the MiXCR reported the clonotypes of each sample, which the unique clonotype was defined as the unique CDR3 amino acid sequences and V-J segments genes. Further bioinformatics analysis of the TCR beta chain and data visualization was performed using the Immunarch package in R (version 4.0.1) (25). The circlize package was used to generate the circos plot of each sample regarding V-J usage (26).

Statistical analyses

All statistical analyses were performed with Prism 9.0.0 (GraphPad Software). Tumor volumes were compared by two-way ANOVA and were expressed as mean tumor volume ± standard error of the mean (SEM). Mouse survival rates were compared with the Kaplan–Meier method and log-rank tests. NanoString data were compared by one-way ANOVA or two-tailed t tests. All other data were compared with two-tailed t tests and expressed as mean value ± SEM. P values of < 0.05 were considered to indicate statistically significant differences.

Results

The combination of NBTXR3 with NF-αCTLA4 and αPD1 immunoradiotherapy leads to improved tumor control and increased survival rates

Mice were challenged with a two-tumor system, as shown in Figure 1A, to simulate a primary tumor and a secondary metastatic site. Mice were then treated with XRT supplemented with the NBTXR3 nanoparticle. Although this treatment was effective at restraining the growth of the primary tumor (Figure 1B), it did not affect the secondary tumors (Figure 1C) and, consequently, only a limited (albeit statistically significant) effect on median survival (+3 days; Figure 1D).

We began by comparing the ability of αCTLA4 vs. NF-αCTLA4 to augment immunoradiotherapeutic control of tumor growth. Mice were next treated with XRT+αPD1 and either αCTLA4 or NF-αCTLA4. XRT+αCTLA4+αPD1 was no better than XRT+NBTXR3 at limiting primary tumor growth (Figure 1B), although it was significantly better at slowing secondary tumor growth (Figure 1C), conferring a significantly improved median survival and an overall survival rate of 25% (2/8 mice; Figure 1D). However, substituting NF-αCTLA4 for αCTLA4 produced better results still; not only control of the

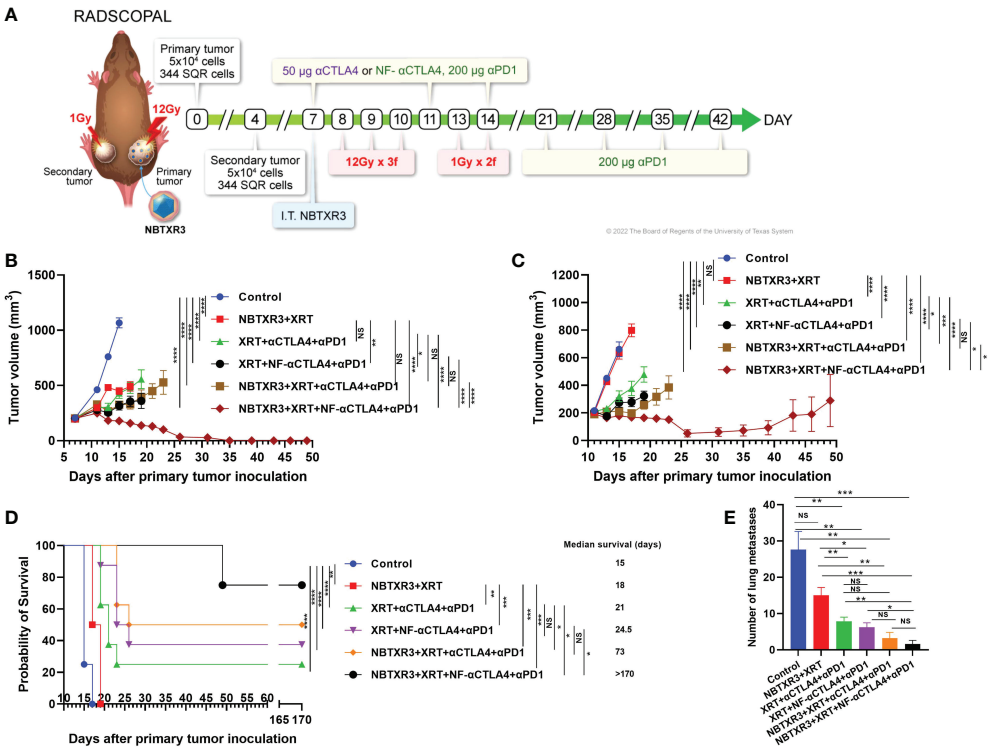


FIGURE 1
NF-αCTLA4, combined with NBTXR3-mediated immunoradiotherapy, significantly improves treatment efficacy in αPD1-resistant lung cancer. **(A)** Treatment schema for combination therapies of NBTXR3, XRT, αCTLA4 or NF-αCTLA4, and αPD1. Mice were subcutaneously injected with 5×10^4 344SQR cells in the right legs on day 0 (to establish primary tumors, to be irradiated with high dose radiation) and in the left legs on day 4 (to establish secondary tumors, to be irradiated with low dose radiation). NBTXR3 was delivered to the primary tumor by intratumoral injection on day 7. Primary tumors were treated with three 12 Gy fractions on days 8, 9, and 10. Secondary tumors were irradiated with two 1 Gy fractions on days 13 and 14. αPD1 (200 μg) and anti-CTLA4 or NF-αCTLA4 (50 μg) were given by intraperitoneal injection on days 7, 11, and 14. αPD1 treatment was continued once a week from day 21 until day 42. For intratumoral injections of NF-αCTLA4, 50 μg of NF-αCTLA4 was intratumorally injected into the primary tumors on days 7 and 11. **(B)** Tumor volumes of the primary tumor. **(C)** Tumor volumes of the secondary tumor. **(D)** Survival rates and median survival time. **(E)** The number of spontaneous lung metastasis on day 16. Data are expressed as means ± SEM. P< 0.05 was considered statistically significant. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001, NS, not significant.

primary tumor was greater than either NBTXR3+XRT or XRT +αCTLA4+αPD1 (Figure 1B), but the growth of the secondary tumor was also significantly slower (Figure 1C), and overall survival was increased to 37.5% (3/8 mice; Figure 1D) (although the difference in median survival between the XRT+αCTLA4 +αPD1 and the XRT+NF-αCTLA4+αPD1 groups was not significant).

We further amplified these two radioimmunological combinations with the NBTXR3 nanoparticle. The addition of NBTXR3 to XRT+αCTLA4+αPD1 improved primary and secondary tumor control to a level matching that achieved by XRT+NF-αCTLA4+αPD1 (Figures 1B, C) and achieved an overall survival rate of 50% (4/8 mice; Figure 1D), with a significantly longer median survival than XRT+αCTLA4 +αPD1. Nevertheless, the most potent combination of all was that of NBTXR3+XRT+NF-αCTLA4+αPD1. This combination, and this combination alone, achieved 100% complete remission of the primary tumors (8/8 mice; Figure 1B, Supplementary Figure 1) and 75% complete remission of the secondary tumors (6/8 mice; Figure 1C, Supplementary Figure 1A). Both overall and median survival was highest in this group, with 75% overall survival and a median survival of >170 days. Thus, the treatment groups displayed an increasing level of tumor control, with NBTXR3 amplifying the local effects of radiation, CPI in the form of αCTLA4 and αPD1 boosting the systemic immune response against the tumors, and the substitution of αCTLA4 with NF-αCTLA4 boosting this even further. This was reflected when we examined the mice's lungs for the presence of metastatic nodules; we found that the number of metastases was reduced roughly in proportion to the relative degree to which the primary and secondary tumors were controlled by each treatment group (Figure 1E). In addition, the treatment efficacy of intratumoral injection of NF-αCTLA4 was evaluated. As shown in Supplementary Figure 1B, intratumoral injection of NF-αCTLA4 and various combinations of XRT, NBTXR3, and αPD1 delayed the growth of both tumors and survival compared

to the control group. Remarkably, compared to two times injection of NF-αCTLA4 without NBTXR3 (XRT+NF-αCTLA4(IT2)+αPD1), single time intratumoral injection of NF-αCTLA4 with NBTXR3 (NBTXR3+XRT+NF-αCTLA4 (IT1)+αPD1) achieved similar primary tumor control, and significantly improved secondary tumor control and increased the survival rate from12.5% to 28.5%. Interestingly, two times injection of NF-αCTLA4 with NBTXR3 (NBTXR3+XRT+NF-αCTLA4(IT2)+αPD1 did not improve tumor control compared to (NBTXR3+XRT+NF-αCTLA4(IT1)+αPD1 but starkly increased the percentage of cured mice (28.5% to 57.1%). Remarkably, the percentage of cured mice achieved by XRT +NF-αCTLA4(IT2)+αPD1 (12.5%) was multiplied by 4.57 times when NBTXR3 was added to this combination (NBTXR3+XRT+NF-αCTLA4(IT2)+αPD1.

NBTXR3, in combination with NF-αCTLA4, reduces Tregs and activates $CD8^+$ T cells in the tumor immune microenvironment

Next, we used flow cytometry to explore how each combination therapy affected the tumor immune microenvironment of both primary and secondary tumors. As shown in Figure 2, radiotherapy consisting of NBTXR3+XRT without CPI produced no significant alterations in any measured cell population in the primary or secondary tumor, other than a reduction in NK cells in the primary tumor. However, a trend towards a decrease in Tregs and an increase in the $CD4^+$ population can be observed in both tumors. XRT, combined with CPI (consisting of αCTLA4+αPD1), also reduced NK cells in the primary tumor. In addition, treatment with XRT +αCTLA4+αPD1 resulted in a significant increase in the percentage of $CD4^+$ T cells (as well as a trend to Treg increase) and a concomitant decrease in the percentage of $CD8^+$ T cells and NK cells within the secondary tumor. Substitution of NF-αCTLA4 for αCTLA4 significantly reduced the percentage of $CD4^+/CD45^+$ and increased $CD8^+/CD45^+$ in the primary tumor. On the secondary, substituting NF-αCTLA4 for αCTLA4 significantly increases Gzm B^+ $CD8^+/CD8^+$ T cells.

Interestingly, adding NBTXR3 to either the XRT+αCTLA4 +αPD1 group or XRT+NF-αCTLA4+αPD1 group led to an almost complete absence of Tregs in the primary tumor as compared to the control group. In the NBTXR3+XRT +αCTLA4+αPD1 group, this came at the cost of significantly reducing overall $CD4^+$ T cells (as compared to the levels measured in the XRT+αCTLA4+αPD1 group). The NBTXR3 +XRT+NF-αCTLA4+αPD1 group, on the other hand, achieved a reduction in Tregs equal to that of the NBTXR3+XRT +αCTLA4+αPD1 group without this cost to the $CD4^+/CD45^+$ overall population. Moreover, the secondary tumors of the NBTXR3+XRT+NF-αCTLA4+αPD1 group boasted the lowest average Treg levels while also displaying significantly higher overall $CD4^+$ levels. This was accompanied by the highest percentage of granzyme B^+ $CD8^+$ T cells in the secondary tumors of any group. Overall, the use of NF-αCTLA4 and NBTXR3 reduced Tregs in both primary and secondary tumors, and both in tandem did so while preserving $CD4^+$ T cell levels and increasing cytotoxic T cell levels in the secondary tumor. The quadruple therapies did not result in significant changes in B cell populations relative to the control.

NBTXR3, in combination with NF-αCTLA4, modulates immune-related gene expression that favors antitumor activity

To better understand how these combination therapies affected immune activities at the genetic level, RNA was isolated from the primary and the secondary tumors harvested on day 18. This RNA was analyzed using the NanoString PanCancer Immune Profiling Panel. As shown in Figure 3A, NBTXR3+XRT tends to enhance immune pathways in the primary tumors, but only B cell function has significantly increased. However, combination therapies, including XRT +αCTLA4+αPD1, XRT+NF-αCTLA4+αPD1, NBTXR3+ XRT +NF-αCTLA4+αPD1 involving CPI succeeded in promoting the activities of various immune pathways, such as the adaptive pathway, antigen processing, B cell function, T cell function, NK cell function, *etc.* Whether CTLA4 blockade was mediated by NF-αCTLA4 or conventional αCTLA4 made no significant difference in any pathways, nor did whether or not NBTXR3 was used. When directly comparing the two quadruple therapies, NBTXR3+XRT+NF-αCTLA4+αPD1 and NBTXR3+XRT +αCTLA4+αPD1 (that is, comparing NF-αCTLA4 and αCTLA4 head-to-head when all other treatment modalities were in play), we observed that the most upregulated genes fell within functional categories predominantly related to innate immune function. These functional categories included the acute phase response, chemotaxis, inflammation, and cell-cell adhesion (Supplementary Figure 2A).

We also analyzed the primary tumor for the enrichment of genes indicative of particular immune cell types. We found that macrophage-related genes were significantly enriched in treatment groups containing NF-αCTLA4 relative to both the control group and treatment groups containing αCTLA4, suggesting superior macrophage enrichment by the NF-αCTLA4 antibody compared to conventional αCTLA4 (Figure 3B). Several other non-statistically significant trends were also visible. Overall, the XRT+αCTLA4+αPD1, XRT +NF-αCTLA4+αPD1, and NBTXR3+XRT+NF-αCTLA4 +αPD1 groups tended to have higher scores in $CD8^+$ T cells, dendritic cells (DCs), T_H1 cells, $CD45^+$ cells, Tregs, neutrophils, and macrophages than the control. Moreover, the XRT

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+αCTLA4+αPD1, XRT+NF-αCTLA4+αPD1, and NBTXR3 +XRT+NF-αCTLA4+αPD1 groups tended to have higher scores in DCs, cytotoxic cells, T_H1 cells, $CD45^+$ cells, and macrophages than the NBTXR3+XRT group. In addition, a higher ratio of CD8 T/Treg was observed in NBTXR3+XRT +NF-αCTLA4+αPD1 as compared to NBTXR3+XRT+αCTLA4 +αPD1 and XRT+NF-αCTLA4+αPD1 (Supplementary Figure 3A). Interestingly, the quadruple therapy with NF-αCTLA4 also led to more abundant total tumor-infiltrating lymphocytes than the control in the primary tumor compared to the quadruple therapy with αCTLA4 (Supplementary Figure 3A).

Examining the individual genes that were specifically altered by our treatments (Figure 3C, Supplementary Table 1), we saw that, compared to NBTXR3+XRT+αCTLA4+αPD1, NBTXR3 +XRT+NF-αCTLA4+αPD1 significantly elevated the expression

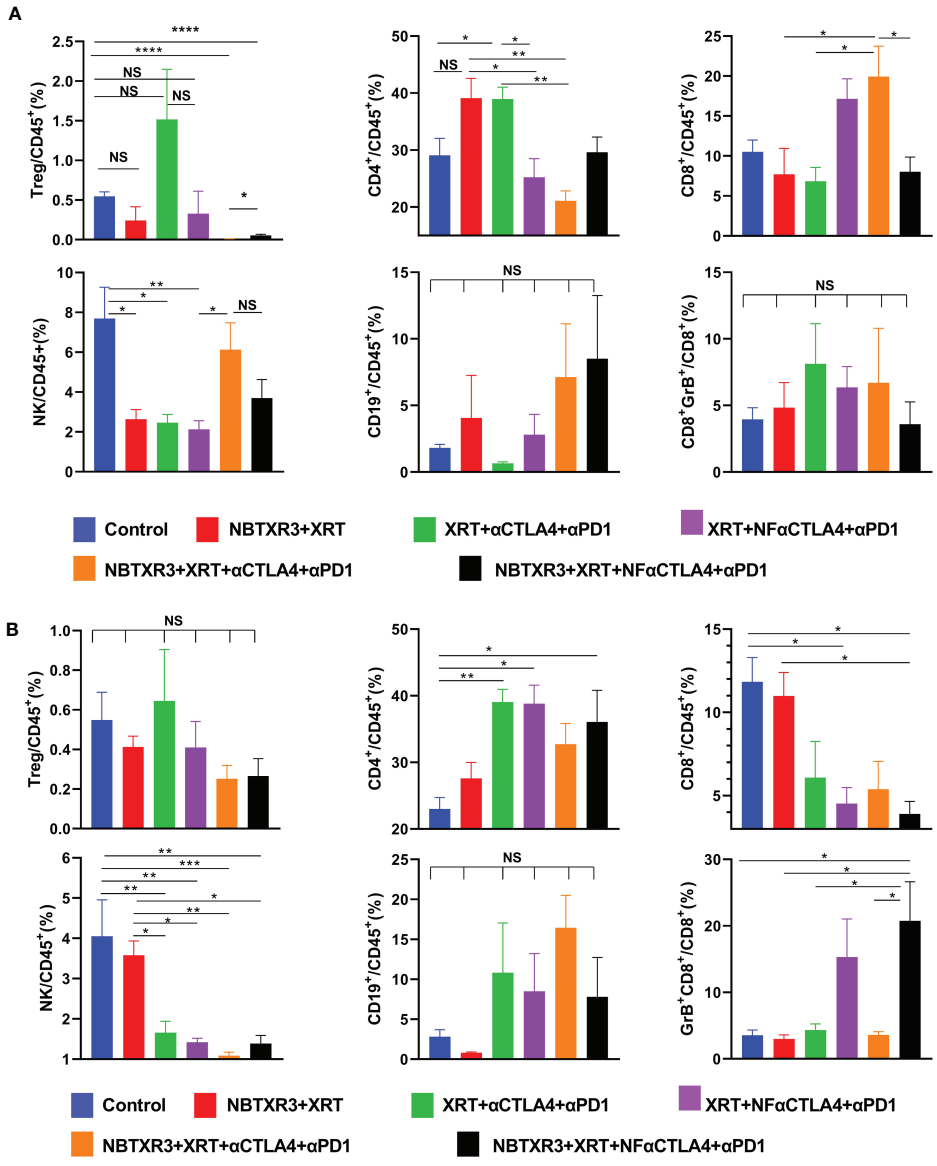

FIGURE 2
NF-αCTLA4 and NBTXR3-mediated immunoradiotherapy significantly reduces Tregs and promotes $CD8^+$ T cell activation in the tumor immune microenvironment. **(A)** Percentages of various immune cells in the primary tumor. **(B)** Percentages of various immune cells in the secondary tumors. The mice (n=5) were treated with various combination therapies as indicated in Figure 1A, and both primary and secondary tumors were harvested on day 16. Immune cell populations, including $CD3^+CD4^+FoxP3^+$(Tregs), $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^-CD49b^+$ (NK cells), $CD3^-CD19^+$ (B cells) and the Granzyme B^+ (Gzm B^+) $CD3^+CD8^+$ T cells subpopulation, were analyzed by flow cytometry. Data are expressed as means ± SEM. $P< 0.05$ was considered statistically significant. *$P< 0.05$, **$P< 0.01$, ***$P< 0.001$, ****$P< 0.0001$, NS, not significant.

