Aus der Medizinische Klinik mit Schwerpunkt Hämatologie, Onkologie und Tumorimmunologie der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

A screen to uncover mediators of resistance to liver X receptor agonistic cancer therapy

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Ermittlung potenzieller Vermittler von Resistenz gegen die Liver-X Rezeptor agonistische Krebstherapie

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LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ApoA1	Apolipoprotein A1
ApoE	Apolipoprotein E
BCRP	Breast cancer resistance protein
CLL	Chronic lymphocytic leukemia
CRC	Colorectal carcinoma
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cells
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
GM-CFS	Granulocyte macrophage colony-stimulating factor
GVAX	Irradiated tumor cell vaccine
HDL	High density lipoprotein
HEV	High endothelial venule
HGF	Hepatocyte growth factor
HSPB7	Heat shock protein family B member 7
ICAM-1	Intracellular adhesion molecule 1
LPCAT3	Lysophosphatidylcholine acyltransferase

LPS	Lipopolysaccharide
LXR	Liver X Receptor
LXRE	LXR-responsive element
MDR-1	Multidrug resistance protein 1
MDSC	Myeloid-derived suppressor cell
MRP-1	Multidrug resistance-associated protein 1
MSI-H	High microsatellite instability
N-CoR	Nuclear receptor co-repressor
NSG	NOD scid gamma
PD1	Programmed cell death protein 1
PSGL-1	P-selectin glycoprotein ligand-1
RCT	Reverse cholesterol transport
RT-qPCR	Real time quantitative PCR
RXR	Retinoid X Receptor
SELL	L-selectin
SREBP	Sterol regulatory element-binding protein
TAM	Tumor-associated macrophage
TGF-β	Tumor growth factor β
TME	Tumor microenvironment
VEGF-A	Vascular endothelial growth factor A

ABSTRACT

Objectives: Colorectal carcinoma (CRC) is one of the most common malignant tumors in the Western population. Liver X receptor (LXR) agonistic therapy has been shown to efficiently reduce tumor growth in colorectal carcinoma and a variety of other cancer types in mice. The potent LXR agonist RGX-104 is currently being tested in a phase 1 clinical trial in humans, potentially becoming a viable therapeutic option for cancer patients. Acquired drug resistance remains a major problem in cancer therapy and the present work examines to what extent such development of resistance to the LXR agonist RGX-104 takes place in colorectal carcinoma and seeks to find potential mediators of resistance to RGX-104.

Methods: In this study, the murine colon carcinoma cell line CT26 was used to develop CT26 cell lines capable of progressing under RGX-104 treatment. Subsequently, *in vitro* and *in vivo* growth behavior of these resistant cell lines was characterized. For *in vivo* testing, experiments were conducted in immunocompetent and immunocompromised mice. Finally, alterations in gene expression levels of these resistant CT26 cell lines were analyzed using RNA-sequencing.

Results: *In vivo* characterization of resistant CT26 cells demonstrated a robust growth advantage in comparison to CT26 cells naïve to LXR agonistic therapy in the presence of RGX-104 treatment and suggested an immune evasive phenotype. Consistent with this, the genetic screen captured both known and novel candidate mediators of immunoevasion. Amongst these novel candidate genes, the present work revealed *Abcg1, Sell* and *Hspb7* to be significantly upregulated in the resistant cells and implicated them in playing an important role in conferring the described growth advantage.

Conclusion: This study introduces *Abcg1, Sell* and *Hspb7* as potential mediators of drug resistance to the LXRβ-agonist RGX-104 and could provide the basis for further research on any of these genes as potential targets in cancer therapy.

Ziele: Das kolorektale Karzinom stellt einen der häufigsten bösartigen Tumore in der westlichen Bevölkerung dar. Es konnte gezeigt werden, dass die Behandlung von Tumoren mit einem Liver-X Rezeptor (LXR) Agonisten das Tumorwachstum von kolorektalen Karzinomen, sowie einer Vielzahl anderer Krebsarten, bei Mäusen effektiv reduzieren kann. Der LXR Agonist RGX-104 wird derzeit in einer klinischen Phase-1-Studie am Menschen getestet und ist somit auf dem Weg zu einer praktikablen therapeutischen Option für Krebspatienten zu werden. Die Entwicklung von Resistenzen gegen zunächst wirksame Krebsmedikamente stellt eine wichtige Problematik in der Krebstherapie dar. In der vorliegenden Arbeit wird die Fragestellung untersucht, inwieweit eine derartige Resistenzentwicklung gegen den LXR Agonisten RGX-104 stattfindet und wodurch diese Resistenz vermittelt wird.

Methode: In dieser Studie wurde die murine Kolonkarzinom-Zelllinie CT26 verwendet, um CT26-Zelllinen zu derivieren, die in der Lage sind, unter LXR-agonistischer Therapie zu wachsen. Anschließend wurde das Wachstumsverhalten dieser resistenten Zellklone sowohl *in vivo* als auch *in vitro* untersucht. Um das *in vivo* Wachstumsverhalten genauer zu charakterisieren wurden Experimente in immunkompetenten und an immungeschwächten Mäusen durchgeführt. Anschließend wurden Veränderungen der Genexpressionslevels dieser resistenten CT26-Zellklone mittels RNA-Sequenzierung analysiert.

