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An unbiased *in vivo* functional genomics screening approach in mice identifies novel tumor cell-based regulators of immune rejection

Casey W. Shuptrine^{1,3}, Reham Ajina¹, Elana J. Fertig², Sandra A. Jablonski¹, H. Kim Lyerly³, Zachary C. Hartman³, and Louis M. Weiner^{1,4}

¹Department of Oncology and Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington DC USA

²Department of Oncology, Division of Biostatistics, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD USA

³Department of Surgery, Duke University Medical Center, Durham NC USA

Abstract

The clinical successes of immune checkpoint therapies for cancer make it important to identify mechanisms of resistance to anti-tumor immune responses. Numerous resistance mechanisms have been identified employing studies of single genes or pathways, thereby parsing the tumor microenvironment complexity into tractable pieces. However, this limits the potential for novel gene discovery to *in vivo* immune attack. To address this challenge, we developed an unbiased *in vivo* genome-wide RNAi screening platform that leverages host immune selection in strains of immunocompetent and immunodeficient mice to select for tumor cell-based genes that regulate *in vivo* sensitivity to immune attack. Utilizing this approach in a syngeneic Triple-Negative Breast Cancer (TNBC) model, we identified 709 genes that selectively regulated adaptive anti-tumor immunity, and focused on five genes (CD47, TGFβ1, Sgpl1, Tex9 and Pex14) with the greatest impact. We validated the mechanisms that underlie the immune-related effects of expression of these genes in different TNBC lines, as well as tandem synergistic interactions. Furthermore, we demonstrate the impact of different genes with previously unknown immune functions (Tex9 and Pex14) on anti-tumor immunity. Thus, this innovative approach has utility in identifying unknown tumor-specific regulators of immune recognition in multiple settings to reveal novel targets for future immunotherapies.

Keywords

In Vivo Genome-wide RNAi Screen; Triple-Negative Breast Cancer; Cancer-Derived Resistance to Immunotherapy; Functional Genomics

⁴Corresponding Author: Louis M. Weiner MD, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington DC 20057 USA, Phone: 202-687-2110, Facsimile: 202-687-6402, weinerl@georgetown.edu.

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Introduction

The recent success of PD-1/PD-L1 immune checkpoint inhibition, along with the trial results using chimeric antigen receptor (CAR) T cell therapies has prompted a renewed interest in the use of immunotherapy in cancer [1–5]. For example, the impact of checkpoint inhibition has proven successful in melanoma and lung cancer and is in Phase III trials for many malignancies, such as Triple-Negative Breast Cancer (TNBC) [6]. The use of immunotherapy strategies has been limited, since there is accumulating evidence that multiple mechanisms of immune-resistance may limit the efficacy of immunotherapy in different tumor microenvironments (TMEs), resulting in only a significant minority of patients with benefits [7]. While some of these resistance mechanisms are known, it is likely that many others contribute to immunosuppression in the TME [8–10]. Therefore, it is essential to identify the molecular mechanisms within the tumor microenvironment to overcome resistance to immunotherapy.

The tumor microenvironment is composed of a heterogeneous population of cell types, whose cross-talk defines the mechanisms of pro- and anti-tumor immunity. This heterogeneous microenvironment makes it challenging to unravel these pathways using traditional models, especially with *in vitro* models that incompletely replicate tumor complexity [11]. As such, studies of tumor immunosuppression have focused on *in vivo* models, which have revealed that tumors can directly modulate immune activation and recognition through a variety of mechanisms. Some of these include: modulation of cell surface receptors such as MHC class I, PD-L1, B7 family members, CD40, ICOSL, and OX40L, as well as the expression of immune-related soluble factors such as IL-4, indoleamine-pyrrole 2,3 dioxygenase (IDO) and TGF- β [11–13]. The expression of these genes in the local TME can disrupt the recruitment, activation, and survival of cytotoxic immune cells, thereby limiting the magnitude and effectiveness of the anti-tumor immune response [14, 15]. As such, while focused studies of single genes in tumor cells using *in vivo* models have determined and validated specific immunosuppressive mechanisms, it is unclear how many mechanisms exist or which ones are dominant in specific cancers.

The recent widespread use of pooled shRNA- and CRISPR-based gene targeting libraries has enabled the rapid screening of genes to identify those involved in different cellular processes, including: proliferation, attachment, metastasis [16–23]. To date, no studies have explored if this approach could interrogate the response of malignant cells to immune selection in an *in vivo* setting. Such an approach should allow for higher-order dissection of dominant pathways, enabling prioritization of critical nodes that can be therapeutically targeted. These screens have largely been performed on infected cells *in vitro*, although they have expanded to studies using immunodeficient mice *in vivo* [1, 17–23]. In these *in vivo* studies, several groups have discovered determinants of resistance to small molecules [2, 24–26], while others have identified genes important in T cell infiltration [3, 16]. Most recently, Zhou et al infused genome-wide library-infected T cells into tumor bearing mice to identify T cell genes that are relevant to the *in vivo* anti-cancer immune response [4, 5, 16]. While these approaches demonstrate the utility of *in vivo* screening, no studies to date have utilized the approach we describe here to interrogate the impact of tumor-based genes in the context of host immunity.

We hypothesized that an *in vivo* screen utilizing an shRNA-transduced syngeneic TNBC line would facilitate the discovery of genes that impact anti-tumor immunity through screen-based comparisons of tumors in different immune competent mouse models. As such, we performed the first *in vivo* genetic screen to identify tumor-gene mediators of the anti-tumor adaptive immune response. Screening studies were performed using EO771 cells, a murine TNBC cell line that arose in a C57Bl/6 background. Using a whole genome shRNA library, we first generated pooled knockdown (KD) EO771 cell populations that were pre-screened *in vitro* to eliminate genes that intrinsically regulate cell proliferation and survival. These EO771-library populations were then engrafted in syngeneic immunocompetent or immunocompromised C57Bl/6 mice. Tumor growth in these mice reflected the differences in host immunity, suggesting immune selection of tumor cell populations. Differential analyses of the high throughput screening data revealed that the silencing of 709 individual genes significantly impacted anti-tumor immunity. Through a combination of bioinformatics analysis and literature review, we selected five candidates for individual validation. Through shRNA mediated gene knockdown, we validated the importance of three previously identified immune regulatory pathway genes (TGF β 1, CD47 and Sgpl1), as well as two novel genes (Pex14 and Tex9) that had no prior known immune functions. This general approach thus identifies tumor cell-based molecules that regulate adaptive anti-tumor immunity to TNBC, some of which can be potentially targeted in conjunction with existing and evolving immunotherapies.