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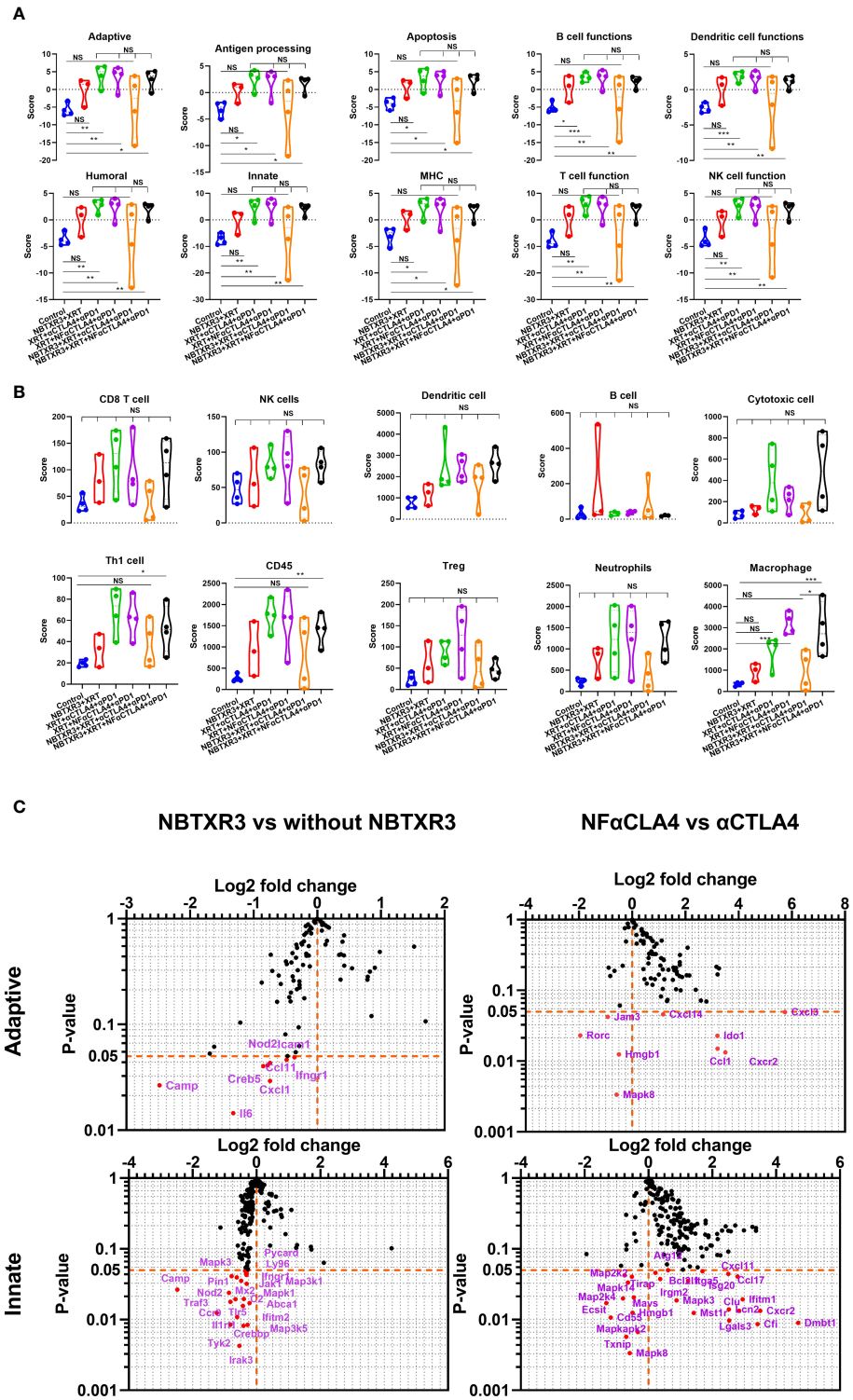

FIGURE 3
NBTXR3, combined with NF-αCTLA4, modulates immune-related gene expression to improve the primary tumor's antitumor immune response. **(A)** Activities of immune pathways in the primary tumors. **(B)** The score of immune cell abundance in the primary tumor. **(C)** Changes in gene expression in adaptive and innate immune pathways. Mice bearing 344SQR tumors were treated with various immunoradiotherapies as indicated in Figure 1A, and the primary tumors were harvested on day 18. The RNA extracted from the tumors was analyzed by a nCounter PanCancer Immune Profiling Panel. Data are expressed as means ± SEM. $P < 0.05$ was considered statistically significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS, not significant.

of several chemokine ligands and receptors (*Ccl1*, *Ccl17*, *Cxcl11*, Cxcl14, *Cxcl3*, *Cxcr2*) as well as a smattering of genes primarily involved in innate immunity, including: *Clu*, the gene for clusterin, an extracellular chaperone that promotes clearance of inflammation and injury-induced immune complexes; >*Fut7*, a carbohydrate involved in cell and matrix adhesion that enables leukocyte accumulation at a site of inflammation; *Ifitm1*, an IFN-induced antiviral protein implicated in cell adhesion and control of cell growth and migration; *Lcn2*, a neutrophil-secreted factor that sequesters iron-containing siderophores; and *Spp1*, a cytokine involved in enhancing the production of IFNγ and IL-12 and reducing the production of IL-10 (Figure 3C). NF-αCTLA4 also significantly downregulated certain genes involved in the TGFβ pathway, such as *Tgfb2*, *Tgfb3*, *Rora*, and *Rorc* (Figure 3C, Supplementary Figure 3B). Interestingly, the addition of NBTXR3 to XRT+NF-αCTLA4+αPD1 led to no increase in gene expression (Figure 3C, Supplementary Table 3) but decreased expression of *Cam*, *Il6*, *Fas*, *Nod2*, *etc.*

As with the primary tumors, in the secondary tumors (Figure 4A), all therapies containing CPI resulted in elevated activities in most pathways relative to groups without CPI. Once again, whether the CTLA4 blocker used was NF-αCTLA4 or αCTLA4 here made little difference, as did whether or not NBTXR3 was used. The difference between the primary and secondary tumors was that, unlike in the primary tumors, NBTXR3+XRT did not cause elevated immune activities in the secondary tumor relative to the control. Quadruple therapies had higher scores associated with immune cell populations across the board than the control group (Figure 4B). In addition, more abundant tumor-infiltrating lymphocytes were observed in mice treated with combination therapies with CPIs (Supplementary Figure 3A). As in the primary tumor, the quadruple therapy with NF-αCTLA4 resulted in a higher CD8/Treg ratio than the quadruple therapy with αCTLA4 in the secondary tumors (Supplementary Figure 3A). To our surprise, however, the quadruple therapy group involving NF-αCTLA4 had, on the whole, lower expression of immune-related genes than did the quadruple therapy involving conventional αCTLA4 (Figure 4C, Supplementary Table 2). Among the downregulated genes were, notably, those related to Treg identity and function. In particular, *Foxp3* expression was significantly downregulated in the NF-αCTLA4 quadruple therapy group compared to the αCTLA4 quadruple therapy group (Figure 4C).

Contrary to what was observed in the primary tumors, using the NBTXR3 nanoparticle in concert with CPI significantly upregulated many genes (Figure 4C, Supplementary Figure 3B, Supplementary Table 4). Among the most highly upregulated groups were cytokines (Supplementary Figure 2C). These exhibited a bimodal distribution, roughly half upregulated and half downregulated (Supplementary Figure 2D). Examining the function of these genes, we found that those cytokine genes that were upregulated with quadruple therapies featuring NBTXR3 – *Il1a, Csf2, Il1b, Spp1, Il12rb1*, and *Il1r2* – were all pro-inflammatory genes. Downregulated cytokine genes also contained pro-inflammatory genes and anti-inflammatory cytokines such as *Il4ra* and *Tgfb3* in the secondary tumors. Also, adding NBTXR3 to XRT+NF-αCTLA4+αPD1 significantly upregulated the *Gzmb* in the secondary tumors.

Quadruple therapies with NBTXR3 and NF-αCTLA4 generate long-term memory and diverse TCR repertoire against lung cancer

To explore if the mice that survived the initial tumor challenge (Figure 1C) maintained long-term antitumor immune memory, these survivor mice were rechallenged with 344SQR lung cancer cells 156 days following administration of the last fraction of radiation. As shown in Figure 5A, none of the survivor mice from any combination therapies developed tumor after rechallenging, while tumor growth was observed in all the control group mice. In addition, numerous lung metastases were detected in all the control mice, but none was found in the survivor mice (Figure 5A).

To understand the immune memory profile of the survivor mice, immune cells, including $CD4^+$ T cells, $CD8^+$ T cells, and B cells, were analyzed by flow cytometry. As shown in Figure 5B, the survivor mice were not significantly different from the control in terms of overall $CD4^+/CD45^+$ ratio, and the control mice had higher percentages of $CD8^+$ T cells in their blood than the survivor mice in the XRT+NF-αCTLA4+αPD1 and NBTXR3+XRT +αCTLA4+αPD1 groups. However, all treatment groups had significantly higher percentages of $CD4^+$ central memory cells ($CD3^+CD4^+CD44^+CD62L^+$), $CD8^+$ central memory cells ($CD3^+CD8^+CD44^+CD62L^+$), and $CD4^+$ effector memory cells ($CD3^+CD4^+CD44^+CD62L^-$) than the control (Figure 5B, Supplementary Figure 5). In addition, both NBTXR3+XRT+NF-αCTLA4+αPD1 and NBTXR3+XRT+αCTLA4+αPD1 had significantly more $CD8^+$ effector memory cells ($CD3^+CD8^+CD44^+CD62L^-$) than the control (Figure 5B, Supplementary Figure 5). There was no difference in B cells, either total or memory, between the control mice and the survivors, except for the XRT+NF-αCTLA4+αPD1 group, which exhibited significantly higher total B cell levels. No group of survivor mice boasted any significant elevation in any memory population relative to the other, indicating that each therapy had successfully established a memory population in the surviving mice.

To evaluate the differences in T cell diversity between the treated mice and the control, TCR repertoires from the blood of the control mice and the survivor mice in NBTXR3+XRT+NF-αCTLA4+αPD1 were analyzed. As shown in Figure 5C, the survivor mice had a significantly lower inverse Simpson index than the control in the beta chain, indicating that the NBTXR3 +NF-αCTLA4-mediated immunoradiotherapy generated a more diverse T cell repertoire.

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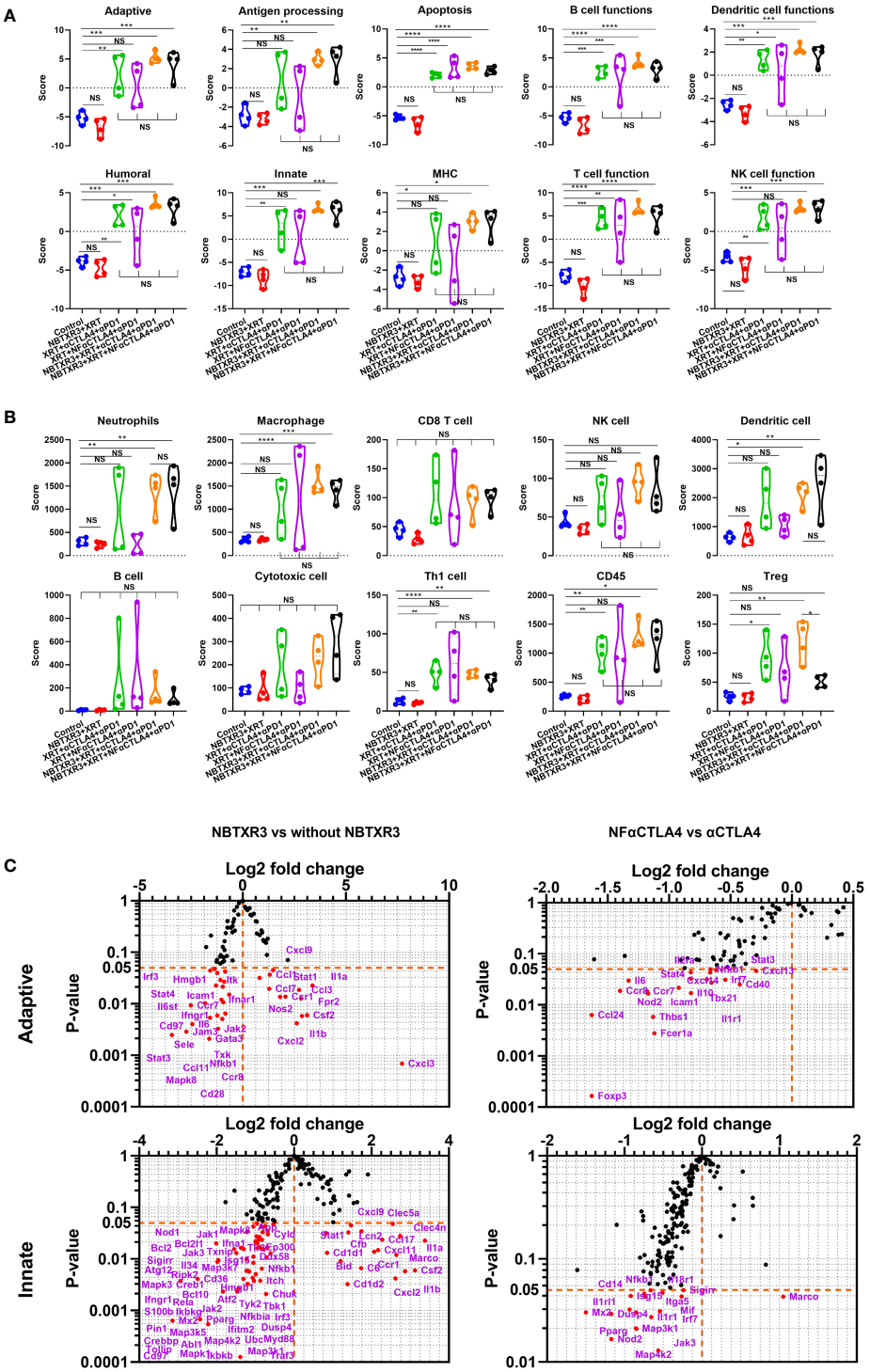

FIGURE 4
NBTXR3, combined with NF-αCTLA4, reduces Treg abundance and promotes antitumor immune response in the secondary tumor. **(A)** Activities of immune pathways in the secondary tumors. **(B)** Score of immune cell abundance in the secondary tumor. **(C)** Changes in gene expression in adaptive and innate immune pathways. Mice bearing 344SQR tumors were treated with various immunoradiotherapies in Figure 1A, and the secondary tumors were harvested on day 18. The RNA extracted from the tumors was analyzed by an nCounter PanCancer Immune Profiling Panel. Data are expressed as means ± SEM. P< 0.05 was considered statistically significant. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001, NS, not significant.

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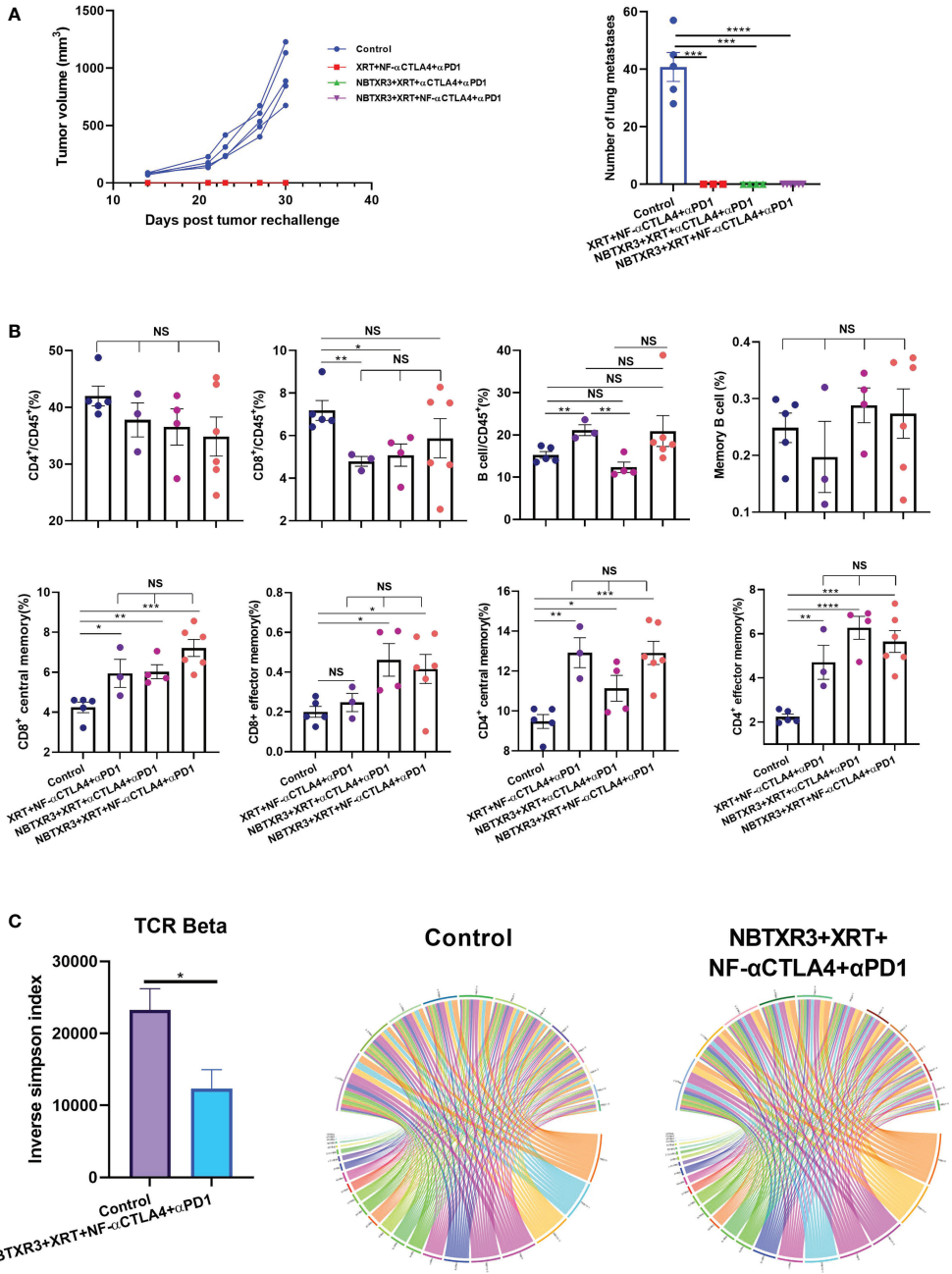

FIGURE 5
Immunoradiotherapies generate long-term antitumor memory immune response. **(A)** Tumor growth curves and the number of lung metastases of survivors after tumor rechallenge. **(B)** Memory immune cells populations in survivor mice. **(C)** Inverse Simpson index of TCRβ and representative circos plots displaying the pairings of V-J gene families of the TCRβ in survivor mice and control mice. The survivor mice cured by different immunoradiotherapies were rechallenged with 344SQR cells 156 days following their final fraction of radiation. Tumor growth was monitored, and the memory immune cells populations were profiled 21 days post tumor rechallenge. Data are expressed as means ± standard error of the mean (SEM). P< 0.05 was considered statistically significant. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001, NS, not significant.