Ergebnisse: *In vivo* zeigte sich unter der Behandlung mit RGX-104 ein signifikanter Wachstumsvorteil der resistenten Zelllinien im Vergleich zu CT26 Zellen, die noch nie mit einem LXR Agonisten behandelt wurden. Zudem wiesen die resistenten CT26 Zellen einen immunevasiven Phänotyp auf. Damit vereinbar identifizierte das genetische Screening sowohl bekannte als auch neue Kandidatengene, die eine Immunevasion begünstigen können. Als Topkandidaten dieser bislang nicht charakterisierten Kandidaten zeigte diese Arbeit, dass *Abcg1*, *Sell* und *Hspb7* in den resistenten Zellen deutlich hochreguliert sind und dass sie eine wichtige Rolle in dem zuvor aufgezeigten Wachstumsvorteil spielen.

Schlussfolgerung: Diese Studie führt *Abcg1*, *Sell* und *Hspb7* als potenzielle Vermittler von Resistenz gegen den LXR Agonisten RGX-104 ein, und bildet die Grundlage zu weiterführender Forschung an diesen Genen in der Krebstherapie.

1. INTRODUCTION

Colorectal carcinoma

Colorectal carcinoma (CRC) is one of the most common malignant tumors in the Western population, ranking third in the US and third among the male population in Germany. Among women in Germany, colorectal carcinoma is the second most common cancer (1,2). Sporadic colorectal carcinoma is typically a disease of the older population with increasing occurrence in patients aged 50 years and older. Improved screening in the population of this age and the associated early detection and removal of cancer precursors has overall led to a decrease in incidence of colorectal carcinoma in recent years. However, among individuals aged 40 or younger the trend is contrary, with an increased incidence of cases (2). Currently, colorectal carcinomas are treated with radical surgery and a chemotherapeutic combination regime, containing oxaliplatin, 5-fluorouracil and leucovorin or capecitabine. In advanced stages, additional targeted therapies are applied, such as antibodies against epidermal growth factor receptor (EGFR) and against vascular endothelial growth factor A (VEGF-A) (3). Colorectal carcinoma with high microsatellite instability (MSI-H) constitutes a subgroup of CRC that exhibits enhanced immunogenicity due to increased neoantigen burden (4). Clinical studies have indicated a high efficacy of checkpoint inhibition in patients with MSI-H colorectal carcinoma, but checkpoint inhibition is not yet commonly used in the clinic for treatment of colorectal carcinoma (5). Randomized and placebo-controlled studies have shown that chemotherapy plus additional treatment with the VEGF-A inhibitor bevacizumab increases progression-free survival of patients with metastatic colorectal carcinoma to a median of 10.6 months, compared to 6.2 months without additional bevacizumab treatment. Nonetheless, almost in all patients, drug treatment eventually fails, resulting in progression of the disease and highlighting the remarkable capacity of colorectal carcinoma cells to develop mechanisms to evade drug treatment (6).

Drug resistance in cancer

Despite the plethora of distinct treatment approaches that have been investigated over the last decades, drug resistance remains a central roadblock to successful cancer therapy. Major factors accounting for the frequent emergence of drug resistance in cancer include genomic instability, tumor heterogeneity, and heterogeneity in the tumor microenvironment (TME). Tumors can be intrinsically resistant, meaning that drug therapy is originally ineffective, or they can acquire resistance during therapy (7).

In the following I will briefly describe some of the most common mechanisms that promote drug resistance in cancer.

Increased drug efflux and drug inactivation

Reducing intracellular drug accumulation by upregulating mediators of drug efflux is an important mechanism of cancer drug resistance. The most studied transporter proteins in this context are members of the ATP binding cassette transporter family. Multidrug resistance protein 1 (MDR-1, also ABCB-1) has been associated with chemoresistance in many cancer types and can mediate the efflux of a variety of chemotherapeutic agents, such as etoposide, vinblastine, doxorubicin, paclitaxel and some targeted therapies. MDR-1 has been shown to be upregulated before cancer treatment, leading to intrinsic resistance, but can also be upregulated upon treatment (8–10). Multidrug resistance-associated protein 1 (MRP-1, also known as ABCC-1) has also been linked to drug resistance in various cancers, and breast cancer resistance protein (BCRP, also ABCG-2) is a mediator of drug resistance in breast cancer and leukemia (11,12). Furthermore, drug resistance can stem from tumor cells failing to activate prodrugs or metabolically inactivating active compounds (13).

DNA damage repair

Many chemotherapeutics, such as cisplatin and etoposide, target the DNA or the DNA replication apparatus of tumor cells, leading to impaired DNA replication, cell cycle arrest, and subsequent apoptotic cell death. To evade this destiny, cancer cells need additional alterations in DNA damage repair pathways. Genes involved in DNA damage response mechanisms are frequently dysregulated or silenced in cancer. The dependence of cancer cells on alternative repair pathways can be therapeutically exploited to combat drug resistance (14).

Deregulation of apoptosis

Many cancer therapeutics result in apoptosis, the programmed cell death of cancer cells. Apoptosis can be induced by the intrinsic pathway, in which p53, BAX and caspase-9 play an important role, and the extrinsic pathway, for which death receptors and caspase-8 are essential (15). There are several pro- and antiapoptotic proteins involved in these pathways, and upregulation of antiapoptotic proteins as well as silencing of proapoptotic proteins, such as p53, is common in many types of cancer (16). Antiapoptotic B-cell lymphoma 2 family members, the protein kinase B (Akt), the caspase-8 inhibitor FLIP and the nuclear factor-κB (NF-κB) have been shown to be overly active antiapoptotic proteins

in a broad set of cancers, for example mediating the survival of tumor cells during chemotherapeutical treatment (17).