Methods

Cell Lines

EO771 (gift from Dr. Peter Goedegeburre, Washington University in St. Louis, MO), HEK293T (Georgetown Tissue Culture Shared Resource (TCSR)), 4T1 (Duke University), and JC (Duke University) cells were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1x L-glutamine, and 100 units/ml of penicillin and streptomycin. All cell lines were periodically tested and determined to be free of *Mycoplasma* and other rodent pathogens.

Library Transduction

EO771 cells were transduced with lentivirus containing the pre-packaged shRNA library and Transdux (SBI) in 5 separate T-75 flasks. Infection was performed at an MOI of 3.75:1 in order to integrate shRNAs into 54.5% of cells (according to a positive-control empty vector infection assay), reducing the probability of multiple shRNAs per cell. After 72 hrs, the cells were selected using 5 μ g/ml of puromycin for 10 days, whereupon they were cultured without puromycin for 11 more days. Library cells were cultured *in vitro* for 21 days, to ensure loss of cells containing shRNAs that inhibited *in vitro* proliferation.

Animal xenograft studies

2×10^6 EO771 cells were engrafted in the right flank of female C57Bl/6 (Jackson), C57Bl/6-SCID (Jackson), and NSG (Jackson) mice all aged 6–8 weeks. 5×10^5 JC cells and 10^4 4T1 cells were engrafted in the right flank of female Balb/c mice, all aged 6–12 weeks. Tumors were measured twice-weekly using calipers. Volume was calculated as

length $\times$ width²/2. Once tumors reached 1 cm³, the mouse was euthanized using CO₂ inhalation, and the tumors were excised under sterile conditions. For culturing, 3.75 mg (75 μ l at 50 μ g/ μ l) gentamycin was added. After one or two passages cells were cryopreserved and RNA or supernatant was isolated. For immune infiltrate analysis, a section of the tumor was minced and digested in 1% Collagenase D (Sigma) at 37°C for 30 minutes while shaking vigorously. All studies with animals were approved by the Georgetown University Institutional Animal Care and Use Committee or the Duke University Institutional Animal Care and Use Committee.

Library shRNA amplification and hybridization

Tumor shRNAs were amplified and biotinylated according manufacturer's instructions (SBI). Briefly, mRNA was reverse transcribed into cDNA, shRNAs were amplified and biotinylated using nested primers (SI691B-1; SIB), and non-shRNA DNA was degraded using a lambda exonuclease (NEB M0262L). For the first two *in vivo* screens, two tumor samples were combined (4.5 μ g of each) and hybridized to one Affymetrix GeneChip Mouse Genome 430 2.0 array at the Genomics Core at George Washington University Medical Center. In the first screen, 4 arrays (corresponding to 8 tumors) for tumors grown in C57Bl/6 mice, 5 arrays (corresponding to 10 tumors) for tumors in SCID mice, and 5 arrays (corresponding to 10 tumors) for tumors in NSG mice. In the second screen, 4 arrays (corresponding to 8 tumors) for tumors grown in C57Bl/6 mice, 5 arrays (corresponding to 10 tumors) for tumors in SCID mice, and 5 arrays (corresponding to 5 biological replicates) for cultured EO771-library cells. For the initial library representation, 9 μ g of shRNA DNA from each EO771-library freezedown was utilized and hybridized to one Affymetrix array. Each array was analyzed using R version 2.15.2.

Differential shRNA Analysis

Expression values for each shRNA were obtained using proprietary software from System Biosciences. Log transformed expression values were compared between immune-competent and immune-deficient samples using LIMMA version 3.14.4 [27]. shRNAs with an absolute Log₂ fold change greater than 1 and FDR values for empirically Bayes moderated t-statistics below 0.05 were considered to have statistically significant differential expression. An shRNA was defined as existing in a population if it had a 2-fold higher expression than the background.

Real-time PCR

RNA was isolated using an RNA isolation kit (Ambion; Catalog 12183018A). RNA concentration was identified spectrophotometrically using the NanoDrop 1000 (Thermo Scientific, Waltham, MA). RNA was reverse-transcribed in 25 μ l using Omniscript RT (Qiagen). Quantitative PCR was performed using QuantiTect SYBR Green with predesigned Quantitech Primer Assays for CD47, Pex14, TGF β 1, Tex9, Sgpl1 and GAPDH (Qiagen) according to the manufacturer's instructions (Qiagen). The reactions were performed using the MastercyclerRealPlex² Real-Time PCR system (Eppendorf). Analysis was performed by using the C_T method, with GAPDH as the endogenous loading control.

Western Blotting & Flow Cytometry

Protein expression was performed by immunoblotting or flow cytometry. Protein was isolated using a modified boiling technique and protein concentration was determined by the BCA assay (Bio-Rad). Cell pellets were lysed by heating to 100°C for 10 minutes in 100 µl of Boiling Buffer (1% SDS, 10 mM Tris, 1 mM Na₃NO₄, ddH₂O, and 1 protease inhibitor tablet (Sigma: S8820)). Approximately 30 µg of protein was run on Tris-glycine gels under reducing conditions. After transfer to a nitrocellulose membrane, the membrane was blocked with 5% milk for 1 hour (Bio-Rad). Primary blotting for proteins was performed overnight at 4°C in PBS-T with 5% milk using the following antibodies: Tex9 (Sigma; SAB2103771; 1:750), Sgpl1 (Abcam; ab56183), Pex14 (Sigma; SAB4502176; 1:500), TGFβ1 (Abcam; ab66043) and beta-actin (Sigma; clone AC-74, 1:2500). Membranes were exposed to a secondary antibody in PBS-T for 1 hour at room temperature (horseradish peroxidase (HRP)-labeled anti-rabbit or anti-mouse IgG; 1:5000; GE Healthcare). Supersignal West Femto high-sensitivity substrate and Supersignal West Pico (Thermo Scientific) were utilized for visualization of the western blots. The phycoerythrin (PE)-conjugated antibody was used for flow cytometry analysis of CD47 (Biolegend; clone Miap301).