Survivor mice in combination therapies of NBTXR3 and NF-αCTLA4 modulate immune gene expression for tumor rejection

To understand how the survivor mice responded to tumor rechallenge at the genetic level, we harvested blood from survivor mice from the NBTXR3+XRT+NF-αCTLA4+αPD1 group 20 days before tumor rechallenge and 7 and 21 days post tumor rechallenge. These blood samples were then analyzed by NanoString, with blood draws from the mice in the control group taken at the same time points for comparison. As shown in Figure 6A, 20 days before tumor rechallenge, the mice within the control and NBTXR3+XRT+NF-αCTLA4+αPD1 groups displayed high interindividual variation. Overall, mice in the NBTXR3+XRT+NF-αCTLA4+αPD1 group exhibited lower activities in the various immune pathways measured relative to the control group. However, seven days post tumor rechallenge, survivor mice from the quadruple therapy achieved comparable levels of immune pathway activities to the control. In addition, 21 days post tumor rechallenge, some of the mice in the NBTXR3+XRT+NF-αCTLA4+αPD1 group had higher activities in pathways, such as Adaptive, Chemokines & Receptors, Innate, *etc.* One mouse from the quadruple therapy had much lower level of activities in most of the pathways, but it had much higher activity in B cell function than the control on both day 7 and day 21.

Analysis of individual gene expression in adaptive, innate, and T cell functional pathways revealed that, prior to tumor rechallenge, survivor mice treated with quadruple therapy had significantly altered expression of only five genes: *Cd28*, which was increased, and *Anxa1*, *Cd27*, *Ifitm1*, and *Il1rap*, which were all downregulated (Figure 6B). Seven days post tumor rechallenge, mice in the quadruple therapy group exhibited several significantly downregulated genes, including *Irf7*, *Irak*, *Stat1*, *Cd40*, *Isg15*, *Itga1*, *etc.* However, 21 days post-rechallenge, the combination therapy group had downregulated genes such as *C3ar1*, *Cd8a*, *Cklf*, *Ccr3*, *Casp8*, *Sell*, *etc.* upregulated genes, such as *Cxcr3*, *Ccl5*, *Isg15*, *Camp*, *Thbs1*, *Mavs*, *etc.* as compared to the control. By tracking the expression of immune-related genes over time (Figures 6C, D), it was found that the survivor mice in the NBTXR3+XRT+NF-αCTLA4+αPD1 group markedly upregulated the activities of immune pathways and increased the abundance of immune cells for coping with the recurring cancer cells.

Discussion

Thanks to the advances in precision radiotherapy and immune checkpoint blockade, immunoradiotherapy has been increasingly used to treat various types of cancers, particularly metastatic ones (27). A successful immunoradiotherapeutic regimen needs to address two critical problems: one is to induce tumor antigen release and subsequent cytotoxic T cell activation; the other is to minimize the immunosuppressive rebound that typically follows the initial inflammatory insult caused by the radiation. Radiotherapy can effectively kill local tumors, releasing tumor antigen and adjuvant signals, thereby priming the immune system for antitumor activity, essentially converting the tumor into an *in situ* vaccine (28). This process can be further enhanced by NBTXR3, a hafnium oxide nanoparticle (29). NBTXR3 initially developed as a radiation enhancer, has been recently utilized for immune priming (17, 18). NBTXR3-mediated radiotherapy not only leads to enhanced direct tumor destruction (29–31), but also can activate the cGAS-STING pathway in cancer cells (32), improve the immunogenic cell death and modulate the immunopeptidome for promoting antitumor immunity (33).

To address immune suppression, CPI is typically used (34). We previously demonstrated that the combination of NBTXR3 +XRT+αCTLA4+αPD1 could potently eradicate both local tumors and metastases, resulting in significantly extend survival (19). However, the application of conventional αCTLA4 is limited due to its side effects in patients (35). Encouragingly, a safer and more efficacious version of αCTLA4, called NF-αCTLA4, has been developed and is now undergoing clinical trials (NCT03110107, NCT04785287) (36). This study shows that NF-αCTLA4 is far more effective at promoting antitumor immunity in combination with XRT and curing mice (50% increase) than αCTLA4. Nevertheless, these treatment results are less effective in the absence of NBTXR3. In fact, the addition of NBTXR3 to αCTLA4 or NF-αCTLA4 markedly improved the efficacy of immunoradiotherapy. The addition of NBTXR3 to αCTLA4+XRT+αPD1 or NF-αCTLA4 +XRT+αPD1 significantly improved the control of both primary and secondary tumors. In both cases, NBTXR3 addition allowed to increase by 100% the number of cured mice and significantly increased median survival, compared to the same treatment without NBTXR3 (i.e., improvement of survival rate from 37.5% to 75% when combined with NF-αCTLA4 and αPD1). These results demonstrate the strong immunomodulatory potential of NBTXR3.

As shown in our previous studies, NBTXR3 directly enhances the tumoricidal properties of XRT. This has the added effect of promoting antitumor immunity through the upregulation of antitumor immune genes and facilitating intratumoral cytotoxic T cell infiltration (17). The significant upregulation of *Gzmb*, *Il1a*, *Il1b*, and *Cxcl2*, *Ccl1*, *etc.*, by NBTXR3, suggests this nanoparticle activates cytotoxic effector cells but also may aid their infiltration into tumors by favorably regulating chemokines.

It is worth noting that NBTXR3+XRT alone did not promote immune activities in the secondary tumor, demonstrating that the addition of CPIs is essential for creating effective antitumor immunes. Profiling of immune populations within the tumors

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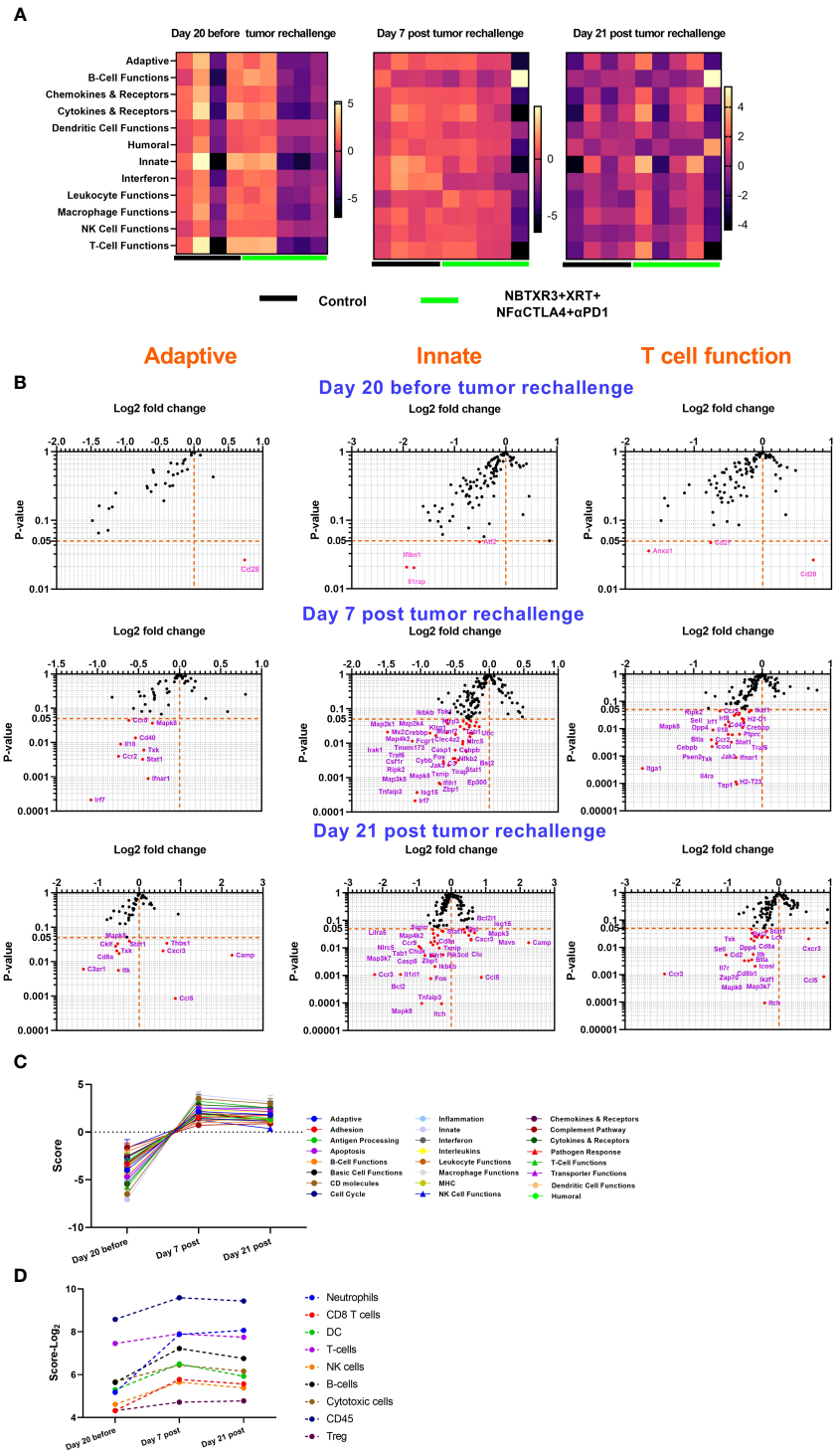

FIGURE 6
Survivor mice cured by NBTXR3 and NF-αCTLA4 mediated immunoradiotherapy upgrade immune response to prevent tumor relapse. **(A)** Comparison of immune pathway activities of survivor and control mice. **(B)** Differences in immune-related gene expression in survivor mice relative to the control. **(C)** Immune pathway activities in survivor mice before and post tumor rechallenge. **(D)** Immune cell abundance in survivor mice before and post tumor rechallenge. The survivor mice in the NBTXR3+XRT+NF-αCTLA4+αPD1 group were rechallenged with 344SQR cells 156 days post the last radiation fraction. RNA from blood harvested 20 days before, 7 and 21 days post tumor rechallenge were analyzed by NanoString.

via FACS revealed that both αCTLA4 and NF-αCTLA4 markedly reduced the number of Tregs in both primary and secondary tumors. The significantly lower Treg score from the NanoString in the NBTXR3+XRT+NF-αCTLA4+αPD1 group relative to the NBTXR3+XRT+αCTLA4+αPD1 group demonstrates that NF-αCTLA4 is more efficacious in depleting Tregs. This is a great significance, as Tregs are one of the primary immune suppression populations (37). The reduction in Treg abundance may increase the activation of $CD8^+$ T cells observed in the FACS data. In addition, significant downregulation of potent inhibitory genes such as *Foxp3*, *Ctla4*, *Lag3*, *Tgfb1*, *Il10*, and *Vegfa*, *etc.* by NF-αCTLA4 compared to αCTLA4 implies that this second generation αCTLA4 can reduce immune suppression by inhibiting a wide range of immune suppressor genes (38–40). The significantly higher percentages of Gzm B^+CD8^+ T cells in the secondary tumors of mice treated with NBTXR3+XRT+NF-αCTLA4+αPD1 compared to those treated with NBTXR3+XRT+αCTLA4 +αPD1 demonstrate that NF-αCTLA4 is more effective than conventional αCTLA4 in improving activation of CD8 T cells.

The efficacy of immunoradiotherapy lies not just in its ability to effectively eradicate existing tumors but also in preventing the relapse of cancers. Our data demonstrate that adding NBTXR3 to NF-αCTLA4-mediated immunoradiotherapy can significantly extend survival and initiate long-term immunological memory that effectively inoculates against tumor reoccurrence. This immunity manifests as elevated levels of memory T cells, including central and effector memory cells of $CD4^+$ and $CD8^+$ lineages. Also of note is that the survivor mice in the NBTXR3+NF-αCTLA4 quadruple therapy exhibited a significantly more diverse T cell repertoire than the untreated mice. Although further testing would be needed to confirm, we speculate that this results from epitope spreading, with tumor neoepitopes being exposed as a result of the combined therapy, prompting the expansion of T cells of multiple specificities.

Conclusion

In conclusion, the combination of NBTXR3 nanoparticle-enhanced XRT with NF-αCTLA4+αPD1 CPI improved the control of local tumors and metastases, resulting in a statistically significantly higher mice survival rate. The addition of XRT-activated NBTXR3 to NF-αCTLA4+αPD1 therapy was able to significantly promote the activities of a wide range of immune pathways and downregulate the activity of Treg for improved antitumor immune response. In addition, all mice cured by this combination therapy were immunized to prevent tumor re-growth by maintaining a durable and sustained antitumor memory response and a more diverse TCR repertoire. These data cast an encouraging light on future clinical trials exploring NBTXR3 with multiple CPIs.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://doi.org/10.6084/m9.figshare.21158677.v1.

Ethics statement

The animal study was reviewed and approved by IACUC, MD Anderson Cancer Center.

Author contributions

YH, SP, MC, and JW designed the study. YH, AH, and HB performed the experiments. YH, GB, SP, and QW analyzed the data. YH, GB, and SP wrote the manuscript. All of the authors discussed the results and reviewed the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

JWW reports research support from GlaxoSmithKline, Bristol Meyers Squibb, Merck, Nanobiotix, RefleXion Alkermes, Artidis, Mavu Pharma, Takeda, Varian, and Checkmate Pharmaceuticals. JWW serves on the scientific advisory board for Legion Healthcare Partners, RefleXion Medical, MolecularMatch, Merck, AstraZeneca, Aileron Therapeutics, OncoResponse, Checkmate Pharmaceuticals, Mavu Pharma, Alpine Immune Sciences, Ventana Medical Systems, Nanobiotix, China Medical Tribune, GI Innovation, Genentech and Nanorobotics. JWW is on Speaking Engagements for Ventana Medical Systems, US Oncology, Alkermes, and Boehringer Ingelheim. He is co-founder of Healios, MolecularMatch, OncoResponse and serves as an advisor to Astra Zeneca, OncoResponse, Merck, MolecularMatch, Incyte, Aileron and Nanobiotix. JWW holds stock or ownership in Alpine Immune Sciences, Checkmate Pharmaceuticals, Healios, Mavu Pharma, Legion Healthcare

Partners, MolecularMatch, Nanorobotics, OncoResponse, and RefleXion. JWW has accepted honoraria in the form of travel costs from Nanobiotix, RefleXion, Varian, Shandong University, The Korea Society of Radiology, Aileron Therapeutics and Ventana. JWW has the following patents; MP470 amuvatinib, MRX34 regulation of PDL1, XRT technique to overcome immune resistance. MD Anderson Cancer Center has a trademark for XRTTM. SP and JS are employees of Nanobiotix.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

This study received funding from Nanobiotix. The funder had the following involvement with the study: experiment design, data analysis, and manuscript writing.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1022011/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) Individual curves of primary and secondary tumors treated with various combination therapies. **(B)** Tumor growth curves and survival rates of mice treated with various combination therapies, in which NF-αCTLA4 was intratumorally injected on day 7 (IT1) or days 7 and 11 (IT2) into the primary tumors.

SUPPLEMENTARY FIGURE 2
Alteration of immune pathway function by NF-αCTLA4 and NBTXR3. Raw transcript abundance was determined using the nCounter MAX Analysis System, as described in the methods. The average $\log_2$ fold-change of each gene was determined in , . Genes significantly up- or downregulated were manually assigned to the functional group. **(A)** Functional groups of genes significantly up- or downregulated within primary tumors between NBTXR3+XRT+NF-αCTLA4+αPD1 and NBTXR3+XRT+αCTLA4+αPD1. **(B)** Functional groups of genes significantly up- or downregulated within secondary tumors between treatment groups containing NF-αCTLA4 and those without. **(C)** Functional groups of genes significantly up- or downregulated within secondary tumors between NBTXR3+XRT+NF-αCTLA4+αPD1 and XRT+NF-αCTLA4+αPD1. **(D)** Genes coding for cytokines or their respective receptors that were significantly up- or downregulated within secondary tumors between treatment groups containing NBTXR3 and those without.

SUPPLEMENTARY FIGURE 3
NBTXR3, in combination with NF-αCTLA4, alters immune-related gene expression in primary tumors. **(A)** Relative immune cells score in the primary tumors. **(B)** Changes in the expression of immune-related genes induced by NF-αCTLA4 or NBTXR3. Mice bearing 344SQR tumors were treated with various immunoradiotherapies in , and the primary tumors were harvested on day 18. The RNA extracted from the tumors was analyzed by a nCounter PanCancer Immune Profiling Panel.

SUPPLEMENTARY FIGURE 4
NBTXR3, in combination with NF-αCTLA4, alters immune-related gene expression in secondary tumors. **(A)** Relative immune cells score in the secondary tumors. **(B)** Changes in the expression of immune-related genes induced by NF-αCTLA4 or NBTXR3. Mice bearing 344SQR tumors were treated with various immunoradiotherapies in , and the secondary tumors were harvested on day 18. The RNA extracted from the tumors was analyzed by a nCounter PanCancer Immune Profiling Panel.

SUPPLEMENTARY FIGURE 5
Representative flow cytometry graphs of memory T cells in the blood. Memory T cells populations 21 days post tumor rechallenge were profiled. The survivor mice cured by different immunoradiotherapies were rechallenged with 344SQR cells 156 days following their final fraction of radiation. Naïve mice were also challenged with the same number of 344SQR cells and served as control.