Drug target alteration

Directly targeting proteins that are specific for the tumor or that are typically dysregulated in tumor cells has revolutionized cancer therapy (18). Although targeted therapies generally are very effective and show fewer side effects than chemotherapeutic agents, their efficacy can be affected by mutations in the drug target or alterations in the expression level of the targeted protein: inhibitors of EGFR, which is commonly upregulated in cancer, had to be re-designed multiple times to overcome resistance caused by EGFR gatekeeper mutations (19). Increased upregulation of the androgen receptor (AR) in prostate cancer resulted in failure of AR inhibitors bicalutamide and leuprolide (20).

Tumor microenvironment

The microenvironment of solid tumors is composed of a variety of cellular and non-cellular components including fibroblasts, immune cells, blood vessels, and the extracellular matrix (ECM). It has long been known that the TME plays a fundamental role in tumor development and cancer progression (21). In addition, recent studies have identified the TME as a mediator of drug resistance. Integrins, which attach cells to the ECM, can modulate intracellular signaling pathways associated with cancer progression and drug resistance, and upregulation of integrins has been linked to reduced response of cancer cells to pharmaceutical treatment (17,22). Cells of the microenvironment can also promote survival of cancer cells upon treatment by releasing cytokines and growth factors, such as the proinflammatory messenger interleukin-6, which has been shown to mediate resistance to doxorubicin treatment in Burkitt's lymphoma, and hepatocyte growth factor (HGF), which can lead to resistance to the BRAF inhibitor PLX4720 (23,24). Furthermore, immunosuppressive cytokines such as interleukin-10 and tumor growth factor (TGF)-β, which can be produced by the tumor or by cells of the microenvironment, can mediate failure of cancer immunotherapy (25). Myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), both of which can dampen immune responses, have been shown to be more abundant in the microenvironment of tumors that are resistant to checkpoint inhibitors (26,27).

Epithelial-mesenchymal transition

As pointed out before, cancer cells in solid tumors are well integrated in the TME. However, to become metastatic they need to overcome cell-cell adhesion by repressing cell adhesion receptors,

such as integrins and cadherins, which occurs during epithelial-mesenchymal transition (EMT). EMT can be induced by many signaling pathways, such as TGF-β, PDGF and EGF signaling (28). Additionally, EMT-cells exhibit a cancer stem cell-like phenotype, including the upregulation of signaling pathways such as Wnt, Notch, and Hedgehog signaling. These signaling pathways promote cell survival and proliferation and can help the tumor cell to evade drug-induced cell death. Cancer stem cells exhibit stem cell-like characteristics that promote resistance to chemotherapeutic agents by combining the features mentioned previously, such as high expression of anti-apoptotic proteins, increased drug efflux, and the use of alternative DNA damage repair mechanisms (29,30).

In conclusion, cancer treatment can become ineffective because of a wide spectrum of resistance mechanisms, eventually leading to tumor progression and increased cancer mortality. Therefore, identifying mediators of drug resistance and finding ways to overcome resistance is essential in improving cancer therapy.

Liver-X nuclear receptors

The role of LXRs in lipid metabolism

The liver X receptors α and β (LXRα and LXRβ, respectively) are members of the family of nuclear hormone receptor transcription factors and play an important role in lipid metabolism. LXRα is highly expressed in the liver and is also abundant in other tissues involved in cholesterol metabolism, especially in the intestine and in adipose tissue, while LXRβ is ubiquitously expressed (31). Both LXRα and LXRβ form permissive heterodimers with the retinoid X receptor (RXR) that bind to specific recognition sequences in the promoters of target genes. These specific recognition sequences are called LXR-responsive elements (LXREs) (32). In the absence of a ligand, LXR/RXR heterodimers form complexes with co-repressors, which inhibits target gene expression. Chen *et al*. identified silencing mediator for retinoid or thyroid-hormone receptors (SMRT) as a co-repressor of RXR, while another important co-repressor of this complex, nuclear receptor co-repressor (N-CoR), was identified by Hörlein and colleagues (33,34). The binding of a ligand leads to a conformational change in the LXR/RXR complex, resulting in an exchange of the bound co-repressor with a co-activator (35). This in turn leads to the expression of the target gene. Important ligands that can activate this complex are cholesterol metabolites, in particular oxysterols. Target genes encode ATP-binding cassette (ABC) transporter isoforms A1, G1, G5 and G8, various apolipoproteins (including

ApoE), cholesteryl ester transfer protein (CETP) 33, fatty acid synthase and cytochrome P450 isoform 7A1 (36).

Studies in *Lxrα*-knockout mice revealed the impact of LXRs on cholesterol homeostasis. *Lxrα* null mice, fed with a diet rich in cholesterol, accumulate cholesterol in the liver due to reduced cholesterol catabolism and bile acid metabolism (37). LXR-mediated expression of *ABCG5* and *ABCG8* in the liver and intestine has been shown to lead to increased sterol secretion and efflux (38,39). Several studies have linked LXR activation with reverse cholesterol transport (RCT), indicating an impact of LXRs on whole-body cholesterol homeostasis (38,40,41). Important mediators of cellular cholesterol efflux – the initial step of RCT – are ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1). ABCA1 transports cholesterol and phospholipids to the transport protein apolipoprotein A1 (ApoA1), and ABCG1 mediates cholesterol efflux to high density lipoproteins (HDL) (42,43). Laffitte and colleagues additionally showed that the expression of apolipoprotein E (ApoE), which plays an important role in cholesterol transport, is regulated by LXRs in macrophages and adipose tissue (44). Hence, increased uptake of cholesterol activates the LXR/RXR complex, inducing expression of the cholesterol efflux proteins ABCA1 and ABCG1 and the cholesterol transport protein ApoE. As such, LXR signaling represents a central pathway in the regulation of intracellular cholesterol levels. It also plays important roles in pathophysiological contexts, as exemplified by its role in macrophages in the prevention of atherosclerosis (45,46).
In addition to their broad role as regulators in cholesterol homeostasis, LXRs are also important in fatty acid and phospholipid metabolism. They regulate de novo lipogenesis in the liver, mainly through induction of the expression of sterol regulatory element-binding protein 1c (SREBP-1c). Additionally, LXR-dependent expression of lysophosphatidylcholine acyltransferase 3 (LPCAT3) mediates phospholipid metabolism and cell membrane homeostasis, especially in the intestine (36).