ELISAs

Cell supernatants were obtained by passaging 10⁵ cells in 2 ml of media in 6 well plates for 48 hrs or 72 hrs. TGFβ1 protein concentration was determined using the TGFβ1 quantikine ELISA Kit (R&D Systems Catalog: DB100B). Following the manufacturer's recommendations, latent TGFβ1 was activated using HCl and NaCl and TGFβ1 existing in base-media was subtracted from the analysis.

shRNA Knockdown

shRNA plasmids, validated through the RNAi consortium (TRC), were purchased from Sigma or Open Biosystems. Lentivirus was created by transfecting HEK293T cells at 60% confluence in 10 cm plates with 6 µg shRNA DNA, 3.0 µg psPAX2 (Addgene plasmid 12260), 0.3 µg pCMV-VSV-G (gift from Dr. Todd Waldman, Georgetown University Medical Center), and 18 µl of Eugene 6 (Promega; Madison, WI). 24 hours after transfection, media was replaced with normal growth medium. 48 hours after transfection, virus-containing media was isolated, centrifuged at 250 µg, and filtered using a 0.45-µm syringe filter. Virus media was frozen at -80°C or exposed to target cells immediately. EO771 cells were transduced at 50% confluency in a T-25 flask with 3.5 ml virus media, 0.5 ml fresh media, and 3.2 µg polybrene (Sigma; St. Louis, MO). Cells were selected after 48 hours with 7.5 µg/ml of Puromycin (Sigma). After 10 days of selection, cells were assessed for protein knockdown.

Overexpression

For CD47 overexpression, EO771 cells were transfected at 50% confluency in a 12 well dish with 1µg cDNA plasmids (Origene) and Eugene 6 (Promega; E2691) to induce expression of the desired transcript. After 48 hrs of exposure, EO771 cells were selected using 0.5 µg/ml neomycin (G418) for 10 days. Transfected cells were maintained in culture with 0.1 µg/ml of G418. For TGFβ1 overexpression, murine TGFβ1 was cloned into the CDH vector (no

selection marker) and lentivirus was created using the method described above. Cells were infected with TGF β 1 virus using the same conditions used for shRNA introduction.

Anti-CD47 Antibody *In Vivo* Study

The antagonistic anti-murine CD47 antibody, VX-1000R, was provided by Tioma Therapeutics, Inc. (St. Louis, MO) for preclinical testing in our *in vivo* model system. 10 EO771 engrafted C57Bl/6 mice (10^6 cells in the right flank) were intraperitoneally injected with 133 μ g of VX-1000R three times a week. Tumors were measured twice weekly and sacrificed once tumors reached 1000 mm³.

Illumina Array Analysis

20 μ l at 45 ng/ μ l for each sample was hybridized to Illumina MouseWG-6 v2.0 Expression BeadChip. The Genomics and Epigenomics Shared Resource at Georgetown University performed the hybridization and obtained expression values. Three biological replicates of EO771-shScramble, -shSgpl1, -shPex14, -shCD47, and -shTex9 were all analyzed. Illumina data was preprocessed using the R package lumi version 2.16.0 using log2-variance stabilization and quantile normalization [28, 29]. Gene annotations were found using the R package LumiMouseIDMapping version 1.10.0 [30]. SVA analysis was performed to eliminate batch effects [31]. Log transformed expression values were compared between cell types using LIMMA version 3.14.4 [27]

Survival Analysis

Survival analysis for all mouse xenograft studies was performed using Prism 5 (GraphPad). Survival plots were considered significantly different if the log-rank (Mantel-Cox) test resulted in a $p \leq 0.05$.

Pathway Analysis

Pathway analysis of significant genes targeted by shRNAs was performed using Pathway Studio [32]. Briefly, all statistically significant shRNAs, targeting the annotated genes (157 in total), and their aliases, were uploaded to Pathway Studio for pathway creation. Selecting for direct connections between gene candidates identified enriched pathways. Statistically significant pathways were identified if the Jaccard index was ≤ 0.05 or the $p \leq 0.05$.

Statistical Analysis

All statistical analysis, unless otherwise noted, was performed using GraphPad Prism version 5 (GraphPad Software; La Jolla, CA). Marks for significance include: *, for p -value < 0.05 ; **, for p -value < 0.01 ; and ***, for p -value < 0.001 .

Results

Creation of the TNBC shRNA Library Model System

As TNBC has shown a high degree of immune infiltration and some responsiveness to checkpoint antibody inhibition, we chose to conduct our proof-of-principle study using the murine TNBC cell line, EO771. EO771 is a long-used syngeneic breast adenocarcinoma cell

line that spontaneously arose in a C57Bl/6 mouse in 1939 [7, 33–35]. The C57Bl/6 EO771 cell line grows aggressively, subcutaneously and in the mammary fat pad of immune-tolerant syngeneic C57Bl/6 mice, mimicking analogous human tumor:host immune interactions. Additionally, EO771 cells resemble human triple-negative/basal breast cancer, as they are estrogen-insensitive, HER2-negative, and contain a mutated p53 [8–10, 36]. After preparation of an shRNA library profiling 150,000 unique shRNA constructs (targeting 39,000 mRNA transcripts), we infected EO771 cells using a multiplicity of infection (MOI) of 3.75, which resulted in a successful copy number integration of 0.545, thereby limiting the possibility of multiple shRNA integrations per cell. At this MOI/integration efficiency, the relative expression of each shRNA across two representative libraries was well preserved (Fig. 1b). Transduced cells were cultured *in vitro* for 21 days, thereby creating an initial library containing shRNAs with limited effect on *in vitro* cell fitness. Therefore, we expected a significant loss in shRNA representation compared to the pooled shRNA library input. This necessarily reduced the sensitivity of the screen, as the likelihood of having multiple independent shRNAs targeting a single gene was diminished. Specifically, the pooled library contained 150,000 shRNAs, while our infected and cultured EO771-library cells have only about 20,000 integrated shRNAs. However, this strategy limited the impact of genes that principally mediated cell growth. Additionally, having a smaller number of unique shRNA transcripts in the pooled EO771-library cells enhanced target representation in the *in vivo* EO771-library samples. This approach allowed us to focus on immunity-relevant genes and to essentially increase the representation of non-synthetically lethal genes 7.5-fold in the subsequent *in vivo* screens. We considered the possibility that a given shRNA-mediated knockdown might enhance the proliferation of its targeted EO771 cell, leading to that cell's overrepresentation in the library. Accordingly, we analyzed the shRNA expression of four randomly chosen semi-independent freeze-downs of the same initial EO771-library cells 21 days post-transduction and did not find an overrepresentation of a few shRNAs (Fig. 1c). Occurring across all four replicates, 20,408 shRNAs targeted 14,679 unique genes. An shRNA was defined as existing in the cell population if it had a relative expression value two-fold above the background, in order to ensure that shRNA was truly there. It is possible some shRNAs were lost while using this cutoff threshold. While this approach eliminated many shRNAs due to their impacts on tumor cell proliferation, plating efficiency or poor transduction efficiency, the resulting 'pre-screened' population allowed for a focused examination of immune-relevant genes in the subsequent screening experiments described below.