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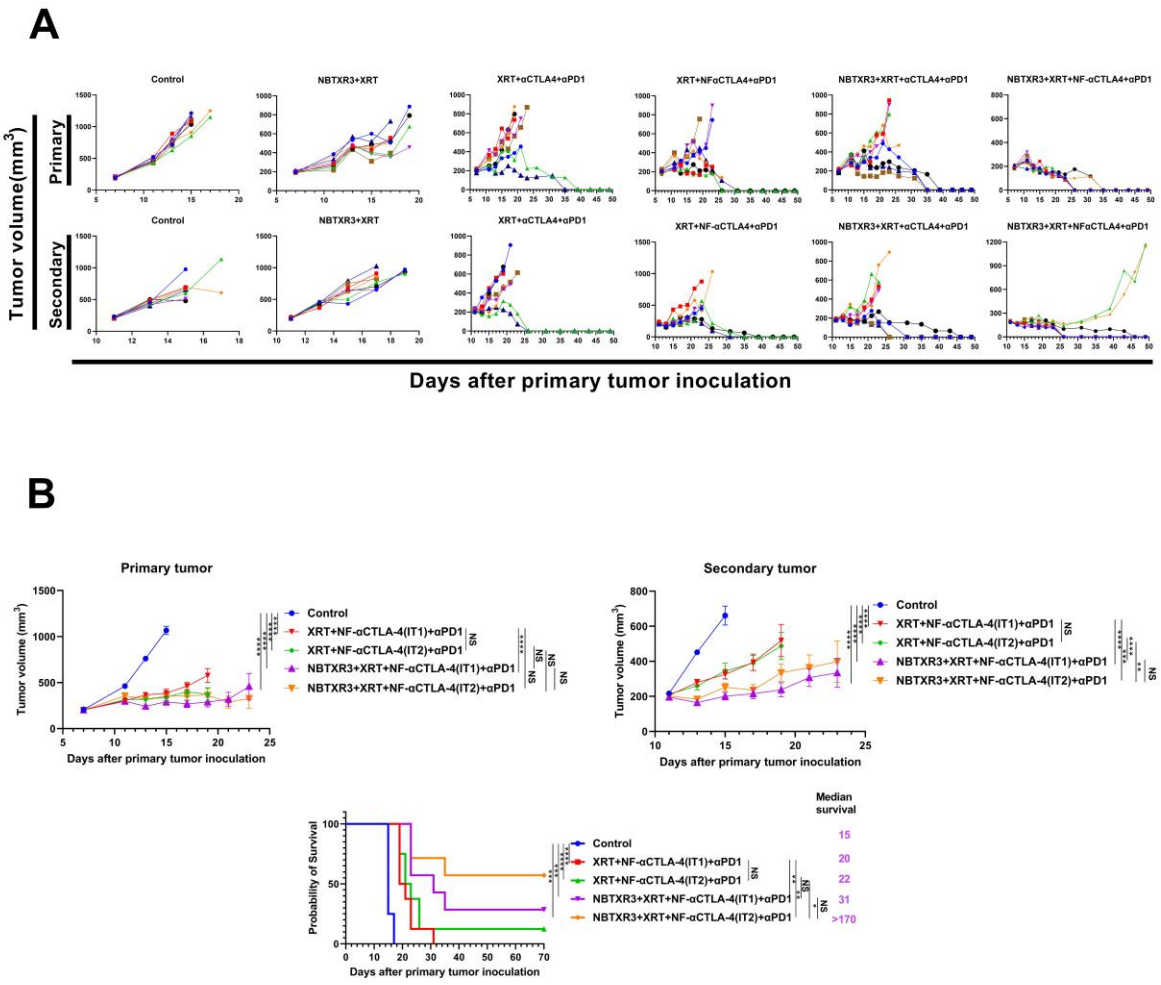
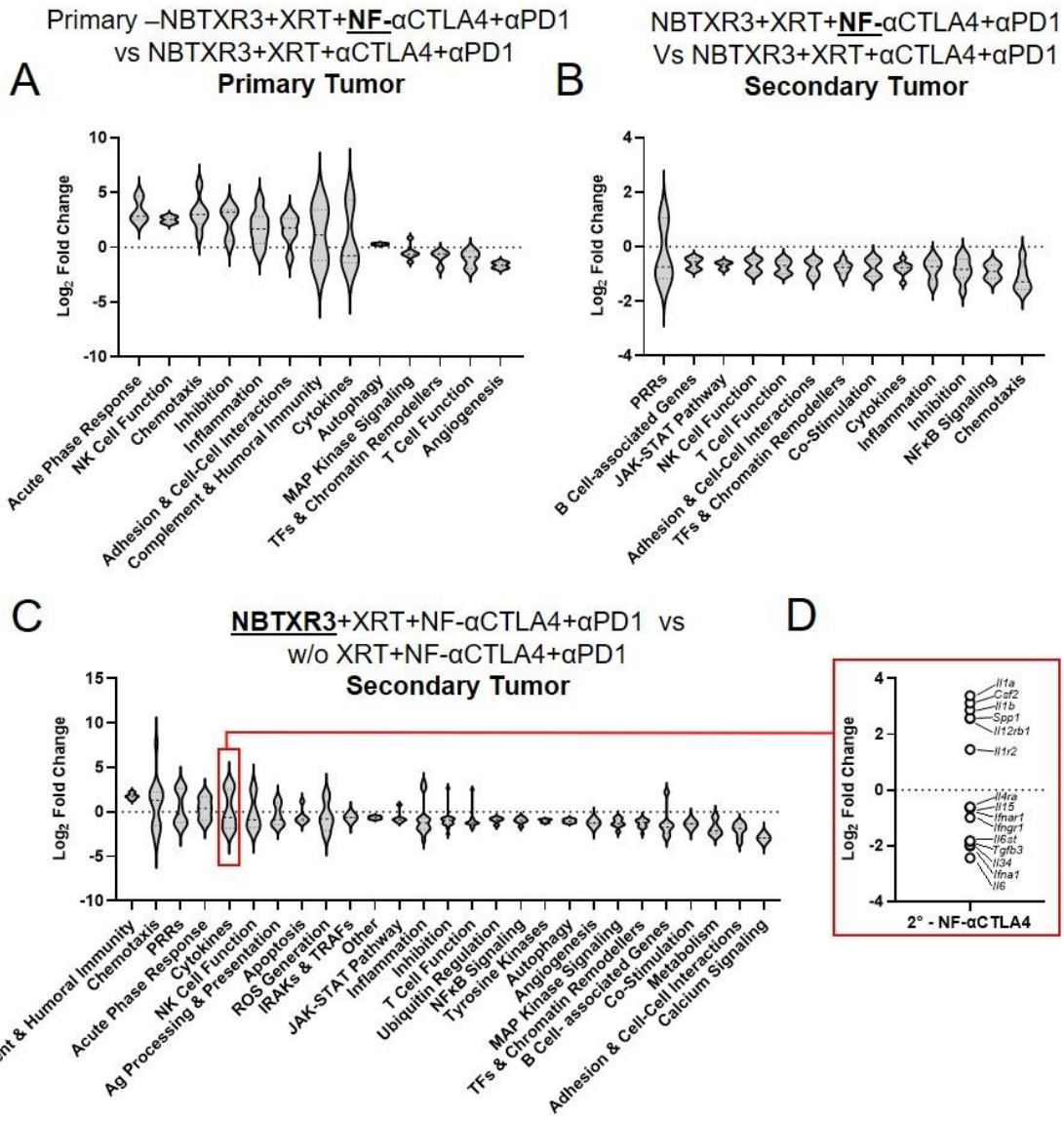
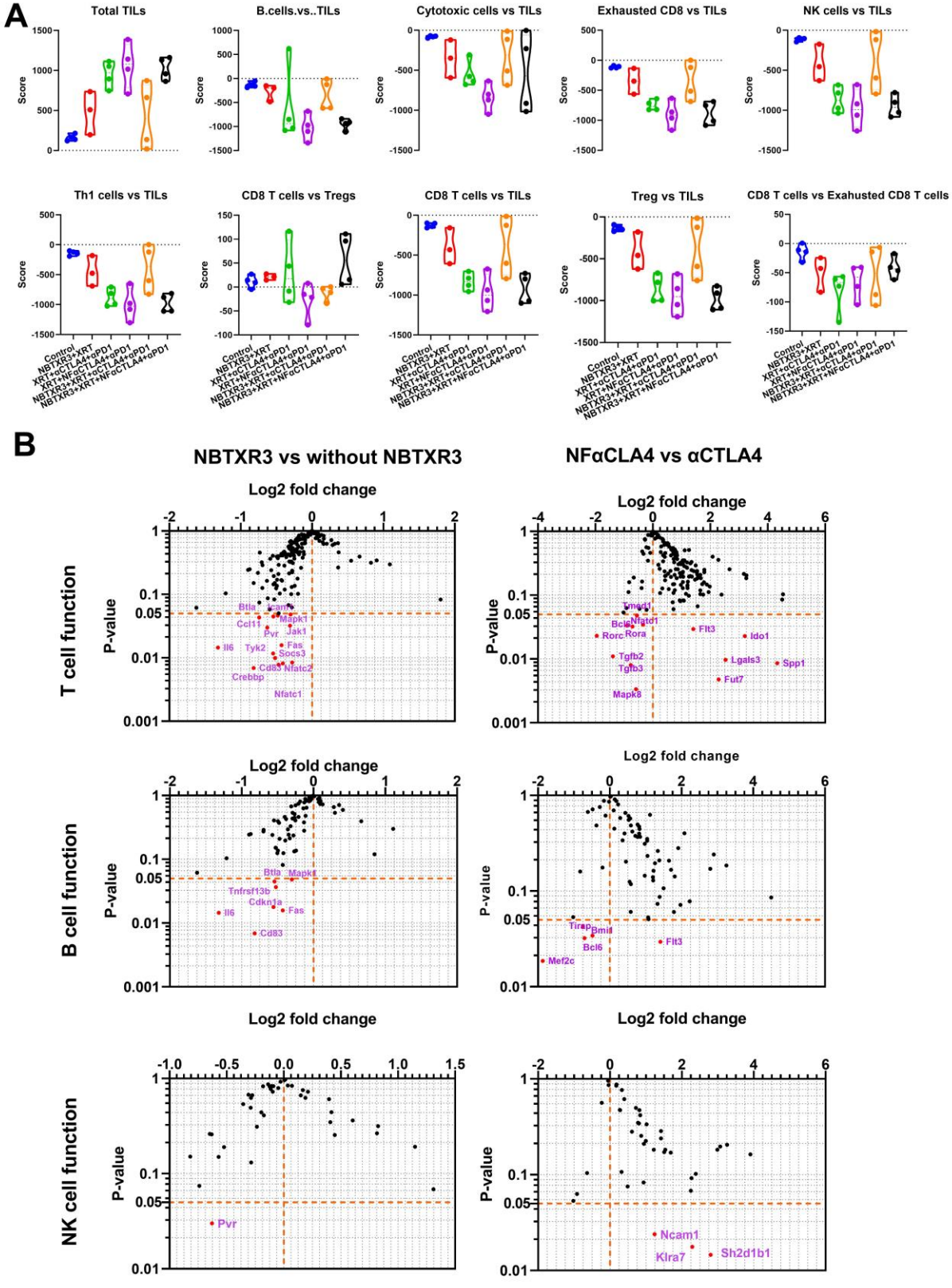


Supplementary Figure 1 | (A) Individual curves of primary and secondary tumors treated with various combination therapies. **(B)** Tumor growth curves and survival rates of mice treated with various combination therapies, in which NF-αCTLA4 was intratumorally injected on day 7 (IT1) or days 7 and 11 (IT2) into the primary tumors.



Supplementary Figure 2 | Alteration of immune pathway function by NF-αCTLA4 and NBTXR3. Raw transcript abundance was determined using the nCounter MAX Analysis System, as described in the methods. The average log_2 fold-change of each gene was determined in , . Genes significantly up- or downregulated were manually assigned to the functional group. **(A)** Functional groups of genes significantly up- or downregulated within primary tumors between NBTXR3+XRT+NF-αCTLA4+αPD1 and NBTXR3+XRT+αCTLA4+αPD1. **(B)** Functional groups of genes significantly up- or downregulated within secondary tumors between treatment groups containing NF-αCTLA4 and those without. **(C)** Functional groups of genes significantly up- or downregulated within secondary tumors between NBTXR3+XRT+NF-αCTLA4+αPD1 and XRT+NF-αCTLA4+αPD1. **(D)** Genes coding for cytokines or their respective receptors that were significantly up- or downregulated within secondary tumors between treatment groups containing NBTXR3 and those without.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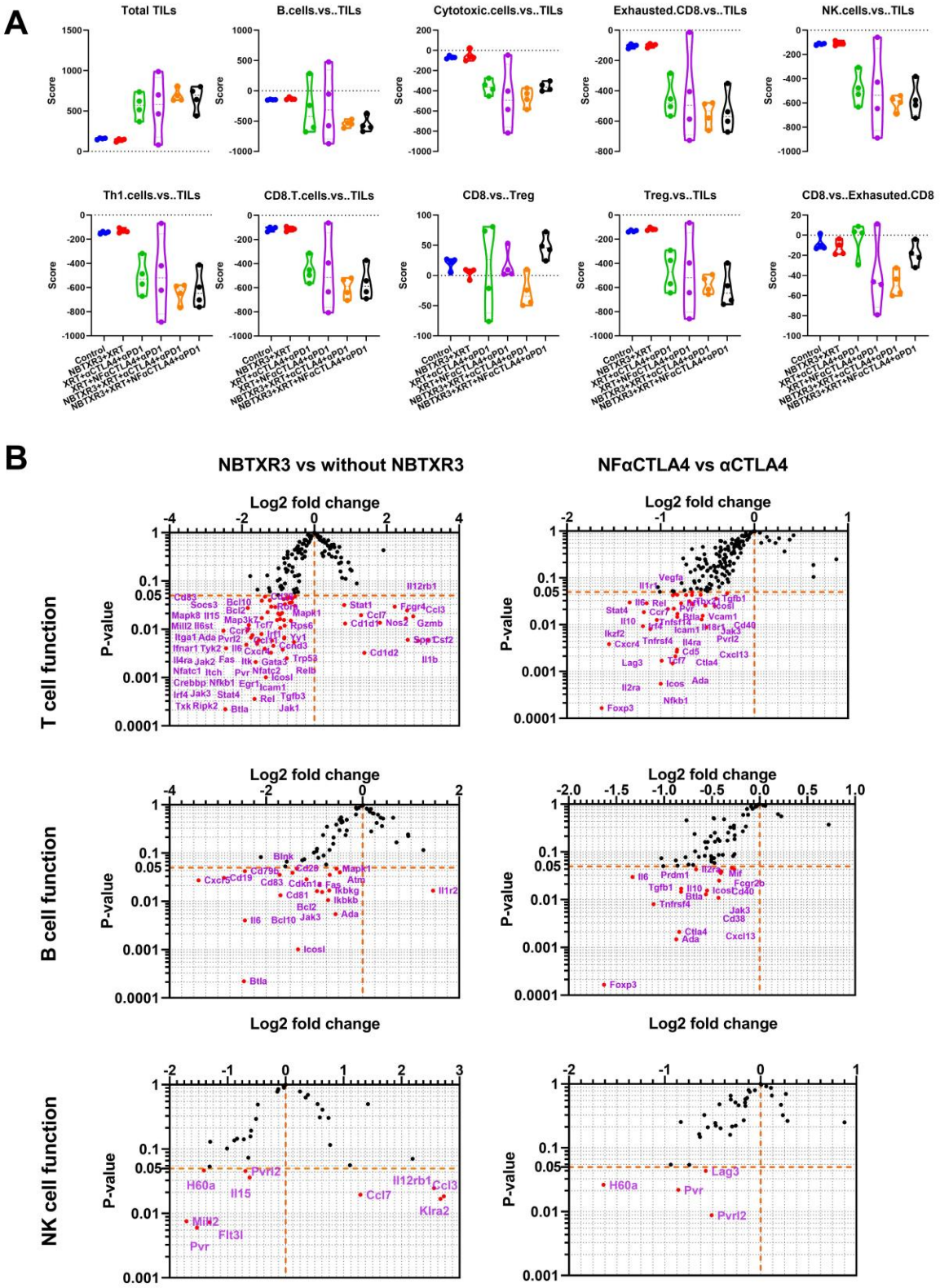
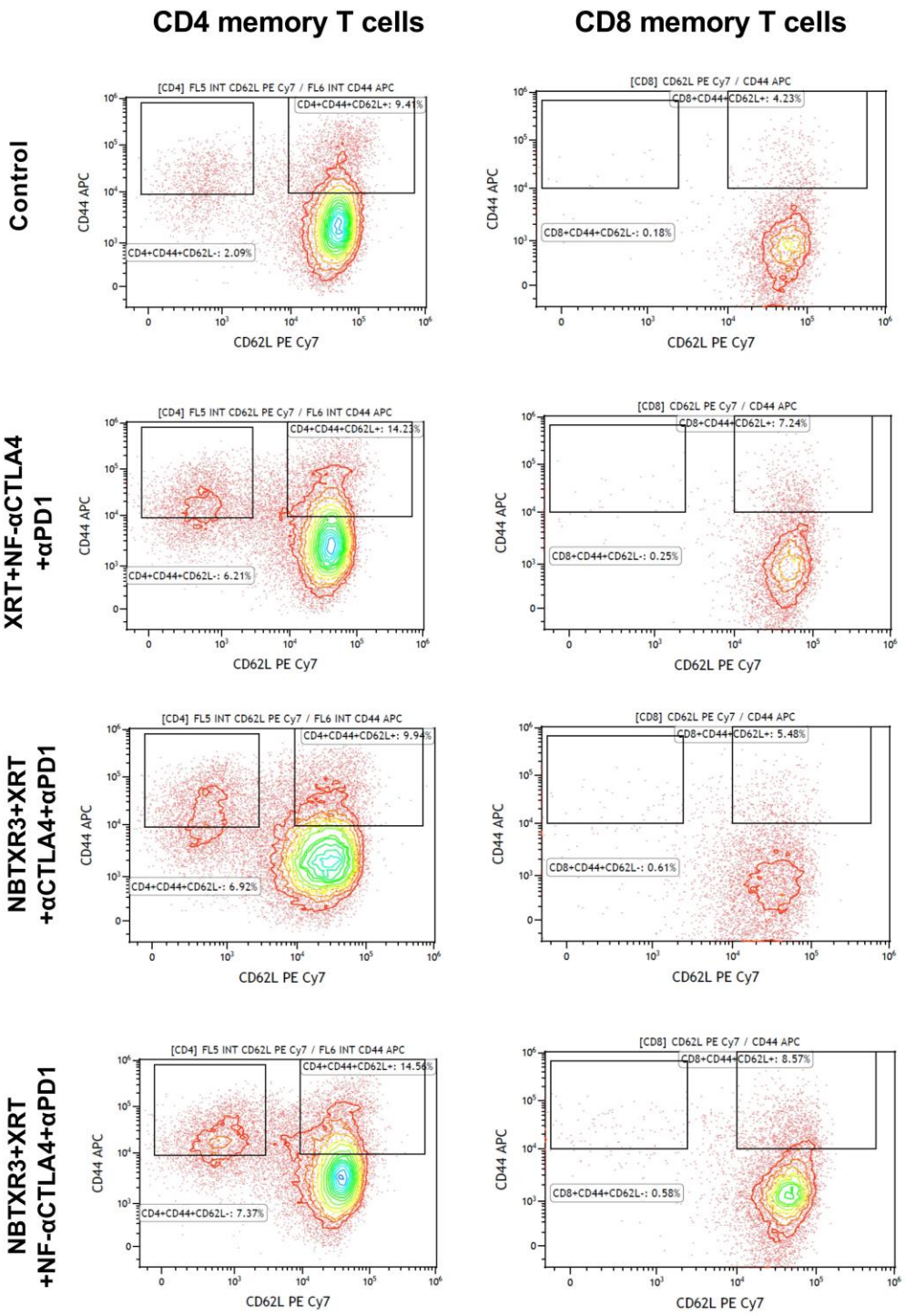
Total TILs
B.cells.vs..TILs
Cytotoxic cells vs TILs
Exhausted CD8 vs TILs
NK cells vs TILs
Th1 cells vs TILs
CD8 T cells vs Tregs
CD8 T cells vs TILs
Treg vs TILs
CD8 T cells vs Exhausted CD8 T cells
Score
Control
NBTXR3+XRT
XRT+αCTLA4+αPD1
XRT+NFαCTLA4+αPD1
NBTXR3+XRT+αCTLA4+αPD1
NBTXR3+XRT+NFαCTLA4+αPD1
B
NBTXR3 vs without NBTXR3
NFαCLA4 vs αCTLA4
Log2 fold change
P-value
T cell function
B cell function
NK cell function
Btla
Icam1
Ccl11
Mapk1
Pvr
Jak1
Il6
Tyk2
Fas
Socs3
Cd83
Nfatc2
Crebbp
Nfatc1
Tmed1
Bcl6
Nfatd1
Rorc
Rora
Flt3
Ido1
Tgfb2
Tgfb3
Lgals3
Spp1
Fut7
Mapk8
Mapk1
Tnfrsf13b
Cdkn1a
Tirap
Bmi1
Mef2c
Ncam1
Klra7
Sh2d1b1

Supplementary Figure 3 | NBTXR3, in combination with NF-αCTLA4, alters immune-related gene expression in primary tumors. **(A)** Relative immune cells score in the primary tumors. **(B)** Changes in the expression of immune-related genes induced by NF-αCTLA4 or NBTXR3. Mice bearing 344SQR tumors were treated with various immunoradiotherapies in , and the primary tumors were harvested on day 18. The RNA extracted from the tumors was analyzed by a nCounter PanCancer Immune Profiling Panel.