The immunological functions of LXRs

In addition to their canonical role in lipid metabolism, LXRs have been shown to exert immunomodulatory effects. The involvement of LXRs in modulating inflammatory responses has been widely studied, but their exact role has not yet been fully resolved. LXRs have been shown to repress inflammatory gene expression in macrophages upon presentation of bacterial lipopolysaccharides (LPS) *in vitro* and can also reduce inflammatory responses *in vivo* (47). In

contrast to these observations in murine systems, Fontaine and colleagues propose a different effect of LXRs on human macrophages, showing that pretreatment of human macrophages with LXR agonists leads to an enhanced inflammatory response upon LPS exposure. This was detectable after 48 hours of pretreatment, while short-term pretreatment resulted in reduction of the inflammatory response, suggesting a time dependence of this effect (48). Consistent with a role of LXRs in promoting host defense, absence of LXRs in *Lxrαβ*$^{-/-}$ mice leads to an increased susceptibility to infection with *Listeria moncytogenes*. This effect was attributed to the decreased expression of apoptosis inhibitor 6 (also SPα) in *Lxrαβ*$^{-/-}$ mice, which is transcriptionally regulated by LXRα and acts as promoter of macrophage survival and antimicrobial activity (49).

The question of how inflammatory gene expression in macrophages is modulated by LXR-signaling is not yet completely understood, as there are no LXREs found in the promoter regions of many of the genes that are regulated by LXRs. Inflammatory gene regulation by LXRs is thought to function through trans-repression where modulation of NF-κB signaling seems to play an important role (50). The role LXRs play in modulating inflammatory responses is not limited to macrophages. Bensinger and colleagues showed that LXRs can inhibit the proliferation of activated T cells and suggest that intracellular sterol levels, regulated by ABCG1, play a role in this effect (51). Additionally, Cui and colleagues linked LXR-dependent induction of SREBP-1 to inhibition of Th17 cell differentiation through suppression of IL-17 transcription. Th17 cells are a subtype of CD4+ T cells, playing a role in activating neutrophils and in the development of autoimmune diseases (52). Intracellular sterol levels also seem to play an important role in dendritic cell (DC) activation. *Abca1/g1*-deficiency in DCs leads to the accumulation of intracellular cholesterol and to increased inflammatory activity, including enlarged lymph nodes and upregulation of CD11b+DC abundance and inflammatory cytokine secretion (53). In 2009, Villablanca and colleagues found that tumors can release ligands that activate LXRα in maturating DCs, leading to the inhibition of C-C chemokine receptor type 7 (CCR7) expression (54). CCR7 expression on DCs has been shown to be an important mediator of DC migration to lymphoid organs, where DCs can then activate T cells (55). These findings suggest an immune-dampening effect of LXRs in DCs. However, the results of Villablanca and colleagues conflict with the findings of Feig and colleagues, whereupon LXR-agonistic treatment of immature DCs leads to increased CCR7 expression, thus increasing the inflammatory response (56).

The pro-inflammatory effects of LXRs on DCs have been underlined by Töröcsik and colleagues, who showed that pre-treatment of DCs with LXR agonists leads to increased expression of

costimulatory molecules CD80 and CD86 and inflammatory cytokines such as TNF-α and IL-12 upon LPS presentation (57). Additionally, Beceiro and colleagues found that efficient DC chemotaxis is dependent on LXR regulated expression of CD38 (58).

In conclusion, the role of LXRs in modulating inflammation is highly complex, with LXR activation resulting in the promotion or inhibition of inflammation depending on the specific context in which it occurs.

LXRs in cancer

LXRs have been described previously to have antitumor effects on a variety of cancers. Fukuchi and colleagues were the first to show the antiproliferative effects of LXR agonists on LNCaP human prostate cancer cells (59). This initial report led to a variety of studies identifying antiproliferative effects of LXR agonists on other sex-specific tumor cells, such as ovarian and breast cancer cells (60,61). Moreover, Lo Sasso and colleagues found that expression of activated LXRα could reduce intestinal tumor formation and slow the growth of xenograft colorectal tumors in mice (62).

ApoE, a target gene of LXR, has been implicated in various diseases such as cardiovascular and Alzheimer's disease (63,64). Recent studies from the Tavazoie laboratory have identified ApoE as a suppressor of metastatic and immune evasive phenotypes in cancer (65–67). Pencheva and colleagues used a systematic *in vivo* selection-based approach to identify microRNAs that are involved in the metastatic formation of melanoma. microRNAs are a set of small non-coding RNAs of 20-22 nucleotides, which have been described to influence gene expression post-transcriptionally and have been implicated in the pathogenesis of cancer and cancer metastasis (68–70). Studying highly metastatic LM2 melanoma cells led to the identification of three miRNAs (miR-1908, miR-199a-3p and miR-199a-5p) as drivers of metastasis in melanoma cells. Pencheva et al. further found that each of these miRNAs negatively regulate *Apoe* gene expression and that suppression of extracellular ApoE leads to increased endothelial recruitment and melanoma cell invasion *in vitro* and *in vivo*, two important metastatic progression phenotypes. Upregulation of ApoE with an LXRβ agonist not only reduced melanoma metastatic progression, but also strongly inhibited melanoma tumor growth and prolonged animal survival. Additional experiments, conducted by Tavazoie and colleagues, revealed that the potent LXRβ agonist RGX-104 could suppress tumor growth not only in melanoma but also in ovarian cancer, glioblastoma, lung cancer, breast cancer, kidney and colon cancer (66). RGX-104 is an orally bioavailable small molecule that activates LXRβ and is currently being tested in a