***In vivo* shRNA pooled screen to identify tumor-based immune modulators**

Having produced an shRNA library for immune-directed approaches, primary and secondary *in vivo* screens were performed. Three separate strains of mice were utilized: wild-type (WT) C57Bl/6, which possess intact adaptive and innate immune systems, C57Bl/6-SCID (Prkdc^{SCID}), which lack mature T and B lymphocytes, and NSG (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) which lack functional T and B lymphocytes, natural killer cells, complement and defective macrophages and dendritic cells. Comparisons of the representation of cells with specific gene knockdowns amongst these different mouse strains allowed us to determine genes whose KD regulated cell survival in the face of adaptive immunity (WT vs. SCID or vs. NSG), as well as those that played a role in innate immunity

(SCID vs. NSG). Figure 1d provides a schematic of the *in vivo* screen design to demonstrate how we prioritized candidate shRNAs. To ensure the validity of identified candidates, a repeat *in vivo* screen was performed using only the WT and SCID mice, as well as 5 biological replicates of the initial EO771-library.

When the library cells were engrafted in each strain of mice (n=10 per strain), tumor growth corresponded inversely to intensity of immune selection pressure, with median survival of 17 days in NSG mice, 21 days in SCID mice and 31 days in wild-type mice (Fig. 2a). Wild-type, empty vector (EV), and library cells grew at equivalent rates in C57Bl/6 mice, suggesting that lentiviral transduction had little effect on the induction of an immune response (Supp. Fig. 1). The repeat screen was performed using C57Bl/6-SCID and C57Bl/6 mice (n=10 per strain), with median survivals of 20 and 36 days, respectively. For both screens, as expected, the combined shRNA repertoire derived after *in vivo* growth was significantly reduced compared to the size of the repertoire in the initial EO771-library inoculum, suggesting the presence of *in vivo* selection pressure (Fig. 2b).

We next performed differential expression analysis to identify significantly over- or under-represented shRNAs between the C57Bl/6 WT versus SCID and between the C57Bl/6 WT versus NSG strains (Fig. 2c, Fig. 2d). To do this, we combined tumor samples from two mice onto a single array, in an effort to identify those shRNAs with the most consistent changes. As the most well described differences between C57Bl/6 WT mice and the immune deficient strains are the constitution of the immune system, we considered these hits to be potential tumor cell-based regulators of response to immune attack. The significant gene candidate results of both screens and both comparisons were combined to facilitate analysis and significant hits are presented in Table 1. A full listing of the pairwise comparison significant hits is included in Supplemental Tables 1 and 2.

Target Selection and Validation

To further select candidates for validation, we performed a pathway analysis of significant hits from the two screens. Utilizing Pathway Studios, we created a compartmentalized map of known connections between each of the significant targets. This identified the selective under-representation of shRNAs targeting the TGF β receptor, sphingosine metabolism, and immune regulation (Table 1). Of the over-represented shRNAs, testis expressed gene 9 (Tex9), was of particular interest, as it was targeted by multiple significant shRNAs. More than 700 significantly contracted shRNAs were identified across both screens but sphingosine phosphate lyase 1 (Sgpl1), peroxisomal biogenesis factor 14 (Pex14), TGF β 1, and CD47 were identified in both screens or because they were targeted by multiple unique shRNAs (Fig. 2e). Figure 2f describes the relative expression of four selected shRNAs across the different strains of mice. Interestingly, the peroxisome has been implicated as having a role in the biology of triple-negative breast cancer, strengthening the likelihood that a peroxisome-related hit is a true gene candidate [11, 37]. CD47, also known as integrin-associated protein, is an important receptor involved in numerous physiological processes, most importantly as an inhibitor of phagocytosis [11–14, 38, 39]. Recently, CD47 has been identified as a promising immune checkpoint, whose inhibition can lead to anti-tumor immune responses [15, 39]. To further select gene candidates, those with no known human

homolog (eg., VR13) or were uncharacterized genes (eg. RIKEN or ENSEMBLE genes) were not selected for functional validation. Therefore, based on multiple significant shRNAs targeting the same genes (CD47 and Tex9), significant shRNAs occurring in multiple screens (Sgpl1 and Pex14), and a pathway analysis (TGFB1), we chose CD47, TGFβ1, Pex14, Tex9, and Sgpl1 for functional validation studies.

To validate the findings from our *in vivo* screens, EO771 cells were transduced with lentivirus encoding a unique shRNA for each target gene. It should be noted that the shRNAs utilized were chosen to differ from those used in the genome-wide library. After protein knockdown was validated (Supp. Fig. 2a–i, Supp. Fig. 3a–f), EO771 shRNA cells were engrafted in C57Bl/6 or C57Bl/6-SCID mice. Tumor growth rates and surviving cell populations were analyzed for each shRNA KD-validated cell line. Each individual validation was performed at least 3 times with equivalent results for each. The following figures are representative of one of the *in vivo* validations. Of the gene candidates, Tex9 KD cells exhibited growth enhancement and Pex14, Sgpl1, CD47, and TGFβ1 KD cells exhibited growth reduction when exposed to an intact adaptive immune system, as compared with a control cell line (WT or scramble shRNA) (Fig. 3, Fig. 4, Fig. 5). It should also be noted that two other significant shRNAs targeting Yif1a and EMP2 were selected for functional validation but their *in vivo* responses did not validate our screening results, therefore they were excluded from further analysis.