Supplementary Figure 4 | NBTXR3, in combination with NF-αCTLA4, alters immune-related gene expression in secondary tumors. **(A)** Relative immune cells score

in the secondary tumors. **(B)** Changes in the expression of immune-related genes induced by NF-αCTLA4 or NBTXR3. Mice bearing 344SQR tumors were treated with various immunoradiotherapies in , and the secondary tumors were harvested on day 18. The RNA extracted from the tumors was analyzed by a nCounter PanCancer Immune Profiling Panel.



Supplementary Figure 5 | Representative flow cytometry graphs of memory T cells in the blood. Memory T cells populations 21 days post tumor rechallenge were profiled. The survivor mice cured by different immunoradiotherapies were rechallenged with 344SQR cells 156 days following their final fraction of radiation. Naïve mice were also challenged with the same number of 344SQR cells and served as control.

SUPPLEMENTAL TABLE 1: Genes significantly differentially regulated between NBTXR3+XRT+NF-αCTLA4+αPD1 and NBTXR3+XRT+αCTLA4+αPD1 treatment groups in the primary tumor.

Gene	Log 2 Fold-Change	P-Value	Full Name	Notable Aliases	Function
Acute Phase Response					
Clu	2.84	0.0133	Clusterin	Apolipoprotein J; Ku70-binding protein 1 (KUB1)	Extracellular chaperone that promotes clearance of inflammation and injury-induced immune complexes; protects cells against apoptosis and against cytolysis by complement
Dmbt1	4.69	0.00901	Deleted in malignant brain tumors 1	Hensin; muclin; salivary agglutinin; glycoprotein 340	Function unknown; may play roles in mucosal defense, cellular immune defense, epithelial differentiation, liver regeneration, cell fate and differentiation, taste, and others
Lcn2	2.52	0.0139	Lipocalin 2	Neutrophil gelatinase-associated lipocalin (NGAL)	Neutrophil-secreted factor that sequesters iron-containing siderophores; also functions as a growth factor
Adhesion & Cell-Cell Interactions					
Fut7	2.29	0.00476	Fucosyltransferase 7	Selectin-ligand synthase	Participates in the biosynthesis of the sialyl Lewis X (sLe(x)), a carbohydrate involved in cell and matrix adhesion that enables leukocyte accumulation at a site of inflammation
Ifitm1	2.95	0.0193	Interferon-induced transmembrane protein 1	CD225	IFN-induced antiviral protein implicated in cell adhesion and control of cell growth and migration
Itga5	1.24	0.0354	Integrin alpha 5		Pairs with ITGB1 to form a receptor for fibronectin and IL-1β
Jam3	-0.918	0.0422	Junctional adhesion molecule C		Immunoglobulin that mediates tight junctions between endothelial cells; mediates transepithelial migration of PMNs; promotes chemotaxis of vascular endothelial cells and stimulates angiogenesis
Lgals3	2.53	0.00971	Galectin 3		Galactose-specific lectin that binds IgE; involved in acute inflammatory responses, including neutrophil activation and adhesion, chemoattraction of monocytes macrophages, opsonization of apoptotic

					neutrophils, and activation of mast cells
Ncam1	1.24	0.0239	Neural cell adhesion molecule 1	CD56	Cell adhesion molecule involved in the expansion of T-, B-, and NK cells; potentiates signal transduction by interacting with fibroblast growth factor receptors, N-cadherin, and other components of the extracellular matrix; required for effecient cytotoxic cell killing in NK cells
Angiogenesis					
Mef2c	-1.87	0.0186	Myocyte enhancer factor 2c		Transcriptional activator that binds specifically to the MEF2 element present in the regulatory regions of many muscle-specific genes; controls cardiac morphogenesis and myogenesis, and is also involved in vascular development; required for B cell survival and proliferation in response to BCR stimulation, efficient IgG1 antibody responses to T cell-dependent antigens, and for normal induction of germinal center B cells
Tgfb2	-1.39	0.011	Transforming growth factor beta 2	Cetermin; polyergin	Multifunctional protein that regulates various processes such as angiogenesis and heart development
Autophagy					
Atg12	0.221	0.0459	Autophagy related 12		Pairs with ATG5 to promote the extension of the phagophoric membrane in autophagic vesicles
Irgm2	0.371	0.0374	Immunity-related GTPase family M member 2	Interferon-inducible protein 1 (IFI1)	Function not fully known, but most likely regulates autophagy and pro-inflammatory cytokine production
B Cell-associated Genes					
Mef2c	-1.87	0.0186	Myocyte enhancer factor 2c		Transcriptional activator that binds specifically to the MEF2 element present in the regulatory regions of many muscle-specific genes; controls cardiac morphogenesis and myogenesis, and is also involved in vascular development; required for B cell

					survival and proliferation in response to BCR stimulation, efficient IgG1 antibody responses to T cell-dependent antigens, and for normal induction of germinal center B cells
Chemotaxis					
Ccl1	3.21	0.015	C-C motif chemokine ligand 1	T lymphocyte-secreted protein I-309; small inducible cytokine A1	Chemoattractant for monocytes but not neutrophils; binds to CCR8
Cxcr2	3.5	0.0133	C-X-C motif chemokine receptor 2	CD182; IL-8 receptor B	Receptor for IL-8 and CXCL3; powerful chemoattractant for neutrophils
Ccl17	2.79	0.0406	C-C motif chemokine ligand 17		Chemoattractant ligand for CCR4 and -8; attracts T cells
Cxcl11	2.5	0.0442			Dominant ligand for CXCR3; attracts activated T cells; strongly induced by IFNγ
Cxcl14	1.16	0.0459	C-X-C motif chemokine ligand 14	Bolekine; macrophage inflammatory protein 2-gamma (MIP2γ)	Potent chemoattractant for neutrophils
Cxcl3	5.74	0.0493	C-X-C motif chemokine ligand 3	GRO3 oncogene	Ligand for CXCR2; attracts neutrophils
Complement & Humoral Immunity					
Cfi	3.42	0.00863	Complement factor I	Konglutinogen-activating factor (KAF)	Serine protease that inhibits all three complement pathways by inactivating C3b and C4b
Cd55	-1.18	0.0107	Cluster of differentiation 55	Complement decay-accelerating factor	Cell surface glycoprotein that interacts with surface-bound C4b and inhibits its conversion of C2 to C2b
Cytokines					
Spp1	4.33	0.00858	Secreted phosphoprotein 1	Osteopontin	Cytokine involved in enhancing production of IFNγ and IL-12 and reducing production of IL-10
Tgfb2	-1.39	0.011	Transforming growth factor beta 2	Cetermin; polyergin	Multifunctional protein that regulates various processes such as angiogenesis and heart development
Tgfb3	-0.767	0.00802	Transforming growth factor beta 3		Multifunctional protein that regulates embryogenesis and cell differentiation
Inflammation					

Clu	2.84	0.0133	Clusterin	Apolipoprotein J; Ku70-binding protein 1 (KUB1)	Extracellular chaperone that promotes clearance of inflammation and injury-induced immune complexes; protects cells against apoptosis and against cytolysis by complement
Irgm2	0.371	0.0374	Immunity-related GTPase family M member 2	Interferon-inducible protein 1 (IFI1)	Function not fully known, but most likely regulates autophagy and pro-inflammatory cytokine production
Isg20	1.69	0.0482	Interferon-stimulated gene 20		IFN-induced antiviral exoribonuclease that acts on ssRNA with minor activity towards ssDNA
Itga5	1.24	0.0354	Integrin alpha 5		Pairs with ITGB1 to form a receptor for fibronectin and IL-1β
Lgals3	2.53	0.00971	Galectin 3		Galactose-specific lectin that binds IgE; involved in acute inflammatory responses, including neutrophil activation and adhesion, chemoattraction of monocytes macrophages, opsonization of apoptotic neutrophils, and activation of mast cells
Mavs	-0.456	0.0206	Mitochondrial antiviral signaling protein	IFNβ promoter stimulator protein 1 (ISP-1)	Intermediary protein involved in the nonclassical inflammasome pathway; acts downstream of DDX58 and IFIH1, leading to the activation of NFκB, IRF3, and IRF7, and the subsequent induction of IFNβ and RANTES
Spp1	4.33	0.00858	Secreted phosphoprotein 1	Osteopontin	Cytokine involved in enhancing production of IFNγ and IL-12 and reducing production of IL-10
Inhibition					
Bcl2l1	0.612	0.0499	B cell lymphoma 2 like 1	Protein phosphatase 1	Potent inhibitor of caspase-mediated cell death
Cfi	3.42	0.00863	Complement factor I	Konglutinogen-activating factor (KAF)	Serine protease that inhibits all three complement pathways by inactivating C3b and C4b
Ido1	3.2	0.0228	Indoleamine 2,3-dioxygenase 1		Initiates catabolism of tryptophan; limits immunopathology by inhibiting T cell division
Macrophage-associated Genes					
Mst1r	1.42	0.0124	Macrophage-stimulating 1 receptor	CD136; RON; protein tyrosine kinase 8 (PTK8)	Transduces intracellular signals upon binding to MST1 ligand; regulates many physiological

					processes including cell survival, migration and differentiation
MAP Kinase Signaling					
Ecsit	-1.32	0.0171	Evolutionarily conserved signaling intermediate in Toll pathway		Adapter protein of the Toll-like and IL-1 receptor signaling pathways; involved in the activation of NFκB *via* MAP3K1; promotes proteolytic activation of MAP3K1; involved in the BMP signaling pathway.
Mapk3	0.878	0.0187	Mitogen-activated protein kinase 3	Extracellular signal-regulated kinase 1 (ERK1)	Serine/threonine kinase that acts as an essential component of the MAP kinase signal transduction pathway
Mapk8	-0.586	0.00335	Mitogen-activated protein kinase 8	c-Jun N-terminal kinase 1 (JNK1); Stress-activated protein kinase 1c (SAPK1)	Serine/threonine-protein kinase involved in various processes such as cell proliferation, differentiation, migration, transformation and programmed cell death; phosphorylates a number of transcription factors, primarily components of AP-1 such as JUN, JDP2, and ATF2, thus regulating AP-1 transcriptional activity; promotes stressed cell apoptosis by phosphorylating key regulatory factors including p53/TP53 and Yes-associates protein YAP1; required for Th1 differentiation
Mapk14	-0.52	0.0405	Mitogen-activated protein kinase 14		One of the four p38 MAPKs; key kinase in the cascades of cellular responses evoked by extracellular stimuli such as proinflammatory cytokines
Map2k2	-0.639	0.0334	Mitogen-activated protein kinase kinase 2	MAPK/ERK kinase 2 (MEK2)	Catalyzes the concomitant phosphorylation of a threonine and a tyrosine residue in a TQY sequence located in MAP kinases; activates ERK1 and -2
Map2k4	-0.795	0.0199	Mitogen-activated protein kinase kinase 4	MAPK/ERK kinase 4 (MEK4); c-Jun N-terminal kinase kinase 1 (JNKK1)	Dual specificity protein kinasethat acts as an essential component of the stress-activated protein/c-Jun N-terminal kinase (SAP/JNK) signaling pathway
Mapkapk2	-0.328	0.00667	MAP kinase-activated protein kinase 2		Serine/threonine-protein kinase involved in cytokine production, endocytosis, reorganization of the cytoskeleton, cell migration, cell cycle control, chromatin remodeling, DNA damage

					response, and transcriptional regulation
NK Cell Function					
Klra7	2.8	0.0146	Killer cell lectin-like receptor, subfamily A, member 7	LGL-1; Ly49G	Potential triggering molecule on murine NK cells
Sh2d1b1	2.29	0.0177	SH2 domain-containing protein 1B	EAT2	Cytoplasmic adapter regulating receptors of the SLAM family; stimulates polarization of the microtubule-organizing center and cytotoxic granules toward the NK cell synapse
Pattern Recognition Receptors					
Tirap	-0.749	0.0423	TIR domain-containing adaptor protein		Adaptor protein involved in TLR2 and TLR4 signaling; acts *via* IRAK2 and TRAF6, leading to the activation of NFκB, MAPK1, MAPK3 and JNK, and resulting in cytokine secretion and the inflammatory response; positively regulates the production of TNFα and IL-6.
ROS Generation					
Txnip	-0.7	0.00572	Thioredoxin interacting protein		Thiol-oxidoreductase; protects cells from oxidative stress by inhibiting thioredoxin
T Cell Function					
Nfatc1	-0.341	0.0346	Nuclear factor of activated T cells, cytoplasmic 1		Inducible nuclear component of the NFAT TF complex; mediates induction of IL-2 and IL-4 in T cells
Rora	-0.899	0.0334	Retinoic acid receptor-related orphan receptor A		Nuclear receptor that binds hormone response elements upstream of several genes to enhance the expression of those genes
Rorc	-1.95	0.0231	Retinoic acid receptor-related orphan receptor C		Plays a key role, downstream of IL-6 and TGFβ, and synergistically with RORA, for lineage specification of uncommitted CD4+ T helper cells into Th17 cells; may inhibit the expression of Fas ligand and IL-2; may also play a role in the pre-TCR activation cascade leading to the maturation of α/β T cells
Transcription Factors & Chromatin Remodellers					

Bcl6	-0.706	0.0322	B cell lymphoma 6 transcriptional repressor		Zing finger TF required for GC and memory formation in both B and T cells
Bmi1	-0.483	0.0342	B lymphoma Mo-murine lymphoma virus insertion region 1 homolog	Polycomb group RING finger protein 4 (PCGF4)	Major component of the polycomb group complex 1 an essential epigenetic repressor of multiple regulatory genes
Hmgb1	-0.499	0.0125	High-mobility group box 1		Remodels chromatin to make DNA more available for transcription
Mef2c	-1.87	0.0186	Myocyte enhancer factor 2c		Transcriptional activator that binds specifically to the MEF2 element present in the regulatory regions of many muscle-specific genes; controls cardiac morphogenesis and myogenesis, and is also involved in vascular development; required for B cell survival and proliferation in response to BCR stimulation, efficient IgG1 antibody responses to T cell-dependent antigens, and for normal induction of germinal center B cells
Tyrosine Kinases					
Mst1r	1.42	0.0124	Macrophage-stimulating 1 receptor	CD136; RON; protein tyrosine kinase 8 (PTK8)	Transduces intracellular signals upon binding to MST1 ligand; regulates many physiological processes including cell survival, migration and differentiation
Other					
Flt3	1.41	0.0294	Fetal liver kinase 3	CD135; stem cell tyrosine kinase 1 (STK1)	Cell-surface receptor for the cytokine FLT3LG; regulates differentiation, proliferation, and survival of hematopoietic progenitor cells and DCs
Tmed1	-0.555	0.048	Transmembrane EMP24 protein transport domain containing 1	Tp24	IL1RL1-interacting protein with potential roles in vesicular protein trafficking in the early secretory pathway, incorporation of secretory cargo molecules into transport vesicles, and cytosolic vesicle coat formation

Supplementary Table 2: Genes significantly differentially regulated between NBTXR3+XRT+NF-αCTLA4+αPD1 and NBTXR3+XRT+αCTLA4+αPD1 treatment groups in the secondary tumor.