multicenter national phase 1 clinical trial in cancer patients (ClinicalTrials.Gov, NCT02922764). Tavazoie and colleagues also showed that treatment of tumor-bearing mice with RGX-104 leads to depletion of MDSCs, resulting in enhanced anti-tumor T cell responses. MDSCs are a heterogeneous subset of immune cells that are known to suppress anti-tumor immune responses, thus mediating the immune escape of cancer cells (71). Immunosuppression in the cancer environment resulting in the evasion of cancer cells is a major hurdle to effective cancer therapy, and pharmacologic approaches to reinvigorate anti-cancer immune responses such as inhibitors of immune checkpoints have started to transform cancer therapy (72).

In conclusion, RGX-104-mediated activation of LXRβ robustly suppresses tumor growth and progression in various tumor types by combating at least three hallmarks of cancer: by reducing endothelial recruitment and the invasion of cancer cells and by restoring immune function (16).

Aim of the study

LXRβ-agonists, such as RGX-104, have been shown to inhibit tumor growth and metastasis in a broad set of cancers through endothelial recruitment and the repression of cancer cell invasion and by enhancing the anti-immune response. Drug resistance remains a major challenge in cancer therapy, and resistance-induced therapeutic failure is a main reason for cancer-related mortality. Cancer cells are prone to adapt to a hostile environment, and as a result, mechanisms of resistance arise frequently. Therefore, it is crucial to find mediators of resistance to cancer drugs in order to identify intrinsically resistant tumors before treatment with a certain drug and to find new potential targets in cancer therapy that can re-sensitize resistant tumor cells. The aim of this study was to find mediators of resistance to RGX-104 in colorectal carcinoma using an unbiased *in vivo* screening method.

2. MATERIALS AND METHODS

Data collection

The results presented in the following study were primarily collected by me during my yearlong research stay in the laboratory of Dr. Sohail Tavazoie at The Rockefeller University. At the beginning of my work in the laboratory, first generation resistant and naïve cell lines had already been derived and validated and gene expression levels had been analyzed using RNA-sequencing. These data, collected by Dr. med. Benjamin Ostendorf, a postdoc in the lab, were kindly provided to me at the beginning of my research in the lab. For the sake of clarity, I present all results of this study in the Results section below. Experiments that were not carried out by me are identified by a footnote.

Tissue culture

The murine colon carcinoma cell line CT26 was obtained from ATCC and cultured in RPMI medium (Gibco RPMI 1640 Medium, ThermoFisher) supplemented with 10% FBS (Fetal Bovine Serum, Sigma-Aldrich) and HEPES Buffer (Gibco HEPES, ThermoFisher) to a final concentration of 10mM.

The 293LTV cell line was purchased from Cell Biolabs and cultured in DMEM (Gibco DMEM–Dulbecco's Modified Eagle Medium, Thermo Fisher) with 10% FBS.
All cells were incubated at 37°C and 5% CO_2. Medium was changed every other day and cells were split once confluency reached 80%.

All cell lines were periodically tested for mycoplasma by PCR.

Mouse experiments

All mouse experiments were conducted in agreement with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at The Rockefeller University.
NOD.Cg-*Prkdc*^{scid} *Il2rg*$^{-/-}$ (NOD scid gamma, NSG) mice were obtained from the Jackson Laboratory (strain number 005557); BALB/c mice were from Charles River (strain 028). BALB/c mice were fed regular rodent chow, while NSG mice received rodent chow supplemented with amoxicillin.

Derivation of naïve and resistant CT26 cell lines[1]

2 x 10^5 CT26 cells were re-suspended in 50 µL PBS, mixed 1:1 with matrigel (356231, Corning) and subcutaneously injected into the lower right flank of 6-8 weeks old male BALB/c mice. Three days after injection the mice were randomly assigned to a chow supplemented with RGX-104 (drug provided by Rgenix, chow supplemented with RGX-104 by Research Diets at 625.8 mg per kg of chow, target dose of 100 mg/kg) or control chow. Mice were palpated every three days for tumor formation and upon formation, tumors were measured every two to three days in two dimensions using digital calipers. Tumor volume was estimated calculating $\pi/6$ x (small diameter) ^{2} x (large diameter).

Once tumor volume exceeded 1500 mm², mice were euthanized by rapid cervical dislocation, and tumors were resected and transferred into one well of a 6-well plate filled with DPBS (Dulbecco's Phosphate-Buffered Saline, Corning) supplemented with Penicillin/Streptomycin (Gibco, Life Technologies) and Amphotericin B (BioWhittaker, Lonza). For dissociation, tumors were cut into small pieces using sterile scalpels, transferred into a 50 mL falcon and spun down at 300 x *g* for 5 minutes at 4°C. For red blood cell lysis, tumor chunks were re-suspended in 5mL of ACK buffer (Lonza), incubated at room temperature for five minutes, neutralized with DPBS and spun down as described above. Tumor pieces were then re-suspended in HBSS+ (Hank's Balanced Salt Solution with Calcium Chloride and Magnesium Chloride, Gibco, life technologies) supplemented with HEPES Buffer (Gibco HEPES, ThermoFisher) to a final concentration of 25 mM, 2% FBS, Pyruvate (Gibco, Life Technologies), Collagenase Type 1 (XOM12195, Worthington Biochemical) to a final concentration of 1.25 mg/mL, Penicillin/Streptomycin and Amphotericin B and incubated for 45 minutes at 37°C with shaking.