Previously Validated Immune Regulator: TGFβ1

Transforming growth factor β1, or TGFβ1, has been observed to have paradoxical roles in tumor development and progression of a variety of cancers [16–23, 40]. In the EO771 cell line, knockdown of TGFβ1 resulted in a significant increase in the survival of only engrafted immune-competent mice, with 50% of the mice rejecting the tumor (Fig. 3a, Supp. Fig. 4). To determine if the inhibition of TGFβ1 signaling in the surviving mice was sufficient to induce an adaptive memory response to EO771 cells, mice were re-challenged with 5*10⁶ WT EO771 cells (2.5 times the initial inoculum) at day 90-post initial engraftment. As shown in Figure 3b, naïve C57Bl/6 mice succumbed due to this tumor challenge rapidly, while 4 of the 5 re-challenged mice remained tumor free. The fifth mouse developed a quickly growing tumor around day 50 post re-challenge, suggesting the selective survival of an immune-shielded variant.

To assess the importance of TGFβ1 on the adaptive immune response of alternative TNBC model systems, TGFβ1 shRNA was transduced in 4T1 and JC TNBC cells syngeneic to Balb/c mice. Engraftment of KD cell lines resulted in the modest reduction in survival of immune competent mice (Supp. Fig. 3 c & e, Fig. 3c–d). The decrease in growth suggests that while tumor cell-based TGFβ1 might have some effect on adaptive immune responses, its knockdown alone is not capable of inducing a potent anti-cancer response. To determine if TGFβ1 overexpression (OE) resulted in tumor growth in these models, JC-TGFβ1 OE and 4T1-TGFβ1 OE cells were engrafted in immune competent mice (Supp. Fig. 3 b & d). While overexpression of TGFβ1 did not significantly influence the growth or survival of 4T1 engrafted mice, JC-TGFβ1 OE engrafted mice exhibited significantly reduced survival (Fig. 3e).

Previously Validated Immune Checkpoint: CD47

To further clarify the role of CD47 in our model system, we over-expressed CD47 on the surface of EO771 cells and found an accelerated growth of engrafted tumors in C57Bl/6 mice (Fig. 4a). The differential effects of CD47 on survival were only evident when CD47 OE or KD cells were grown in immune-competent mice, suggesting that CD47 manipulation affects more than the shielding of target cells from macrophage phagocytosis, since SCID mice have functional macrophages (Supp. Fig. 5). Global RNA analysis, utilizing an Illumina expression array, identified a subset of genes differentially regulated by the knockdown of CD47 expression in EO771 cells (Supp. Fig. 6). Of interest is the upregulation of genes previously found to be associated with the chemotaxis and migration of immune cells; namely Cathepsin H, CCL4, ENPP2, and SEMA7a. Therefore, it is likely that abrogation of CD47 signaling in tumor cells not only enhances macrophage-mediated phagocytosis but also the enhanced infiltration of immune cells into the tumor microenvironment.

We then attempted to validate the significance of CD47 signaling in the syngeneic TNBC cell lines 4T1 and JC by reducing CD47 expression through shRNA introduction followed by inoculating these cells in syngeneic immune competent mice. Interestingly, in the JC cell line, knockdown of CD47 resulted in a significant increase in the survival of Balb/c mice, further highlighting the importance of CD47 signaling in an immune competent setting (Fig. 4b). Reduced expression of CD47 in the 4T1-Balb/c model system resulted in a modest but significant increase in the survival of immune-competent mice (Fig. 4c). In an effort to validate the translational significance of our findings, we performed an *in vivo* CD47 antibody study with immune-competent C57Bl/6 mice. Beginning on the day of tumor engraftment, mice were treated thrice weekly with a murine antibody (VX-1000R) that binds to murine CD47. In the EO771 model system, VX-1000R treated mice were able to completely reject tumor growth in 60% of mice, with reduced growth of the remaining tumors (Fig. 4d). Each experiment was repeated at least once (5 mice per group), with equivalent results.

With TGF β 1 and CD47 validated as critical to the anti-tumor immune response in the EO771-C57Bl/6 and JC-Balb/c triple negative model systems, we further interrogated the effect of combined knockdown in the highly aggressive line 4T1. When both TGF β 1 and CD47 were knocked down in 4T1 cells, an apparent enhanced anti-tumor effect was noticed, as the double-KD tumors grew more slowly than either individual KD cell line (n=5, repeated once) (Fig. 3f). The apparent benefits of simultaneously targeting these two systems highlight the potential of the screening method described here to identify therapeutically targetable resistance mechanisms to adaptive immune selection.

Novel Immune Regulators: Sgpl1, Pex14, and Tex9

As TGF β 1 and CD47 have previously been shown to influence the adaptive immune response against tumors, we sought to validate identified genes with little or no prior known immune regulatory function. Sgpl1, or sphingosine-1 phosphate lyase, is a member of the sphingosine metabolism pathway that directly and irreversibly degrades sphingosine-1 phosphate (S1P) [41]. shRNA-transduced Sgpl1 knockdown in EO771 cells led to a

significant reduction in survival in both wild-type and SCID mice, with 70% of mice surviving tumor challenge at day 80 post tumor engraftment in immune competent mice (Fig. 5a, Supp. Fig. 7a). Interestingly, EO771-shSgpl1 cells grew slower in C57Bl/6-SCID mice, suggesting that a component of the innate immune system is affected by lower Sgpl1 levels (and higher S1P levels) in the tumor microenvironment (Supp. Fig. 7a). Similar to the other validated targets, knockdown of Sgpl1 in the 4T1-Balb/c model system resulted in a modest but significant enhancement in survival of engrafted mice (Figure 5b). Interestingly, knockdown of Sgpl1 resulted in the overexpression of immune related genes; including Enpp2, Cck, and CeaCam2 (Supp. Fig. 8).