Gene	Log 2 Fold-Change	P-Value	Full Name	Notable Aliases	Function
Adhesion & Cell-Cell Interactions					
Icam1	-0.922	0.0216	Intracellular adhesion molecule 1		Cell surface glycoprotein that serves as strong adhesive ligand for LFA-1; important for leukocyte mobility and costimulation
Itga5	-0.509	0.047	Integrin alpha 5		Pairs with ITGB1 to form a receptor for fibronectin and IL-1β
Thbs1	-1.13	0.0058	Thrombospondin 1		Adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions; ligand for CD36
Vcam1	-0.523	0.0256	Vascular cell adhesion molecule 1	CD106	Endothelial-cell adhesion molecule that binds to ITGA4/ITGB1 on leukocytes and mediates both adhesion and signal transduction
Angiogenesis					
Vegfa	-0.869	0.0425	Vascular endothelial growth factor A		Glysosylated mitogen that promotes vascular permeability, vasculogenesis, angiogenesis, and cell migration
Antigen Processing & Presentation					
H60a	-1.64	0.0264	Histocompatibility 60a		Enables NK cell lectin-like receptor binding activity
B Cell-associated Genes					
Btla	-0.822	0.0146	B and T lymphocyte attenuator	CD272	Inhibitory cell surface protein that inhibits T cell function by binding to B7H4 and TNFRSF14
Cd5	-0.828	0.00265	Cluster of differentiation 5	LEU1	Type-I transmembrane glycoprotein found on the surface of T and B cells; may act as a receptor in regulating T cell proliferation
Fcgr2b	-0.399	0.0391	Fc fragment of immunoglobulin gamma receptor IIb	CD32	Low affinity receptor for the Fc region of complexed or aggregated γ-Igs; involved in a variety of effector and regulatory functions such as phagocytosis of immune complexes and modulation of antibody production by B cells
Icosl	-0.551	0.0155	Inducible T cell costimulator ligand	CD275	Ligand for T cell-specific co-receptor ICOS; also induces B cell proliferation and plasma cell differentiation
Prdm1	-0.419	0.0403	Positive regulatory domain I-binding factor	B lymphocyte-induced maturation	Transcription factor that plays a role in the development, retention, and long-term establishment of T cell,

				protein (BLIMP1)	NK cell, and NK-T cells in non-lymphoid organs; drives the maturation of B cell into Ig secreting cells
Chemotaxis					
Ccl24	-1.63	0.00634	C-C motif chemokine ligand 24	Eotaxin-2	Chemoattractant for resting T cells and eosinophils
Ccr7	-1.18	0.0185	C-C chemokine receptor type 7	CD197	Chemokine receptor that activates B and T cells and promotes their homing to secondary lymphoid organs; also stimulates DC expression of MHC class I and II
Ccr8	-1.4	0.0188	C-C motif chemokine receptor 8		Receptor for CCL1; may regulate monocyte chemotaxis and thymic cell line apoptosis
Cxcl13	-0.291	0.0464	C-X-C motif chemokine ligand 13	BLC, BCA-1	B cell chemokine induced by type I interferons; participates in germinal center formation
Cxcl14	-0.82	0.0326	C-X-C motif chemokine ligand 14	Bolekine; macrophage inflammatory protein 2-gamma (MIP2γ)	Potent chemoattractant for neutrophils
Cxcr4	-1.55	0.00386	Chemokine receptor CXCR4	Fusin; CD184	Alpha-chemokine receptor specific for SDF1 *aka* CXCL12
Complement & Humoral Immunity					
Fcer1a	-1.12	0.00278	Fc epsilon receptor 1a		High affinity receptor for IgE; responsible for initiating the allergic response
Co-Stimulation					
Cd40	-0.424	0.025	Cluster of differentiation 40		APC-expressed co-stimulatory protein that binds to CD40L on CD4+ T cells, causing activation of both
Icosl	-0.551	0.0155	Inducible T cell costimulator ligand	CD275	Ligand for T cell-specific co-receptor ICOS; also induces B cell proliferation and plasma cell differentiation
Tnfrsf4	-1.11	0.00805	TNF receptor superfamily member 4	OX40; CD134	Receptor for TNFSF4/OX40L/GP34; costimulatory molecule implicated in long-term T cell immunity; activates NFκB through its interaction with adaptor proteins TRAF2 and TRAF5; suppresses apoptosis through upregulation of BCL2
Tnfsf14	-1.04	0.0126	TNF receptor superfamily member 14	CD270	Receptor for four distinct ligands: LIGHT, lymphotoxin-α, BTLA, and CD160, altogether defining a complex stimulatory and inhibitory

					signaling network; signals *via* the TRAF2-TRAF3 E3 ligase pathway to promote immune cell survival and differentiation; participates in bidirectional cell-cell contact signaling between APCs and lymphocytes; delivers costimulatory signals to T cells, promoting cell proliferation and effector functions; interacts with CD160 on NK cells, enhancing IFNγ production and anti-tumor immune response; upon binding to CD160 on activated CD4+ T cells, downregulates CD28 costimulatory signaling; participates in *cis* or *trans* reactions with BTLA - *cis* interactions seem to promote quiescence; *trans* interactions seem to promote survival
Cytokines					
Tgfb1	-0.408	0.0361	Transforming growth factor beta 1		Multifunctional protein that regulates the growth and differentiation of various cell types and is involved in various processes, such as normal development, immune function, microglia function and responses to neurodegeneration; can induce EMT and cell migration in various cell types; frequently acts as an immunosuppressive cytokine in the TME
Il1r1	-0.655	0.0273	Interleukin 1 receptor type I	CD121a	Receptor for IL-1α and IL-1β; drives several cytokine-induced and inflammatory responses through activation of NFκB and MAPK; recruits TOLLIP, MyD88, IRAK1, and IRAK2
Il2ra	-0.665	0.0431	Interleukin 2 receptor subunit alpha	CD25	Alpha chain of the IL-2 receptor
Il18r1	-0.728	0.0431	Interleukin 18 receptor 1	CD218a	Receptor for IL-18
Il10	-0.821	0.017	Interleukin 10	Cytokine synthesis inhibitory factor (CSIF)	Major immunoregulatory cytokine that inhibits production of pro-inflammatory cytokines, including GM-CSF, G-CSF, IL-1α, IL-1β, IL-6, IL-8, and TNFα; also interferes with antigen presentation by reducing expression of MHC class II and co-stimulatory molecules, thereby inhibiting their ability to induce T cell activation

Il4ra	-0.825	0.00293	Interleukin 4 receptor subunit alpha	CD124	Alpha chain for the IL-4 and IL-13 receptors; involved in Th2 differentiation and IgE production
Il1rl1	-0.935	0.0323	Interleukin 1 receptor-like 1		Receptor for IL-33; recruits MyD88, IRAK1, IRAK4, and TRAF6; activates ERK1, ERK2, and MAPK14
Il6	-1.33	0.0298	Interleukin 6		Pro-inflammatory cytokine that signals through the JAK and STAT pathways
Inflammation					
Cd38	-0.431	0.0109	Cluster of differentiation 38	ADP-ribosyl cyclase 1	Synthesizes the second messengers cyclic ADP-ribose and NADPH; appears to play a critical role in inflammation, although its exact immunological function(s) remain(s) poorly defined
Il1r1	-0.655	0.0273	Interleukin 1 receptor type I	CD121a	Receptor for IL-1α and IL-1β; drives several cytokine-induced and inflammatory responses through activation of NFκB and MAPK; recruits TOLLIP, MyD88, IRAK1, and IRAK2
Il6	-1.33	0.0298	Interleukin 6		Pro-inflammatory cytokine that signals through the JAK and STAT pathways
Il18r1	-0.728	0.0431	Interleukin 18 receptor 1	CD218a	Receptor for IL-18
Irf1	-0.78	0.0288	Interferon regulatory factor 1		Transcriptional regulator that promotes inflammatory innate and adaptive immune responses
Irf4	-1.19	0.00931	Interferon regulatory factor 4		Transcriptional activator that complexes with BATF and binds ISREs within the promoters of multiple genes involved in inflammation
Irf7	-0.543	0.0311	Interferon regulatory factor 7		Key transcriptional regulator of type I IFN-dependent immune responses; promotes transcription of IFNα and -β
Isg15	-0.918	0.0437	Interferon-stimulated gene 15		Ubiquitin-like protein that binds intracellular target proteins upon activation by IFNα or β; can also be secreted to induce NK cell proliferation, act as a chemoattractant for neutrophils, and induce IFNγ upon binding to ITGAL/ITGB2
Itga5	-0.509	0.047	Integrin alpha 5		Pairs with ITGB1 to form a receptor for fibronectin and IL-1β

Mif	-0.264	0.0432	Macrophage migration inhibitory factor	L-dopachrome tautomerase	Pro-inflammatory cytokine that promotes macrophage function through suppression of anti-inflammatory effects of glucocorticoids
Mx2	-1.5	0.0302	Myxovirus resistance protein 2		IFN-induced dynamin-like GTPase with potent antiviral activity against HIV-1
Inhibition					
Btla	-0.822	0.0146	B and T lymphocyte attenuator	CD272	Inhibitory cell surface protein that inhibits T cell function by binding to B7H4 and TNFRSF14
Ctla4	-0.842	0.00212	Cytotoxic T lymphocyte antigen 4		Inhibitory receptor that blocks CD28 co-stimulation by competitively binding its ligands CD80 and CD86
Dusp4	-1.17	0.0292	Dual specificity phosphatase 4		Inactivates ERK1, ERK2, and JNK
Foxp3	-1.63	0.000165	Forkhead box P3	DIETER	Master transcription factor for regulatory T cells (Tregs); represses expression of *Il2 and Ifng*; activates expression of *Tnfrsf18*, *Il2ra*, and *Ctla4*
Il10	-0.821	0.017	Interleukin 10	Cytokine synthesis inhibitory factor (CSIF)	Major immunoregulatory cytokine that inhibits production of pro-inflammatory cytokines, including GM-CSF, G-CSF, IL-1α, IL-1β, IL-6, IL-8, and TNFα; also interferes with antigen presentation by reducing expression of MHC class II and co-stimulatory molecules, thereby inhibiting their ability to induce T cell activation
Lag3	-0.572	0.0436	Lymphocyte activating gene 3	CD223	Inhibitory receptor on activated T cells; binds to ligands, such as FGL1; constitutively expressed on a subset of regulatory Tregs and contributes to their suppressive function; acts as a negative regulator of plasmacytoid dendritic cell (pDCs) activation
Sigirr	-0.239	0.0499	Single Ig domain-containing IL-1R-related protein		Negative regulator of the Toll-like and IL-1R receptor signaling pathways; attenuates the recruitment of receptor-proximal signaling components to the TLR4 receptor; interferes with the heterodimerization of Il1R1 and IL1RAP
Tgfb1	-0.408	0.0361	Transforming growth factor beta 1		Multifunctional protein that regulates the growth and differentiation of various cell types and is involved in various processes, such as normal

					development, immune function, microglia function and responses to neurodegeneration; can induce EMT and cell migration in various cell types; frequently acts as an immunosuppressive cytokine in the TME
Tnfsf14	-1.04	0.0126	TNF receptor superfamily member 14	CD270	Receptor for four distinct ligands: LIGHT, lymphotoxin-α, BTLA, and CD160, altogether defining a complex stimulatory and inhibitory signaling network; signals *via* the TRAF2-TRAF3 E3 ligase pathway to promote immune cell survival and differentiation; participates in bidirectional cell-cell contact signaling between APCs and lymphocytes; delivers costimulatory signals to T cells, promoting cell proliferation and effector functions; interacts with CD160 on NK cells, enhancing IFNγ production and anti-tumor immune response; upon binding to CD160 on activated CD4+ T cells, downregulates CD28 costimulatory signaling; participates in *cis* or *trans* reactions with BTLA - *cis* interactions seem to promote quiescence; *trans* interactions seem to promote survival
JAK-STAT Pathway					
Jak3	-0.563	0.0129	Janus kinase 3		Non-receptor tyrosine kinase involved in various processes such as cell growth, development, or differentiation; mediates essential signaling events in both innate and adaptive immunity
Stat3	-0.616	0.0484	Signal transducer and activator of transcription 3		Transcriptional activator of genes involved in cell growth and apoptosis; activated by JAKs
Stat4	-0.823	0.0435	Signal transducer and activator of transcription 4		Essential TF for Th1 CD4+ T cell development and IFNγ production; also promotes expression of MyD88
MAP Kinase Signaling					
Map3k1	-0.852	0.0212	Mitogen-activated protein kinase kinase kinase 1		Serine/threonine kinase that activates the ERK and JNK kinase pathways by phosphorylation of MAP2K1 and MAP2K4; also activates CHUK and IKBKB, the central protein kinases of the NFκB pathway

Map4k2	-0.846	0.021	Mitogen-activated protein kinase kinase kinase kinase 2		Essential component of the MAP kinase signal transduction pathway downstream of TRAF6; upstream activator of the SAP/JNK signaling pathway
Metabolism					
Ada	-0.872	0.00148	Adenosine deaminase		Key enzyme in purine metabolism; primarily involved in the development and maintenance of the immune system in humans
Pparg	-0.85	0.0303	Peroxisome proliferator activated receptor gamma		Nuclear receptor that binds peroxisome proliferators such as hypolipidemic drugs and fatty acids; once activated binds to specific PPAR response elements (PPRE) and modulates the transcription of its target genes, such as acyl-CoA oxidase, thereby controlling the peroxisomal beta-oxidation pathway of fatty acids
NFκB Signaling					
Nfkb1	-0.663	0.0497	Nuclear factor kappa B subunit 1	p105/p50	One of the NFκB family TFs; inhibits inflammation
Rel	-1.15	0.0287	Avian reticuloendotheliosis viral oncogene homolog	c-Rel	One of the NFκB family TFs; important for B cell and Treg development
NK Cell Function					
Prdm1	-0.419	0.0403	Positive regulatory domain I-binding factor	B lymphocyte-induced maturation protein (BLIMP1)	Transcription factor that plays a role in the development, retention, and long-term establishment of T cell, NK cell, and NK-T cells in non-lymphoid organs; drives the maturation of B cell into Ig secreting cells
Pvr	-0.86	0.0219	Poliovirus receptor	CD155	Mediates NK cell adhesion and triggers NK cell effector functions; binds CD96 and CD226, leading to the formation of a mature immunological synapse between NK cell and target cell
Pvrl2	-0.51	0.00878	Poliovirus receptor-related protein 2	Nectin-2	Variable costimulator/coinhibitor of T cell function, depending on which receptor it binds to: stimulates T cell proliferation and cytokine production upon binding to CD226; inhibits T cell proliferation upon interaction with PVRIG
Tcf7	-0.989	0.0017	Transcription factor 7		HMG box TF predominantly expressed by T cells that drives their development, although also

					involved in NK cell development; activates transcription through a Wnt/β-catenin signaling pathway
Pattern Recognition Receptors					
Cd14	-0.745	0.0458	Cluster of differentiation 14		PRR that recognizes LPS; mostly found on macrophages
Marco	1.05	0.0431	Macrophage receptor with collagenous structure		A PRR that recognizes LDL
Nod2	-1.17	0.0167	Nucleotide-binding oligomerization domain containing 2		PRR specific for muramyl dipeptide (MDP); upon binding to its ligand, recruits RIPK2 and triggers MAPK and NFκB signaling
T Cell Function					
Icos	-0.999	0.000546	Inducible T cell costimulator	CD278	Enhances all basic T cell responses to foreign antigen; essential both for efficient interaction between T and B cells and for normal antibody responses to T cell-dependent Ags
Icosl	-0.551	0.0155	Inducible T cell costimulator ligand	CD275	Ligand for T cell-specific co-receptor ICOS; also induces B cell proliferation and plasma cell differentiation
Prdm1	-0.419	0.0403	Positive regulatory domain I-binding factor	B lymphocyte-induced maturation protein (BLIMP1)	Transcription factor that plays a role in the development, retention, and long-term establishment of T cell, NK cell, and NK-T cells in non-lymphoid organs; drives the maturation of B cell into Ig secreting cells
Tbx21	-0.692	0.0316	T-box transcription factor 21		Initiates Th1 lineage development from naïve Th precursor cells both by activating Th1 genetic programs and by repressing the opposing Th2 and Th17 genetic programs
Tcf7	-0.989	0.0017	Transcription factor 7		HMG box TF predominantly expressed by T cells that drives their development, although also involved in NK cell development; activates transcription through a Wnt/β-catenin signaling pathway
Transcription Factors & Chromatin Remodellers					
Ikzf2	-0.851	0.0459	IKAROS family zinc finger protein 2		Heamtopoietic cell-specific TF involved in early hematopoietic development
Nfkb1	-0.663	0.0497	Nuclear factor kappa B subunit 1	p105/p50	One of the NFκB family TFs; inhibits inflammation
Prdm1	-0.419	0.0403	Positive regulatory domain I-binding factor	B lymphocyte-induced maturation	Transcription factor that plays a role in the development, retention, and long-term establishment of T cell,

				protein (BLIMP1)	NK cell, and NK-T cells in non-lymphoid organs; drives the maturation of B cell into Ig secreting cells
Rel	-1.15	0.0287	Avian reticuloendotheliosis viral oncogene homolog	c-Rel	One of the NFκB family TFs; important for B cell and Treg development
Stat3	-0.616	0.0484	Signal transducer and activator of transcription 3		Transcriptional activator of genes involved in cell growth and apoptosis; activated by JAKs
Stat4	-0.823	0.0435	Signal transducer and activator of transcription 4		Essential TF for Th1 CD4+ T cell development and IFNγ production; also promotes expression of MyD88
Tbx21	-0.692	0.0316	T-box transcription factor 21		Initiates Th1 lineage development from naïve Th precursor cells both by activating Th1 genetic programs and by repressing the opposing Th2 and Th17 genetic programs
Tcf7	-0.989	0.0017	Transcription factor 7		HMG box TF predominantly expressed by T cells that drives their development, although also involved in NK cell development; activates transcription through a Wnt/β-catenin signaling pathway

Supplementary Table 3: Genes significantly differentially regulated between treatment groups possessing and lacking NBTXR3 in the primary tumor.

Gene	Log 2 Fold-Change	P-Value	Full Name	Notable Aliases	Function
Ifngr1	-0.359	0.0489	Interferon gamma receptor 1	CD54	One of the two components of the IFNγ receptor; stimulates activation of the JAK/STAT signaling pathway
Il6	-1.32	0.0145	Interleukin 6		Pro-inflammatory cytokine that signals through the JAK and STAT pathways; induces VEGF to promote angiogenesis
Icam1	-0.481	0.0464	Intracellular adhesion molecule 1		Cell surface glycoprotein that serves as strong adhesive ligand for LFA-1; important for leukocyte mobility and costimulation
Ccl11	-0.742	0.0433	C-C motif chemokine ligand 11	Eotaxin	Chemoattractant for eosinophils
Cxcl1	-0.744	0.0292	C-X-C motif chemokine ligand 1	GRO1 oncogene	Chemoattractant ligand for CXCR2; plays a role in inflammation and as a chemoattractant for neutrophils
Nod2	-0.779	0.0411	Nucleotide-binding oligomerization domain containing 2		PRR specific for muramyl dipeptide (MDP); upon binding to its ligand, recruits RIPK2 and triggers MAPK and NFκB signaling
Creb5	-0.849	0.0404	CAMP responsive element binding protein 5		Phosphorylation-dependent transcription factor that stimulates transcription upon binding to the DNA cAMP response element (CRE), which is found in the promoter regions of several immune-related genes, including *Il2*, *Il6*, *Il10*, and *Tnfa*; regulates diverse cellular responses, including proliferation, survival, and differentiation
Camp	-2.48	0.0266	Cathelicidin antimicrobial peptide		Polypeptide stored in the lysosomes of macrophages and PMNs that digests phagocytosed cells

Supplementary Table 4: Genes significantly differentially regulated between treatment groups possessing and lacking NBTXR3 in the secondary tumor.