The solution was then spun down, re-suspended in 7 mL Trypsin (0.25% Trypsin-EDTA, Gibco, life technologies), incubated for 10 minutes at 37°C with shaking and then neutralized in RPMI (Gibco™ RPMI 1640 Medium, ThermoFisher) supplemented with 10% FBS, HEPES and Penicillin/Streptomycin and Amphotericin B (R10FH) . The cells were filtered through a 70 µM

[1] Naïve and first-generation resistant cell lines were derived by Dr. med. Benjamin Ostendorf.

strainer and transferred to a flask.
Cells were kept in culture for 3-4 passages to deplete non-cancerous stromal cells and then frozen down.

For the derivation of second-generation resistant cell lines, 2 x 10^5 resistant CT26 cells were prepared as described above and injected into the lower flank of 6-8 weeks old BALB/c mice. Starting three days after injection, mice were administered a chow supplemented with RGX-104.
Formation and growth of the tumors were measured as described above and once the estimated tumor volume exceeded 1500 mm^3, mice were euthanized and tumor cells were dissociated as described above.

Validation of tumor growth advantage in resistant CT26 cell lines[2]
2 x 10^5 cells of each of the seven naïve and five resistant CT26 cell lines were prepared and injected as described above into 6-8 weeks old male BALB/c mice. Three days after injection, one group of mice injected with naïve cell lines and one group injected with resistant cell lines were assigned to chow containing RGX-104, and the second group of mice injected with naïve cell lines and the second group of mice injected with the resistant cell lines were fed a control chow. Tumor growth was measured as described above.

Tumor growth of resistant and naïve cell lines in an immunocompromised mouse model
As an immunocompromised mouse model NSG mice were used. 2 x 10^5 cells of each of the seven naïve and five resistant CT26 cell lines were prepared and injected as described above into 6-8 weeks old male BALB/c and NSG mice. Three days after injection, all mice were administered a chow supplemented with RGX-104. Tumor growth was measured as described above.

In vitro cell proliferation assay
To determine whether the derived resistant cell lines exhibited a proliferative advantage in comparison to the derived naïve cell lines, for each CT26 cell line duplicates of 2 x 10^4 cells were seeded on a 6-well plate. After one day, cells were cultured in the presence of either DMSO (Dimethyl sulfoxide,

[2] Conducted by Dr. med. Benjamin Ostendorf.

Sigma-Aldrich) only or RGX-104 at 1 μM in DMSO vehicle. After 4 days, the number of viable cells was counted using a hematocytometer and excluding dead cells by trypan blue staining (72-57-1, Sigma-Aldrich).

Staining of naïve and resistant tumors for CD8a

Six to eight weeks old BALB/c mice were injected with the previously derived cell lines (five resistant and seven naïve, one mouse per cell line). Cells were prepared and injected, and tumors were measured as described above. Three days after injection, mice were administered a chow supplemented with RGX-104. On day 17 after injection, all mice were euthanized by rapid cervical dislocation, and tumors were resected, embedded in O.C.T. Compound (Fisher Healthcare, Tissue plus) and snap frozen by submerging into 2-Methylbutane (Isopentane, Sigma-Aldrich, 78-78-4) cooled with liquid nitrogen. Snap frozen tumors were stored at -80°C until further utilization.

Tumors were sectioned into sections of 5 μm thickness using a Leica cryotome, mounted on a Superfrost Plus Microscope Slide (Fisher Scientific), and stored at -80°C overnight.

The next day sections were allowed to thaw at room temperature for one hour and then incubated in acetone (Fisher Scientific, 67-64-1) at -20°C for five minutes. They were subsequently immersed in acetone with 50% methanol (Methanol HPLC Grade, Fisher Chemical, 67-56-1) at -20°C for five minutes followed by another five-minute incubational step in acetone at -20°C.

Sections were washed three times in DPBS at room temperature and blocked in DPBS with 5% donkey serum (Sigma Aldrich) for 30 minutes at room temperature. CD8a Antibody (Anti-Mouse CD8a, Clone 53-6.7, eBioscience, Fisher Scientific) was diluted 1:100 in DPBS supplemented with 5% donkey serum and sections, covered with the diluted antibody solution, were incubated at 4°C overnight. The next day, sections were washed three times in DPBS. As a secondary antibody donkey anti-Rat IgG, Alexa Fluor 488, was diluted 1:200 in DPBS supplemented with 5% donkey serum. Sections were incubated with the diluted secondary antibody for 45 minutes at room temperature in the dark. Sections were washed once in DPBS and counterstained with DAPI nuclear stain (4,6-Diamidine-2-phenylindole-dihydrochloride, Roche) at a final concentration of 1 μg/mL in DPBS for five minutes followed by another wash in DPBS. For mounting, ~30 μL Prolong Gold Antifade Reagent (Invitrogen) was added to each section, covered using a cover slip and left to dry overnight at room temperature in the dark.

Fluorescence was measured using a laser scanning confocal microscope (Zeiss LSM 510) at 20X magnification. Five pictures were taken of each sample (locations: upper right and left, lower right

and left, middle) and the number of CD8a positive cells was quantified using FiJi software and CellProfiler software using a custom pipeline. The average number of CD8a positive cells in 5 pictures of one sample was calculated and normalized to the average number of DAPI positive cells.