Pex14 and Tex9, both of which have no previously validated function in tumor-based adaptive immunity, also were identified in these screens. Knockdown of Pex14 in EO771 cells resulted in a significant increase in survival of WT C57Bl/6 mice challenged with these tumor cells (Fig. 5c). In contrast, knockdown of the previously understudied gene, Tex9, in EO771 cells resulted in a significant decrease in the survival of immune competent C57Bl/6 mice (Fig. 5d). In both of these cell lines, the effect was only seen in immune competent mice, with little to no effect in SCID mice (Supp. Fig 7b–c). Global RNA analysis, utilizing an Illumina expression array, identified a subset of genes differentially regulated by the knockdown of Pex14 and Tex9 expression in EO771 cells (Supp. Fig. 9a–b). Of interest is the upregulation of genes previously found to be associated with the immune response; namely Tiam1, Enpp2, and IL13ra1. Therefore, this screening method can identify tumor-based genes that enhance or reduce the adaptive immune response against TNBC. Each experiment was performed three times with equivalent results using 10 mice per immune competent group and 5 per immune deficient group.

Discussion

This is the first description of a high-throughput *in vivo* screening approach to identify tumor genes that regulate the anti-tumor immune responses. In our study, we utilized a pre-screening strategy to first eliminate shRNAs targeting genes essential for cell growth, and then subjected this population to *in vivo* immune selection pressures through their implantation into different immune-competent mouse models. Differential analysis revealed 709 genes that were potentially relevant to the anti-tumor adaptive immune response in TNBC. Pathway analysis of these genes revealed the over-representation of multiple interconnected processes/pathways (TGF- β signaling, MHC Class I antigen presentation, immune regulation, sphingolipid metabolism, and leukocyte adhesion) that are essential to tumor immune modulation. To confirm that these targets were important for immune function in TNBC, we validated selected hits using shRNAs in multiple mouse TNBC lines. These studies validated the importance of these pathways in suppressing anti-tumor immunity across different lines and mouse strains.

TGF β 1 signaling was validated as a critical regulator of anti-tumor immunity in these studies, and its reduction induced immune memory, as cured mice were able to resist tumor-rechallenge (Fig. 3b). Attempts to pharmacologically inhibit TGF β 1 have been attempted clinically, but have not yet elicited striking anti-tumor immunity on a consistent basis [42]. This could be due to a variety of factors that include the stage of tumor being treated (our

studies employed tumor cells with suppressed TGF β 1 expression at the time of implantation), as well as heterogeneity within tumors. To this point, our studies also revealed a less pronounced anti-tumor phenotype after TGF β 1 suppression in certain TNBC lines, such as the highly aggressive 4T1 line. In 4T1 cells, TGF β 1 inhibition is not sufficient to elicit anti-tumor immunity but is highly effective when combined with CD47 inhibition. As these pathways have been linked in different settings, it is likely that compensatory signaling could offset the anti-tumor effect of single pathway suppression in certain contexts [43, 44]. This suggests that combinatorial approaches may be needed for effective TGF- β targeted immunotherapy. Thus, this work suggests that TGF β 1 may be an excellent target for combinatorial approaches to elicit anti-tumor adaptive immunity. The dual KD of CD47 and TGF β 1 likely works through the perturbation of the interaction of both proteins with the secreted protein, thrombospondin 1 (TSP1). TSP1 is a direct binding partner of CD47 and has been shown to inhibit angiogenesis, modulate extracellular matrix formation and cell migration, suppress tumor, and most importantly, activate latent TGF β 1 [45, 46]. Thus, a reduction in CD47 on the cell surface of cells allows for higher micro environmental levels of TSP1, potentially leading to the activation of more TGF β 1, which has been directly shown in murine osteoblasts [44]. Therefore, in our model system, co-inhibition of both TGF β 1 after CD47 inhibition, likely inhibits the potential for compensatory TGF β 1 mechanisms from occurring.

This work also revealed that a cluster of genes with known immune regulatory function could have a profound effect on anti-tumor adaptive immunity. CD47 is one of these genes and has previously been shown to inhibit macrophage-mediated lysis through binding to SIRP α on macrophages. In previous studies, antagonism of CD47 led to enhancement in tumor cell lysis by macrophages as well as an increase in CD8⁺ T cells in a syngeneic murine model [47]. However, the impact of CD47 blockade on immunity in our studies was largely T-cell mediated, as knockdown or forced overexpression of CD47 did not alter the growth of EO771 tumors in C57Bl/6-SCID mice, which contain macrophages but lack T-cells. As CD47 is a highly expressed cell surface molecule on many tumors [38], we also tested if antibody-mediated blockade could elicit an anti-tumor phenotype. We found that while one CD47-directed antibody had an anti-tumor effect, it was less effective compared to knockdown of CD47, suggesting functions for CD47 that cannot be blocked by antibodies, or a need for more intense antibody dosing to overcome the large *in vivo* CD47 sink in mice.

Another critical pathway revealed in this work is that of sphingolipid metabolism. While studies have demonstrated that this metabolic process impacts immunity, its relation to the anti-tumor immune response is less clear. Our studies focused on sphingosine-1-phosphate lyase 1 (Sgpl1), which has been shown to cleave phosphorylated sphingoid bases, mainly S1P, which influence T-cell migration [48, 49]. We found that suppression of Sgpl1 had a significant, selective effect on resistance to anti-tumor immune attack in immune competent mice and that this effect is present in multiple TNBC lines and mouse strains. A recent report by van der Weyden et al identified a number of genes important for the metastasis of melanoma and TNBC cells in an immune competent setting [50]. They employed an *in vivo* RNA-seq metastasis screen in immune competent mice across a variety of cell lines and mouse strains, and identified the importance of sphingosine-1-phosphate (S1P) in local