Gene	Log 2 Fold-Change	P-Value	Full Name	Notable Aliases	Function
Acute Phase Response					
App	-0.956	0.0489	Amyloid-beta precursor protein		A secreted antimicrobial peptide
Lcn2	1.74	0.0342	Lipocalin 2	Neutrophil gelatinase-associated lipocalin (NGAL)	Neutrophil-secreted factor that sequesters iron-containing siderophores; also functions as a growth factor
Adhesion & Cell-Cell Interactions					
Abl1	-1.23	0.00999	Abelson tyrosine-protein kinase 1	Proto-oncogene C-ABL	Plays a role in many key processes linked to cell growth and survival such as cytoskeleton remodeling, cell motility and adhesion, receptor endocytosis, autophagy, DNA damage response, and apoptosis; regulates T cell differentiation by phosphorylating TBX21, leading to its enhancement
Cd97	-1.23	0.00591	Cluster of differentiation 97	BL-Ac[F2]	GPCR that promotes granulocyte adhesion and migration; activates T cells *via* binding to CD55; stimulates angiogenesis through binding integrin counterreceptors on endothelial cells
Itga1	-1.88	0.00479	Integrin subunity alpha 1	CD49a; Very late activation protein 1 (VLA-1)	Alpha 1 subunit for common integrin receptors; pairs with the β1 subunit to form a cell-surface receptor for collagen and laminin; involved in cell-cell adhesion and may play a role in inflammation and fibrosis
Jam3	-2.73	0.00287	Junctional adhesion molecule C		Immunoglobulin that mediates tight junctions between endothelial cells; mediates transepithelial migration of PMNs
Mill2	-1.71	0.00755	MHC class I-like protein		Heterodimer with β2-microglobulin with MHC class I; orthologous to human MICA and MICB
Sele	-3.43	0.00248	E-selectin	CD62E; endothelial leukocyte adhesion molecule 1 (ELAM-1)	Cell-surface glycoprotein that mediates in the adhesion of blood neutrophils in cytokine-activated endothelium through interaction with SELPLG/PSGL1

Apoptosis					
Abl1	-1.23	0.00999	Abelson tyrosine-protein kinase 1	Proto-oncogene C-ABL	Plays a role in many key processes linked to cell growth and survival such as cytoskeleton remodeling, cell motility and adhesion, receptor endocytosis, autophagy, DNA damage response, and apoptosis; regulates T cell differentiation by phosphorylating TBX21, leading to its enhancement
Bid	1.2	0.00909	BH3 interacting domain death agonist	Desmocollin type 4, apoptic death agonist	Induces caspases and apoptosis; counters the protective effect of BCL2, allowing release of cytochrome C
Fas	-0.893	0.0218	Fragment apoptosis stimulating		Cell surface death receptor; interaction with FAS-ligand triggers an apoptotic signaling cascade; also activates NFκB, ERK1, and MAPK8
Ifitm2	-0.971	0.0275	Interferon-induced transmembrane protein 2		IFN-induced antiviral protein which inhibits the entry of viruses to the host cell cytoplasm; induces cell cycle arrest and mediates p53-independent apoptosis through caspase activation
Trp53	-0.761	0.0025	Transformation-related protein 53		TF that induces cell-cycle arrest and apoptosis through stimulation of Fas expression
Angiogenesis					
Cd97	-1.23	0.00591	Cluster of differentiation 97	BL-Ac[F2]	GPCR that promotes granulocyte adhesion and migration; activates T cells *via* binding to CD55; stimulates angiogenesis through binding integrin counterreceptors on endothelial cells
Antigen Processing & Presentation					
Cd1d1	0.852	0.0131	Antigen-presenting glycoprotein CD1d1		Murine non-classical class I MHC; primarily presents lipid and glycolipid Ags
Cd1d2	1.38	0.00326	Antigen-presenting glycoprotein CD1d2		Pairs with CD1d1 to form the murine non-classical class I MHC, CD1d; primarily presents lipid and glycolipid Ags; essential for NKT cell development; presents shorter acyl chain Ags than CD1d1
Cd83	-1.45	0.0391	Cluster of differentiation 83		APC surface marker; may be involved in the regulation of Ag presentation

Icam1	-1.29	0.0226	Intracellular adhesion molecule 1		Cell surface glycoprotein that serves as strong adhesive ligand for LFA-1; important for leukocyte mobility and costimulation
H60a	-1.41	0.0467	Histocompatibility 60a		Enables NK cell lectin-like receptor binding activity
Autophagy					
Abl1	-1.23	0.00999	Abelson tyrosine-protein kinase 1	Proto-oncogene C-ABL	Plays a role in many key processes linked to cell growth and survival such as cytoskeleton remodeling, cell motility and adhesion, receptor endocytosis, autophagy, DNA damage response, and apoptosis; regulates T cell differentiation by phosphorylating TBX21, leading to its enhancement
Atg12	-0.785	0.0153	Autophagy related 12		Pairs with ATG5 to promote the extension of the phagophoric membrane in autophagic vesicles
B Cell-associated Genes					
Blnk	-1.72	0.0354	B cell linker	Src homology 1 domain-containing leukocyte protein of 65 kDa (SLP-65); Ly57	Functions as a central linker protein downstream of the B cell receptor, bridging SYK kinase to a multitude of signaling pathways and regulating biological outcomes of B cell function and development; plays a role in the activation of ERK/EPHB2, MAP kinase p38 and JNK; modulates AP1 activation; important for the activation of NFκB and NFAT
Cd19	-2.87	0.0306	Cluster of differentiation 19	B-lymphocyte surface antigen B4	BCR coreceptor; activates signaling pathways that lead to the activation of PI3K and Ca2+ flux
Cd79b	-2.44	0.0421	Cluster of differentiation 79b	B29	One of the two flanking proteins that initiate signaling downstream of the BCR
Cd81	-1.7	0.0133	Cluster of differentiation 81	Tetraspanin-28	Acts as a platform for receptor clustering and signaling; essential for trafficking and compartmentalization of CD19 receptor on the surface of activated B cells; facilitates the localization of CD3ζ at antigen-induced synapses with B cells; may also play a role in antigen presentation

Fcgr4	2.22	0.0295	Fragment crystallizable gamma receptor 4	Fc receptor-like 3 (Fcrl3); CD16-2	Putative mouse ortholog to human FcγRIIIA
Icosl	-1.34	0.00101	Inducible T cell costimulator ligand	CD275	Ligand for T cell-specific co-receptor ICOS; also induces B cell proliferation and plasma cell differentiation
Calcium Signaling					
S100b	-2.93	0.00372	S100 Ca2+ binding protein B		Weakly binds Ca2+ but binds zinc very tightly; may mediate Ca2+-dependent regulation of many physiological processes by interacting with other proteins, such as TPR-containing proteins, and modulating their activity
Chemotaxis					
Ccl1	1.31	0.0367	C-C motif chemokine ligand 1	T lymphocyte-secreted protein I-309; small inducible cytokine A1	Chemoattractant for monocytes but not neutrophils; binds to CCR8
Ccl11	-1.58	0.00536	C-C motif chemokine ligand 11	Eotaxin	Chemoattractant for eosinophils
Ccl17	2.28	0.0238	C-C motif chemokine ligand 17		Chemoattractant ligand for CCR4 and -8; attracts T cells
Ccl3	2.73	0.0185		C-C motif chemokine ligand 3	Macrophage inflammatory protein 1α (MIP1α)
Ccl7	1.29	0.0196	C-C motif chemokine ligand 7	Monocte chemotactic protein 3 (MCP3)	General chemokine that recruits leukocytes to infected tissues; mainly observed in monocyte mobilization
Ccnd3	-1.13	0.00455	Cyclin D3		Regulatory component of the cyclin D3-CDK4 complex that inhibitively phosphorylates members of the retinoblastoma protein family; regulates the cell-cycle during G(1)/S transition
Ccr1	2.07	0.0137	C-C motif chemokine receptor 1	MIP1α receptor	Receptor for CCL3, -5, -7, and -23
Ccr7	-2.51	0.00929	C-C chemokine receptor type 7	CD197	Chemokine receptor that activates B and T cells and promotes their homing to secondary lymphoid organs; also stimulates DC expression of MHC class I and II
Ccr8	-1.57	0.044	C-C motif chemokine receptor 8		Receptor for CCL1; may regulate monocyte chemotaxis and thymic cell line apoptosis

Cxcl11	2.17	0.0148	C-X-C motif chemokine ligand 11		Dominant ligand for CXCR3; attracts activated T cells; strongly induced by IFNγ
Cxcl2	2.62	0.0042	C-X-C motif chemokine ligand 2	Macrophage inflammatory protein 2-alpha (MIP2α); GRO2 oncogene	Chemokine produced by activated monocytes and neutrophils and expressed at sites of inflammation
Cxcl3	7.72	0.000688	C-X-C motif chemokine ligand 3	GRO3 oncogene	Ligand for CXCR2; attracts neutrophils
Cxcl9	1.47	0.0449	C-X-C motif chemokine ligand 9	Humig	Chemoattractant ligand for CXCR3; attracts activated T cells
Cxcr4	-1.75	0.00672	C-X-C motif chemokine receptor 4	Fusin; CD184	Alpha-chemokine receptor specific for SDF1 *aka* CXCL12
Cxcr5	-3.4	0.0272	C-X-C motif chemokine receptor 5	CD185; Burkitt's lymphoma receptor 1 (BLR1)	Cytokine receptor that binds to B lymphocyte chemoattractant (BLC); involved in B cell migration into splenic follicles and Peyer's patches
Complement & Humoral Immunity					
C6	1.73	0.00661	Complement component 6		Part of the membrane attack complex
Cfb	1.4	0.0327	Complement factor B		Alternate complement pathway component; when cleaved, produces a serine protease that binds to C3b to form C3 convertase
Fcgr4	2.22	0.0295	Fragment crystallizable gamma receptor 4	Fc receptor-like 3 (Fcrl3); CD16-2	Putative mouse ortholog to human FcγRIIIA
Co-stimulation					
Icosl	-1.34	0.00101	Inducible T cell costimulator ligand	CD275	Ligand for T cell-specific co-receptor ICOS; also induces B cell proliferation and plasma cell differentiation
Cytokines					
Il1a	3.38	0.0226	Interleukin 1 alpha	Hematopoietin-1	Cytokine produced by monocytes and macrophages in response to cell injury; stimulates thymocyte proliferation by inducing IL-2 release; also stimulates B cell maturation and proliferation, and fibroblast growth factor activity
Csf2	3.12	0.00602	Colony-stimulating factor 2	Sargramostim	Cytokine that stimulates the growth and differentiation of hematopoietic precursor cells from various lineages, including granulocytes, macrophages, eosinophils, and erythrocytes

Il1b	2.87	0.00578	Interleukin 1 beta	Catabolin	One of the two primary inflammatory cytokines produced by the inflammasome (the other one being IL-18); induces neutrophil influx and activation,T cell activation and cytokine production, B-cell activation and antibody production, fibroblast proliferation, and collagen production; synergizes with IL-12 to induce IFNγ synthesis from Th1 cells
Spp1	2.58	0.00606	Secreted phosphoprotein 1	Osteopontin	Cytokine involved in enhancing production of IFNγ and IL-12 and reducing production of IL-10
Il12rb1	2.56	0.0247	Interleukin 12 receptor subunit beta 1	CD212	Cytokine receptor component that associates with IL12RB2 to IL23R
Il1r2	1.46	0.0166	Interleukin 1 receptor type II	CD121b	Non-signaling receptor for IL-1α, -β, and RN; serves as a decoy receptor by competetive binding to IL-1β and preventing its binding to IL1R1
Il4ra	-0.609	0.0435	Interleukin 4 receptor subunit alpha	CD124	Alpha chain for the IL-4 and IL-13 receptors; involved in Th2 differentiation and IgE production
Il15	-0.621	0.0362	Interleukin 15		Stimulates T cell proliferation and phagocytosis in neutrophils
Ifnar1	-0.651	0.0151	Interferon-alpha/beta receptor alpha chain		Component of the receptor for type I IFNs, binding of which activates the JAK-STAT pathway
Ifngr1	-0.991	0.00506	Interferon gamma receptor 1	CD54	One of the two components of the IFNγ receptor; stimulates activation of the JAK/STAT signaling pathway
Il6st	-1.82	0.0103	Interleukin 6 cytokine family signal transducer	Glycoprotein 130 (Gp130); CD130	Transmembrane protein that acts a component in several cytokine receptors, including IL-6
Tgfb3	-1.84	0.0276	Transforming growth factor beta 3		Multifunctional protein that regulates embryogenesis and cell differentiation
Il34	-1.98	0.0088	Interleukin 34		Cytokine that promotes the proliferation, survival and differentiation of monocytes and macrophages, as well as the release of proinflammatory chemokines
Ifna1	-2.01	0.0201	Interferon alpha 1		Macrophage-produced antiviral, pro-inflammatory cytokine
Il6	-2.44	0.00404	Interleukin 6		Pro-inflammatory cytokine that signals through the JAK and STAT pathways

Inflammation					
Ifna1	-2.01	0.0201	Interferon alpha 1		Macrophage-produced antiviral, pro-inflammatory cytokine
Ifnar1	-0.651	0.0151	Interferon-alpha/beta receptor alpha chain		Component of the receptor for type I IFNs, binding of which activates the JAK-STAT pathway
Ifngr1	-0.991	0.00506	Interferon gamma receptor 1	CD54	One of the two components of the IFNγ receptor; stimulates activation of the JAK/STAT signaling pathway
Il1a	3.38	0.0226	Interleukin 1 alpha	Hematopoietin-1	Cytokine produced by monocytes and macrophages in response to cell injury; stimulates thymocyte proliferation by inducing IL-2 release; also stimulates B cell maturation and proliferation, and fibroblast growth factor activity
Il1b	2.87	0.00578	Interleukin 1 beta	Catabolin	One of the two primary inflammatory cytokines produced by the inflammasome (the other one being IL-18); induces neutrophil influx and activation,T cell activation and cytokine production, B-cell activation and antibody production, fibroblast proliferation, and collagen production; synergizes with IL-12 to induce IFNγ synthesis from Th1 cells
Il6	-2.44	0.00404	Interleukin 6		Pro-inflammatory cytokine that signals through the JAK and STAT pathways
Il6st	-1.82	0.0103	Interleukin 6 cytokine family signal transducer	Glycoprotein 130 (Gp130); CD130	Transmembrane protein that acts a component in several cytokine receptors, including IL-6
Irf1	-1.46	0.00796	Interferon regulatory factor 1		Transcriptional regulator that promotes inflammatory innate and adaptive immune responses
Irf3	-0.894	0.0268	Interferon regulatory factor 3		Complexes with CREBBP to translocate to the nucleus and transcriptionally activate type I IFNs
Irf4	-1.58	0.005	Interferon regulatory factor 4		Transcriptional activator that complexes with BATF and binds ISREs within the promoters of multiple genes involved in inflammation
Isg15	-1.96	0.00941	Interferon-stimulated gene 15		Ubiquitin-like protein that binds intracellular target proteins upon activation by IFNα or β; can also be secreted to induce NK cell proliferation, act as a

					chemoattractant for neutrophils, and induce IFNγ upon binding to ITGAL/ITGB2
Mx2	-3.14	0.000635	Myxovirus resistance protein 2		IFN-induced dynamin-like GTPase with potent antiviral activity against HIV-1
Pin1	-1.84	0.00234	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1		Acts as a molecular switch in multiple cellular processes; inhibits mitosis presumably by interacting with NIMA and attenuating its mitosis-promoting activity; downregulates kinase activity of BTK; induces IRAK3 stabilization, nuclear translocation, and expression of pro-inflammatory genes in dendritic cells
Spp1	2.58	0.00606	Secreted phosphoprotein 1	Osteopontin	Cytokine involved in enhancing production of IFNγ and IL-12 and reducing production of IL-10
Tbk1	-0.829	0.0235	TANK-binding kinase 1		Coordinates the activation of IRF3 and NFκB and induction of type I IFNs
Inhibition					
Bcl2	-0.946	0.0163	B cell lymphoma 2		Outer mitochondrial membrane protein that inhibits apoptosis and autophagy; may attenuate inflammation by impairing inflammasome formation
Bcl2l1	-1.22	0.0333	B cell lymphoma 2 like 1	Protein phosphatase 1	Potent inhibitor of caspase-mediated cell death
Btla	-2.46	0.000221	B and T lymphocyte attenuator	CD272	Inhibitory cell surface protein that inhibits T cell function by binding to B7H4 and TNFRSF14
Chuk	-0.722	0.00208	Conserved helix-loop-helix ubiquitous kinase	Inhibitor of NFκB kinase subunit alpha (IKKα)	Part of the IKK complex that inhibits IκBα and permits NFκB nuclear localization
Cyld	-0.68	0.0301	Cylindromatosis lysine 63 deubiquitinase		Inhibits NFκB activation by deubiquitinating upstream signaling factors; inhibits Wnt signaling; restricts polyubiquitination of RIPK1 and -2, thereby limiting necroptosis
Cd28	-1.36	0.0473	Cluster of differentiation 28		Essential T cell co-receptor that enhances T cell activation, proliferation, cytokine production, and survival; binds to CD80 and CD86
Cdkn1a	-1.16	0.0286	Cyclin dependent kinase inhibitor 1A	p21; CDK-interaction protein 1 (CIP1)	Binds to and inhibits cyclin-dependent kinase activity, preventing phosphorylation of

					critical cyclin-dependent kinase substrates and blocking cell cycle progression
Dusp4	-1.3	0.0156	Dual specificity phosphatase 4		Inactivates ERK1, ERK2, and JNK
Ikbkb	-0.715	0.0106	Inhibitor of nuclear factor kappa B kinase subunit beta		Part of the IKK complex that inhibits IκBα and permits NFκB nuclear localization
Ikbkg	-0.692	0.0168	Inhibitor of nuclear factor kappa B kinase subunit gamma	NFκB essential modifier (NEMO)	Regulatory subunit of the IKK core complex that phosphorylates inhibitors of NFκB thus leading to the dissociation of the inhibitor/NFκB complex and ultimately the degradation of the inhibitor
Klra2	2.67	0.0168	Killer cell lectin-like receptor subfamily A member 2	Ly49b	Inhibitor receptor on NK cells for MHC class I; recruits SHP1, -2, and SHIP phosphatases upon binding to its ligand
Nfkbia	-0.911	0.0239	Nuclear factor kappa B inhibitor alpha		Inhibits activity of REL dimers by masking of their nuclear localization signals
Pin1	-1.84	0.00234	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1		Acts as a molecular switch in multiple cellular processes; inhibits mitosis presumably by interacting with NIMA and attenuating its mitosis-promoting activity; downregulates kinase activity of BTK; induces IRAK3 stabilization, nuclear translocation, and expression of pro-inflammatory genes in dendritic cells
Pvrl2	-0.693	0.0455	Poliovirus receptor-related protein 2	Nectin-2	Variable costimulator/coinhibitor of T cell function, depending on which receptor it binds to: stimulates T cell proliferation and cytokine production upon binding to CD226; inhibits T cell proliferation upon interaction with PVRIG
Sigirr	-1.31	0.00408	Single Ig domain-containing IL-1R-related protein		Negative regulator of the Toll-like and IL-1R receptor signaling pathways; attenuates the recruitment of receptor-proximal signaling components to the TLR4 receptor; interferes with the heterodimerization of Il1R1 and IL1RAP
Socs3	-1.45	0.0171	Suppressor of cytokine signaling 3		Inhibits IL6ST and JAK2; negative regulator of IL-6
IRAKs & TRAFs					