RNA-sequencing[3]

Library preparation and sequencing for RNA-sequencing

Early-passage (between passage three to seven) naïve and resistant cell lines were seeded onto 6-well plates and RNA was isolated the next day using the Total RNA Purification kit according to the manufacturer's instructions (Norgen total RNA Purification Kit). For digestion of DNA, DNAseI treatment was performed using the DNAse I kit according to the manufacturer's instructions (RNAse-free DNAse I kit, Norgen Biotek). Subsequently, libraries for RNA-sequencing were prepared using the Scriptseq Complete kit according to the manufacturer's instructions (Epicentre ScriptSeq Complete). As part of this protocol, ribosomal RNA was depleted using the RiboZero Gold kit according to the manufacturer's instructions (Illumina). Cleanup of RNA was performed using Ampure XP beads (Beckman Coulter). The concentration of libraries was measured using an Agilent Bioanalyzer 2100. RNA libraries were pooled and sequenced on one lane of an Illumina NextSeq sequencer (75 basepair single-end reads).

Analysis of RNA-sequencing

Adapter sequences of RNAseq reads were trimmed using cutadapt (`options: -m 15 -q 20 -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC`). Reads were aligned to the mm10 mouse genome using STAR aligner (73) with default settings and restricting output to uniquely mapping transcripts (`--outFilterMultimapNmax 1`). Reads were then quantified using featurecounts (74). Downstream analysis was performed in R using RStudio. Differential gene expression between

[3] Library preparation and analysis of RNA-sequencing results were conducted by Dr. med. Benjamin Ostendorf.

naïve and resistant cell lines was assessed using DESeq2 (75). For ranking of differentially expressed genes, the moderated log-fold change was used.

Gene expression analysis by qRT-PCR

Cells were seeded in triplicates on a 6-well plate and RNA was extracted the next day using the Total RNA Purification Kit according to the manufacturer's instructions (Norgen Total RNA Purification Kit).

1 µg of RNA was reverse transcribed into cDNA using the Superscript III First Strand Synthesis System (Invitrogen, 18080051) and 10 ng of resulting cDNA was used for quantitative real-time PCR (qRT-PCR). For real-time PCR amplification, cDNA was mixed with 300 nM of the respective primers and Fast SYBR® green PCR Master Mix (Applied Biosystems, 4385612).

An ABI Prism 7900HT Real-Time PCR System (Applied Biosystems) was used to quantify the mRNA expression levels. Technical replicates were performed in quadruplicates for each reaction, and the expression of *Gapdh* was assessed as an endogenous control. The average of the quadruplicates of each sample was calculated and gene expression was normalized to *Gapdh* calculating $X^{Ct(\text{Target Gene})}/X^{Ct(\textit{Gapdh})}$ (X = 2 for primers with 100% efficiency).

List of primer sequences for qRT-PCR

Gene-ID	Primer Sequence	
Gapdh	Fwd	GCACAGTCAAGGCCGAGAAT
	Rev	GCCTTCTCCATGGTGGTGAA
Abcg1	Fwd	GTGGATGAGGTTGAGACAGACC
	Rev	CCTCGGGTACAGAGTAGGAAAG
Aldh3a1	Fwd	TGGCAAAGACTCGTCAGACC
	Rev	AGTTCCAAGCACCTATGACAAG
Ceacam1	Fwd	TCACGGGGCAAGCATACAG
	Rev	TCGCCTGAGTACGACGATAGT
Dpys	Fwd	CCACAGGGACGACTTCTCATC
	Rev	CGCTGCATCTAGGATTCGCA
Hspb7	Fwd	ATGAGCCATCGGACCTCCTC
	Rev	CGAGTGGGGCAGCATAAAACT

Prkcb	Fwd	AGCGAGACACCTCCAACTTC
	Rev	CAGTGGGAGTCAGTTCCACAG
Sell	Fwd	TACATTGCCCAAAAGCCCTTAT
	Rev	CATCGTTCCATTTCCCAGAGTC
Sncg	Fwd	GGTCAGCAGCGTCAACACA
	Rev	TCTTGGCCTCTTCATTCTCCTC
Tnfrsf9	Fwd	CGTGCAGAACTCCTGTGATAAC
	Rev	GTCCACCTATGCTGGAGAAGG
2010300C02Rik	Fwd	AGCCTCCTAATAGGGACACATC
	Rev	CACTTTGGTCCTGTCTCTCTGTA

Primer sequences for *Abcg1, Aldh3a1, Ceacam1, Dpys, Hspb7, Prkcb, Sell, Tnf*rsf9 and *2010300C02Rik* were obtained from the Havard Primer Bank; primer sequence for *Sncg* was used as published by Jia and colleagues (76,77).

Lentiviral knockdown of mRNA

shRNA

shRNA plasmid constructs in bacterial glycerol stock were purchased from Sigma-Aldrich. A small amount of bacterial stock was scraped off using a sterile pipette tip and re-suspended in 70 µl of S.O.C. solution (Thermo Fisher) at room temperature. The solution was spread on a pre-warmed LB agar plate supplemented with 100 µg/mL of Ampicillin, and bacteria were left to grow overnight at 37°C. On the next day single colonies from each plate were picked using a sterile pipette tip, re-suspended in 3mL of Luria Broth (LB) medium supplemented with 100 µg/mL of Ampicillin and incubated in an orbital shaker (37°C) for 8 hours. Subsequently, 50 µL of bacteria/LB medium solution was mixed with 50 mL of LB medium supplemented with 100 µg/mL of Ampicillin and incubated overnight (37°C, shaking). The next day plasmid DNA was purified using a Plasmid Plus Midi Kit according to the manufacturer's instructions and eluted in H2O (Qiagen, 12943).