immune cell trafficking. Blocking the release of S1P through the genetic deletion of its extracellular transporter, S1P transporter spinster homologue 2 (Spns2) led to an increased infiltration of effector T cells and natural killer (NK) cells into the metastatic tumor site, lowering the metastatic burden. Since the target Sgpl1, identified and validated in our *in vivo* screens, directly and irreversibly degrades S1P and results in a similar phenotype, our findings are consistent with an impact of Sgpl1 knockdown on its immune stimulated phenotype [50]. Therefore, we hypothesize that knockdown of Sgpl1 works to enhance immune cell tumor destruction by increasing the microenvironment levels of S1P, leading to an increase in NK cell and CD8 T cell trafficking into the tumor. It should also be noted that EO771-shSgpl1 cells grown in SCID mice have a significant delay in growth as compared with NSG mice, suggesting that NK cells are capable of slowing the growth of these tumors alone (Supp. Fig. 7a). While this pathway is known to have inflammatory and immune involvement, it has not yet been targeted in tumor immunotherapeutic approaches. As such, these findings suggest that pharmacologic inhibition of this metabolic pathway could enhance anti-tumor immunity. In line with the previous effect of CD47 and TGFB1 co-KD, it is likely that combining TGFB1 KD with Sgpl1 knockdown will also have a dramatic effect on tumor growth, since similar compensatory mechanisms exist between S1P and TGFB signaling. Both TGFB1 and Sgpl1's binding target S1P, activate the phosphorylation of SMAD3 in cultured cells [51–53]. On top of this TGFB1 has been shown to increase S1P mRNA expression and S1P can transactivate the TGFB1 pathway [54], suggesting a level of crosstalk between the two pathways. In our model system, KD of Sgpl1 enhances S1P expression and can potentially lead to an increase in TGFB1 signaling. Therefore, activation of the TGFB1 pathway might be a compensatory mechanism in TNBC cancer to Sgpl1 elimination, suggesting the combination as potentially synergistic.

Finally, this screening approach also identified a plethora of genes now implicated in immunity-related control of tumor growth that have not yet been linked with any specific immune related functions. We explored this class of genes by focusing on the impact of two genes, Tex9 and Pex14, which were shown to play contrasting roles in tumor immunosuppression. Tex9 has no known function, only being first identified in the Mammalian Gene Collection [55]. We found that knockdown of Tex9 enhanced growth in immunocompetent but not immunocompromised mice. This pattern suggests that Tex9 could function as a tumor antigen in EO771 tumors, but this possibility has not been formally explored. In contrast, Pex14 knockdown reduces the representation of targeted cells in immunocompetent mice, and thus may play a role in tumor immunosuppression. Pex14 is a peroxisomal membrane protein that plays a role in peroxisomal import machinery and can also act as a transcriptional co-repressor [56, 57]. While Pex14 has been implicated in TNBC [37], it has not been associated with any role in tumor immunosuppression. Our results suggest that it may be important for tumor immunosuppression in TNBC. These validation studies demonstrate the ability of this screening approach to identify novel regulators of tumor immunity, thereby providing a path to the identification of new targets for immunotherapy.

By utilizing an RNAi screening approach in an *in vivo* setting, we have identified multiple conserved pathways that underlie tumor-based immune evasion, without introducing bias towards an individual cell type or interaction, and have identified genes whose knockdown

promotes tumor rejection in immunocompetent, but not immunodeficient syngeneic mice. The potential of this functional screening approach is underscored by the identification of several major pathways and novel molecules that regulate the response to anti-tumor immune attack. This screening approach can be used with more powerful immune selection imposed by immune checkpoint inhibition, with the potential to identify novel resistance mechanisms *in vivo*; such studies are ongoing in our laboratory. Alternatively, some genes that regulate anti-tumor immune responses in these and related screens might serve as prognostic or predictive biomarkers when tested in the human setting. Therefore, the preclinical and clinical utility of this screening method could have important implications for the discovery and development of novel immune therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BCA	Bicinchoninic acid assay
CaeCam2	Carcinoembryonic Antigen related cell adhesion molecule 2
CCK	Cholecystokinin
CCL4	C-C motif chemokine ligand 4
CO₂	Carbon Dioxide
CRISPR	Clustered regularly interspaced short palindromic repeats
ENPP2	Ectonucleotide Pyrophosphatase/phosphodiesterase 2
FDR	False Discovery Rate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCL	Hydrogen Chloride
IDO	Indoleamine-pyrrole 2,3 dioxygenase
IgG	Immunoglobulin G

IL-4	Interleukin 4
IL13Ra1	interleukin 13 receptor subunit alpha 1
KD	Knocked Down
LIMMA	Linear Models for Microarray Data
MOI	Multiplicity of Infection
NSG	NOD-SCID-IL2 Gamma Chain knock-out
NaCl	Sodium Chloride
OE	Overexpression
OX40L	Tumor Necrosis Factor (ligand) superfamily, member 4
P53	Tumor Protein P53
Pex14	Peroxisomal Membrane Protein 14
RNAi	RNA interference
S1P	Sphingosine-1-phosphate
SBI	System Biosciences
SEMA7a	Semaphorin 7a
Sgpl1	Sphingosine-1-phosphate ligase 1
shRNA	Short Harpin RNA
SMAD3	SMAD Family member 3
Spns2	S1P transporter spinster homologue 2
Tex9	Testis Expressed Gene 9
Tiam1	T-Cell Lymphoma invasion and metastasis 1
TME	Tumor Microenvironment
TNBC	Triple-Negative Breast Cancer
TSP1	Thrombospondin 1
WT	Wild-Type

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Figure 1.

Schematic of *in vivo* RNAi screening approach to identify tumor-based modulators of immune selection. **a)** EO771 cells were infected with a lentivirus delivered genome-wide shRNA library in order to have only 1 shRNA integration per cell. EO771-library cells were engrafted in mice with varying levels of immunity; C57Bl/6, C57Bl/6-SCID, and NSG mice. Differential analysis of barcoded was used to identify functional mediators of the anti-tumor immune response. **b)** Raw shRNA expression values from two separate replicates of EO771-library cells. The similarity of relative expression values for each shRNA is represented by the identity line $y=x$, represented in red. **c)** Integration of shRNA and gene targets from four separate replicates of EO771-library cells describes the overlap of gene targets found in each. **d)** EO771-library cells that contain an shRNA that enhances survival in WT but not in immune-deficient mice are represented as red containing cells. Blue shRNA containing cells are preferentially eliminated in immune-competent mice. shRNAs with no effect on survival from the immune system will expand at similar rates in both mice (green).

Figure 2.