Traf3	-0.605	0.0128	Tumor necrosis factor receptor-associated factor 3		Adaptor protein that acts in the CD40 signaling cascade; induces NFκB and MAPK activation
JAK-STAT Pathway					
Jak1	-0.811	0.0331	Janus kinase 1		Essential tyrosine kinase involved signal transduction in type I and II cytokines and IFNs
Jak2	-1.2	0.00328	Janus kinase 2		Tyrosine kinase that participates in IFN and IL6ST signaling cascades
Jak3	-0.839	0.0155	Janus kinase 3		Non-receptor tyrosine kinase involved in various processes such as cell growth, development, or differentiation; mediates essential signaling events in both innate and adaptive immunity
Stat1	0.824	0.0317	Signal transducer and activator of transcription 1		Transcriptional activator that mediates cellular responses to IFNs, cytokines, and other growth factors
Stat3	-1.03	0.012	Signal transducer and activator of transcription 3		Transcriptional activator of genes involved in cell growth and apoptosis; activated by JAKs
Stat4	-0.94	0.0107	Signal transducer and activator of transcription 4		Essential TF for Th1 CD4+ T cell development and IFNγ production; also promotes expression of MyD88
MAP Kinase Signaling					
Map3k1	-1.39	0.000125	Mitogen-activated protein kinase kinase kinase 1		Serine/threonine kinase that activates the ERK and JNK kinase pathways by phosphorylation of MAP2K1 and MAP2K4; also activates CHUK and IKBKB, the central protein kinases of the NFκB pathway
Map3k5	-1.44	0.00264	Mitogen-activated protein kinase kinase kinase 5	Apoptosis signal-regulating kinase 1	Essential component of the MAP kinase signal transduction pathway; mediates signaling for determination of cell fate such as differentiation and survival; plays a crucial role in the apoptosis signal transduction pathway through mitochondria-dependent caspase activation; required for the innate immune response; mediates signal transduction of receptor-mediated inflammatory signals, such as TNF or LPS
Map3k7	-0.743	0.0428	Mitogen-activated protein kinase kinase kinase 7	TGFβ-activated kinase (TAK1)	Signal transducer downstream of TGFβ and BMP; controls a variety of cell functions, including

					transcription regulation and apoptosis
Map4k2	-2.22	0.000542	Mitogen-activated protein kinase kinase kinase kinase 2		Essential component of the MAP kinase signal transduction pathway downstream of TRAF6; upstream activator of the SAP/JNK signaling pathway
Mapk1	-0.537	0.0472	Mitogen-activated protein kinase 1	Extracellular signal-regulated kinase 2 (ERK2)	Serine/threonine kinase that acts as an essential component of the MAP kinase signal transduction pathway
Mapk3	-1.47	0.00233	Mitogen-activated protein kinase 3	Extracellular signal-regulated kinase 1 (ERK1)	Serine/threonine kinase that acts as an essential component of the MAP kinase signal transduction pathway
Mapk8	-0.848	0.0423	Mitogen-activated protein kinase 8	c-Jun N-terminal kinase 1 (JNK1); Stress-activated protein kinase 1c (SAPK1)	Serine/threonine-protein kinase involved in various processes such as cell proliferation, differentiation, migration, transformation and programmed cell death; phosphorylates a number of transcription factors, primarily components of AP-1 such as JUN, JDP2, and ATF2, thus regulating AP-1 transcriptional activity; promotes stressed cell apoptosis by phosphorylating key regulatory factors including p53/TP53 and Yes-associates protein YAP1; required for Th1 differentiation
Metabolism					
Ada	-0.562	0.00542	Adenosine deaminase		Key enzyme in purine metabolism; primarily involved in the development and maintenance of the immune system in humans
Cd36	-2.49	0.00409	Cluster of differentiation 36	Fatty acid translocase (FAT)	Class B scavenger receptor that mediates fatty acid uptake
Pparg	-2.43	0.000677	Peroxisome proliferator activated receptor gamma		Nuclear receptor that binds peroxisome proliferators such as hypolipidemic drugs and fatty acids; once activated binds to specific PPAR response elements (PPRE) and modulates the transcription of its target genes, such as acyl-CoA oxidase, thereby controlling the peroxisomal beta-oxidation pathway of fatty acids
NFκB Signaling					

Bcl10	-0.682	0.0354	B cell lymphoma/leukemia 10		Activates NFκB *via* ubiquitination of IKKγ
Nfkb1	-0.834	0.0065	Nuclear factor kappa B subunit 1	p105/p50	One of the NFκB family TFs; inhibits inflammation
Rel	-1.65	0.000363	Avian reticuloendotheliosis viral oncogene homolog	c-Rel	One of the NFκB family TFs; important for B cell and Treg development
Rela	-1.16	0.00571	Avian reticuloendotheliosis viral oncogene homolog A	p65	One of the NFκB family TFs; major driver of inflammation
Relb	-0.537	0.0306	Avian reticuloendotheliosis viral oncogene homolog B		One of the NFκB family TFs; controls lymphoid development, DC biology, and noncanonical NFκB signaling
Ripk2	-1	0.0212	Receptor-interacting serine/threonine-protein kinase 2		RIP kinase that potentiates signals downstream of NOD1 and -2, leading to NFκB activation; promotes BCL10 phosphorylation and subsequent NFκB activation following TCR engagement
NK Cell Function					
Gzmb	2.53	0.0166	Granzyme B		Abundant protease in the cytosolic granules of cytotoxic T and NK cells that activates caspase-mediated cell death when delivered into the target cell through the immunological synapse
Pvr	-1.53	0.006	Poliovirus receptor	CD155	Mediates NK cell adhesion and triggers NK cell effector functions; binds CD96 and CD226, leading to the formation of a mature immunological synapse between NK cell and target cell
Tcf7	-1.81	0.0123	Transcription factor 7		HMG box TF predominantly expressed by T cells that drives their development, although also involved in NK cell development; activates transcription through a Wnt/β-catenin signaling pathway
Pattern Recognition Receptors					
Clec4n	2.74	0.0278	C-type lectin domain family 4, member N	Dectin-2	PRR specific for Mycobacterial mannose-capped lipoarabinomannan
Clec5a	2.55	0.048	C-Type lectin domain family 5, member A	Myeloid DAP12-associating lectin-1	Critical macrophage receptor for dengue virus serotypes 1-4; positive regulator of osteoclastogenesis

Ddx58	-1.05	0.0111	DExD/H-box helicase 58	Retinoic acid-inducible gene I (RIG-I)	Cytoplasmic PRR that recognizes dsRNA; can promote T cell-independent B cell activation; uses MAVS as an adaptor
Fpr2	2.83	0.0126	Formyl peptide receptor 2	Lipoxin A4 receptor	Low affinity receptor for N-formyl-methionyl peptides; activates neutrophils
Marco	2.64	0.012	Macrophage receptor with collagenous structure		A PRR that recognizes LDL
Myd88	-1.55	0.0156	Myeloid differentiation primary response 88		Key adaptor in the TLR signaling pathways; interacts with all TLRs except TLR3; activates NFκB and IRFs
Nod1	-0.994	0.0221	Nucleotide binding oligomerization domain containing 1		Intracellular PRR that recognizes peptidoglycan-derived muropeptides and *Shigella* effector proteins
Tlr3	-1.37	0.0164	Toll-like receptor 3	CD283	Endosomic PRR that recognizes dsRNA
Tollip	-1.05	0.00843	Toll interacting protein		Inhibitory adaptor protein that acts upon TLR2
ROS Generation					
Nos2	1.81	0.0136	Inducible nitric oxide synthase (iNOS)		Produces reactive oxygen species and contributes to inflammatory cytokine production
Pparg	-2.43	0.000677	Peroxisome proliferator activated receptor gamma		Nuclear receptor that binds peroxisome proliferators such as hypolipidemic drugs and fatty acids; once activated binds to specific PPAR response elements (PPRE) and modulates the transcription of its target genes, such as acyl-CoA oxidase, thereby controlling the peroxisomal beta-oxidation pathway of fatty acids
Txnip	-1.05	0.0426	Thioredoxin interacting protein		Thiol-oxidoreductase; protects cells from oxidative stress by inhibiting thioredoxin
T Cell Function					
Abl1	-1.23	0.00999	Abelson tyrosine-protein kinase 1	Proto-oncogene C-ABL	Plays a role in many key processes linked to cell growth and survival such as cytoskeleton remodeling, cell motility and adhesion, receptor endocytosis, autophagy, DNA damage response, and apoptosis; regulates T cell differentiation by phosphorylating TBX21, leading to its enhancement

Cd97	-1.23	0.00591	Cluster of differentiation 97	BL-Ac[F2]	GPCR that promotes granulocyte adhesion and migration; activates T cells *via* binding to CD55; stimulates angiogenesis through binding integrin counterreceptors on endothelial cells
Gata3	-1.62	0.00209	GATA binding protein 3		Transcriptional activator that binds to the enhancer of the TCR α and δ genes; required for Th2 differentiation following immune and inflammatory responses
Gzmb	2.53	0.0166	Granzyme B		Abundant protease in the cytosolic granules of cytotoxic T and NK cells that activates caspase-mediated cell death when delivered into the target cell through the immunological synapse
Icosl	-1.34	0.00101	Inducible T cell costimulator ligand	CD275	Ligand for T cell-specific co-receptor ICOS; also induces B cell proliferation and plasma cell differentiation
Nfatc1	-1.37	0.00394	Nuclear factor of activated T cells, cytoplasmic 1		Inducible nuclear component of the NFAT TF complex; mediates induction of IL-2 and IL-4 in T cells
Nfatc2	-1.03	0.0156	Nuclear factor of activated T cells, cytoplasmic 2		Cytosolic component of the NFAT TF complex; mediates induction of IL-2, IL-3, IL-4, TNFα, and GM-CSF
Pvrl2	-0.693	0.0455	Poliovirus receptor-related protein 2	Nectin-2	Variable costimulator/coinhibitor of T cell function, depending on which receptor it binds to: stimulates T cell proliferation and cytokine production upon binding to CD226; inhibits T cell proliferation upon interaction with PVRIG
Rora	-1.19	0.0294	Retinoic acid receptor-related orphan receptor A		Nuclear receptor that binds hormone response elements upstream of several genes to enhance the expression of those genes
Tcf7	-1.81	0.0123	Transcription factor 7		HMG box TF predominantly expressed by T cells that drives their development, although also involved in NK cell development; activates transcription through a Wnt/β-catenin signaling pathway
Txk	-1.23	0.0397	TXK tyrosine kinase		Regulates the development, function, and differentiation of conventional T cells and nonconventional NK-T cells;

					contributes to signaling from many receptors and participates in multiple downstream pathways, including regulation of the actin cytoskeleton; can phosphorylate PLCγ1, leading to its localization in lipid rafts and activation, followed by subsequent cleavage of its substrates
Transcription Factors & Chromatin Remodellers					
Atf2	-1.04	0.00375	Activating transcription factor 2	Cyclic AMP-responsive element-binding protein 2 (CREB2)	Regulates transcription of various genes involved in anti-apoptosis, cell growth, and DNA damage response; in the nucleus, contributes to global transcription and the DNA damage response, in addition to specific transcriptional activities that are related to cell development, proliferation and death; in the cytoplasm, impairs mitochondrial membrane potential, inducing mitochondrial leakage and promoting cell death; phosphorylated form (mediated by ATM) plays a role in the DNA damage response
Creb1	-1.07	0.00431	CAMP responsive element binding protein 1		Phosphorylation-dependent transcription factor that stimulates transcription upon binding to the DNA cAMP response element (CRE), which is found in the promoter regions of several immune-related genes, including IL-2, IL-6, IL-10, and TNF-α; regulates diverse cellular responses, including proliferation, survival, and differentiation
Crebbp	-1.17	0.00584	CREB binding protein	KAT3A	Binds specifically to phosphorylated CREB and enhances its transcriptional activity toward cAMP-responsive genes; also acetylates histones, giving a specific tag for transcriptional activation
Egr1	-1.85	0.00937	Early growth response 1	Zinc finger protein 268 (ZNF286)	Transcriptional repressor of genes involved in differentiations and mitogenesis; activates expression of p53
Ep300	-0.887	0.0169	Adenovirus early region 1A-associated protein p300		Histone acetyltransferase; participates in chromatin remodeling to facilitate gene accessibility

Gata3	-1.62	0.00209	GATA binding protein 3		Transcriptional activator that binds to the enhancer of the TCR α and δ genes; required for Th2 differentiation following immune and inflammatory responses
Hmgb1	-0.961	0.0214	High-mobility group box 1		Remodels chromatin to make DNA more available for transcription
Irf1	-1.46	0.00796	Interferon regulatory factor 1		Transcriptional regulator that promotes inflammatory innate and adaptive immune responses
Irf3	-0.894	0.0268	Interferon regulatory factor 3		Complexes with CREBBP to translocate to the nucleus and transcriptionally activate type I IFNs
Irf4	-1.58	0.005	Interferon regulatory factor 4		Transcriptional activator that complexes with BATF and binds ISREs within the promoters of multiple genes involved in inflammation
Nfkb1	-0.834	0.0065	Nuclear factor kappa B subunit 1	p105/p50	One of the NFκB family TFs; inhibits inflammation
Pparg	-2.43	0.000677	Peroxisome proliferator activated receptor gamma		Nuclear receptor that binds peroxisome proliferators such as hypolipidemic drugs and fatty acids; once activated binds to specific PPAR response elements (PPRE) and modulates the transcription of its target genes, such as acyl-CoA oxidase, thereby controlling the peroxisomal beta-oxidation pathway of fatty acids
Rel	-1.65	0.000363	Avian reticuloendotheliosis viral oncogene homolog	c-Rel	One of the NFκB family TFs; important for B cell and Treg development
Rela	-1.16	0.00571	Avian reticuloendotheliosis viral oncogene homolog A	p65	One of the NFκB family TFs; major driver of inflammation
Relb	-0.537	0.0306	Avian reticuloendotheliosis viral oncogene homolog B		One of the NFκB family TFs; controls lymphoid development, DC biology, and noncanonical NFκB signaling
Tcf7	-1.81	0.0123	Transcription factor 7		HMG box TF predominantly expressed by T cells that drives their development, although also involved in NK cell development; activates transcription through a Wnt/β-catenin signaling pathway

Yy1	-0.827	0.0068	Yin yang 1		Ubiquitous factor that serves as a transcriptional "switch", either promoting or repressing the transcription of numerous genes through the selective recruitment of either histone deacetylases or acetyltransferases; plays a fundamental role in diverse processes, such as differentiation, replication, and cellular proliferation
Tyrosine Kinases					
Itk	-1.08	0.0289	Interleukin-2-inducible T cell kinase	LYK	Key actor in the TCR signaling cascade; phosphorylates PLCγ1, LAT, and LCP2
Jak1	-0.811	0.0331	Janus kinase 1		Essential tyrosine kinase involved signal transduction in type I and II cytokines and IFNs
Jak2	-1.2	0.00328	Janus kinase 2		Tyrosine kinase that participates in IFN and IL6ST signaling cascades
Jak3	-0.839	0.0155	Janus kinase 3		Non-receptor tyrosine kinase involved in various processes such as cell growth, development, or differentiation; mediates essential signaling events in both innate and adaptive immunity
Tbk1	-0.829	0.0235	TANK-binding kinase 1		Coordinates the activation of IRF3 and NFκB and induction of type I IFNs
Txk	-1.23	0.0397	TXK tyrosine kinase		Regulates the development, function, and differentiation of conventional T cells and nonconventional NK-T cells; contributes to signaling from many receptors and participates in multiple downstream pathways, including regulation of the actin cytoskeleton; can phosphorylate PLCγ1, leading to its localization in lipid rafts and activation, followed by subsequent cleavage of its substrates
Tyk2	-0.947	0.0201	Tyrosine kinase 2	JTK1	Plays both structural and catalytic roles in numerous cytokines and interferons signaling; associates with cytokine and growth factor receptors and activate STAT family members including STAT1, STAT3, STAT4, or STAT6
Ubiquitin Regulation					

Bcl10	-0.682	0.0354	B cell lymphoma/leukemia 10		Activates NFκB *via* ubiquitination of IKKγ
Cyld	-0.68	0.0301	Cylindromatosis lysine 63 deubiquitinase		Inhibits NFκB activation by deubiquitinating upstream signaling factors; inhibits Wnt signaling; restricts polyubiquitination of RIPK1 and -2, thereby limiting necroptosis
Itch	-0.872	0.00379	Itchy E3 ubiquitin protein ligase		Participates with TNFAIP3 in a ubiquitin-editing complex that marks components of inflammatory signaling pathways such as JUNB and CXCR4 for degradation
Ubc	-1.49	0.013	Polyubiquitin C		Serves various roles, including immate immunity, DNA repair, and stimulation of autophagy and the proteasomal response
Other					
Atm	-0.471	0.04	Ataxia telangiectasia mutated		Serine/threonine protein kinase that activates checkpoint signaling upon DSBs, apoptosis, and genotoxic stresses; acts as a master controller for cell cycle checkpoint signaling pathways required for the DNA damage response and genomic stability
Rps6	-0.821	0.0119	Ribosomal protein 6		Component of the 40S small ribosomal subunit; plays an important role in controlling cell growth and proliferation through the selective translation of particular classes of mRNA