In vivo RNAi screen identifies shRNAs gained or lost when EO771-library tumor cells are grown in an immune competent host. All results shown are from the initial *in vivo* screen and include analysis of all arrays run for each *in vivo* condition (4 arrays for C57BL/6, 5 for SCID, 5 for NSG). **a**) Survival of mice engrafted with EO771-library cells. Both BL6 vs. SCID and BL6 vs. NSG were statistically significant. * = Mantel-Cox test with $p < 0.05$. **b**) Total number of genes targeted by shRNAs across each tumor strain. **c**) Waterfall plot of relative fold change of each shRNA in the C57Bl/6 vs. NSG analysis. Green circles represent those shRNAs with an adjusted p value (q value) < 0.05 . **d**) Volcano plot of each shRNA by log2 fold change and q value. Green circles represent those hits with a absolute log2 fold change > 1 and $q < 0.05$. **e**) Combined results of significant shRNAs from the two independent RNAi screens. **f**) Relative levels of shRNAs from the first screen in each individual tumor found through the Affymetrix arrays. B8 initial represents the starting

shRNA repertoire. The red line represents background array expression. The blue line is considered to be the detection limit for an shRNA in the tumor. Each screen was performed with 10 mice per group and two tumors (equal amounts) were combined for each array. WT= wild-type mouse, SCID = severe combined immunodeficient mouse, NSG = NOD SCID Gamma mouse. * indicates a $q < 0.05$; ** indicates a $q < 0.01$; *** indicates a $q < 0.001$.

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Figure 3.

Loss of TGFB1 enhances *in vivo* adaptive immune response to Triple Negative Breast Cancer cell lines in multiple mouse strains. **a)** *In vivo* analysis of C57Bl/6 mice engrafted with EO771 (2×10^6) cells with KD of TGFB1. **b)** Surviving mice from **a)** and naïve C57Bl/6 mice were challenged at day 90 post initial tumor engraftment with 2.5 times the initial inoculum of EO771-WT cells (5×10^6 cells) to assess for an anti-tumor adaptive memory response. Representative *in vivo* analysis of Balb/c mice engrafted with 5×10^5 JC TGF β 1 KD cells (**c**) and 10^4 4T1 TGF β 1 KD cells (**d**). **e)** Overexpression of TGF β 1 in JC cells results in rapid growth of tumors in immune competent Balb/c mice. **f)** Engraftment of 4T1 cells with double KD of TGF β 1 and CD47 in WT Balb/c mice results in the retardation of tumor growth. N = 5 mice per group. Each panel is a representative survival and growth kinetics analysis (panel insert) from one animal study and was repeated two times with equivalent results. The dotted lines and circle markers indicate cells expressing the shScramble, while solid lines and square markers represent the cells with the indicated shRNA knockdown or protein overexpression.

Figure 4.

Abrogation of CD47 signaling enhances *in vivo* adaptive immune response to Triple Negative Breast Cancer cell lines in multiple mouse strains. **a)** *In vivo* analysis of WT C57Bl/6 mice engrafted with 2×10^6 EO771 cells with CD47 shRNA KD, a scrambled shRNA control, or CD47 overexpression (OE). **b)** and **c)** *In vivo* analysis of WT Balb/c mice engrafted with 5×10^5 JC cells with CD47 KD and 10^4 4T1 cells with CD47 KD, respectively. **d)** Representative *in vivo* analysis of C57Bl/6 mice engrafted with 10^6 EO771-WT cells treated with PBS or the anti-CD47 antibody VX-1000R. N = 10 mice per group for **a)** and **d)**. N = 5 mice per group for **b)** and **c)**. Each panel is a representative survival and growth kinetics analysis (panel insert) from one animal study and was repeated at least once with equivalent results. The dotted lines and circle markers indicate mice engrafted with cells expressing the shScramble, solid lines and square markers represent those mice engrafted with cells with CD47 shRNA knockdown, dashed lines and triangle markers indicate mice engrafted with cells with CD47 overexpression.

Figure 5.

Knockdown of three novel genes (Sgpl1, Pex14, and Tex9) augment the antitumor immune response in multiple models of TNBC in an immune competent setting. **a)** and **b)** *In vivo* analysis of C57Bl/6 and Balb/c mice, respectively, engrafted with 2×10^6 EO771 or 10^4 4T1 cells, with scrambled shRNA or Sgpl1 shRNA integration. N = 10 mice per group for **a)** and **b)**. **c)** and **d)** *In vivo* analysis of C57Bl/6 mice, engrafted with 2×10^6 EO771 cells, with scrambled shRNA, Pex14 shRNA (**c**), or Tex9 shRNA integration (**d**). N = 10 mice per group. Each panel is a representative survival and growth kinetics analysis (panel insert) from one animal study and was repeated at least once with equivalent results (3 times for EO771 C57Bl/6 studies, 2 times for 4T1 Balb/c studies). The dotted lines and circle markers indicate mice engrafted with cells expressing the shScramble, solid lines and square markers represent those mice engrafted with cells with the targeted gene shRNA knockdown.

Table 1

Combined results of significant shRNAs from two independent in vivo screens

	Average # in C57Bl/6 tumors	Average # in SCID tumors	Average # in NSG tumors	Gained in C57Bl/6 vs. SCID + NSG	Lost in C57Bl/6 vs. SCID + NSG
Screen 1					
Genes targeted	7978	9253	8609	14	196
Unique shRNAs	9828	11457	10834	13	184
Screen 2					
Genes targeted	15186	11264	N/A	3	565
Unique shRNAs	23813	14416	N/A	3	541
Enriched pathways	Significant genes in indicated pathway				
TGF- β R signaling	Tgfb1, Elk1, Rps6ka5, Smad3, Smad5, Mef2d				
Immune regulation	Cd47 (2), Igkc2 (2)*, Il17, Ifnb1, H2-Q5, H2-Q6				
Sphingolipid metabolism	Sgpl1*, Smpd2, Gltd2, B4galnt2				
Leukocyte adhesion to endothelial cells	Cd99, Vcam1, Cxcl11, Sele				
MHC class I antigen presentation	Psmid8 (2)*, Uba52, Ubc, Rps27a				

Genes highlighted by a * were significant in both screens. A (2) following the gene name indicates two unique shRNAs were identified as significant