Transfection

For generation of viral particles, 293-LTV cells were seeded on a 10 cm dish and transfected at 60 – 90% confluency. For transfection 2 µg of pCGPV, 2 µg of VSV-G and 2 µg of RSV-Rev were mixed with 6 µg of the respective shRNA construct and 35 µg of PEI (Polyethylenimine, Polysciences, 9002-

98-6). After 20 minutes of incubation at room temperature, the complex was added dropwise to 293-LTV cells. The next day medium on the cells was replaced with fresh medium and 48h later virus was harvested by taking off the cell medium, spinning it down at 300 x g for 5 minutes and filtering the supernatant through a 0.45 μm filter (Corning, 430314).

Lentiviral transduction

For lentiviral transduction, 200,000 CT26 cells were transduced with 2 mL of appropriate virus in the presence of 10μg/mL of polybrene (Sigma-Aldrich, TR-1003-G) for 16h. 48h after adding the virus, 13 μg of puromycin (Thermo Fisher Scientific, A1113803) was added to the cell medium for lentiviral selection. Cells were cultured in the presence of Puromycin until all cells on a non-transduced control plate were dead. The level of mRNA knockdown was confirmed by qRT-PCR as described above.

List of shRNA sequences

Target	shRNA Sequence
Abcg1	CCGGCCGGGTTGAAACTGTTCGTTTCTCGAGAAACGAACAGTTTCAACCCGGTTTTTG
Ceacam1	CCGGCTTTGCTTGGTACAAGGGAAACTCGAGTTTCCCTTGTACCAAGCAAAGTTTTTG
Dpys	CCGGGCACATGCAATTCCCGTTCATCTCGAGATGAACGGGAATTGCATGTGCTTTTTG
Hspb7	CCGGGCCCAATTCAAAGCATTCTAACTCGAGTTAGAATGCTTTGAATTGGGCTTTTTG
Prkcb	CCGGCGGTATATTGACTGGGAGAAACTCGAGTTTCTCCCAGTCAATATACCGTTTTT
Sell	CCGGGCATCTGTGATGCAGGGTATTCTCGAGAATACCCTGCATCACAGATGCTTTTTG
Sncg	CCGGGCACACTGAATGCCCTGCCTACTCGAGTAGGCAGGGCATTCAGTGTGCTTTTTG
2010300C02Rik	CCGGTGTGAAACTTACGATTGATTTCTCGAGAAATCAATCGTAAGTTTCACATTTTTG

Statistical analysis

With the exception of the statistical analysis of the RNA-sequencing results (analysis of RNA-sequencing described above), all statistical analyses were performed using the Student's t-test. To explore whether knockdown of target genes would reduce tumor growth rate, one-tailed t-tests were performed; for all other statistical comparisons, two-tailed t-tests were used. Statistical analyses were carried out using the computer software GraphPad Prism 6.0. Statistical significance was concluded at $P < 0.05$. Statistical designations used throughout all figures: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. All results show the mean with standard error of the mean. For all *in vivo* experiments, statistical analysis was performed without adjusting for multiple testing.

3. RESULTS

In vivo selection of cell lines resistant to LXR agonistic cancer therapy

Previous experiments in the Tavazoie lab showed that RGX-104 exhibits robust anti-tumor activity in many cancer models (66,67). However, most tumors eventually grow out under treatment with RGX-104, indicating the development of drug resistance. To confirm these findings and generate cell lines resistant to RGX-104 to investigate mediators of resistance, CT26 cells were selected *in vivo* under LXR agonistic pressure.

In order to select *in vivo* for cells exhibiting resistance to LXR-agonistic therapy, the murine colorectal carcinoma cell line CT26 was injected into syngeneic BALB/c mice. Subsequently, mice were administered control chow or chow supplemented with the LXRβ-agonist RGX-104. CT26 tumors were allowed to form and, upon reaching a pre-defined size, tumors were resected, and tumor cells were dissociated, yielding seven naïve (non-treated) cell lines, as well as five cell lines that grew out under LXR agonistic treatment (from here on referred to as resistant) (Fig. 1a-b) [4]. Consistent with the development of resistance to RGX-104, the resistant cell lines initially exhibited robust growth suppression under LXR-agonistic therapy, followed by outgrowth despite ongoing therapy.

CT26 cell lines selected *in vivo* under LXR-agonistic pressure show growth benefit *in vivo* but not *in vitro*

To validate whether cell lines that grew out under LXR treatment showed a therapy-resistant phenotype, both naïve and resistant cell lines were re-injected into BALB/c mice fed either control or LXR agonist-supplemented chow. This experiment showed a growth advantage of resistant CT26 cell lines in BALB/c mice that was significant in mice treated with RGX-104 (Fig. 2 a-b; $P = 0.34$ and $P = 0.044$ for control and RGX-104 treated cohorts, respectively, two-tailed t-test)[4]. This growth advantage of the resistant CT26 cell lines could be due to various reasons. There could be a direct evasion mechanism to the drug or its therapeutic effects. Alternatively, the cells could exhibit

[4] Experiment conducted by Dr. med. Benjamin Ostendorf.

enhanced survival fitness through enhanced proliferative activity, increased endothelial recruitment, or immune evasion.

To determine first whether increased tumor growth was due to a cell-autonomous or non-cell-autonomous mechanism, *in vitro* cell proliferation assays were performed. To this end, resistant and naïve CT26 cell lines were cultured in the presence of DMSO or RGX-104 in DMSO vehicle. Cell count after 4 days showed no *in vitro* growth advantage of the resistant cell lines under RGX-104 treatment or DMSO, suggesting a microenvironment-dependent mechanism of resistance (Fig. 2c).

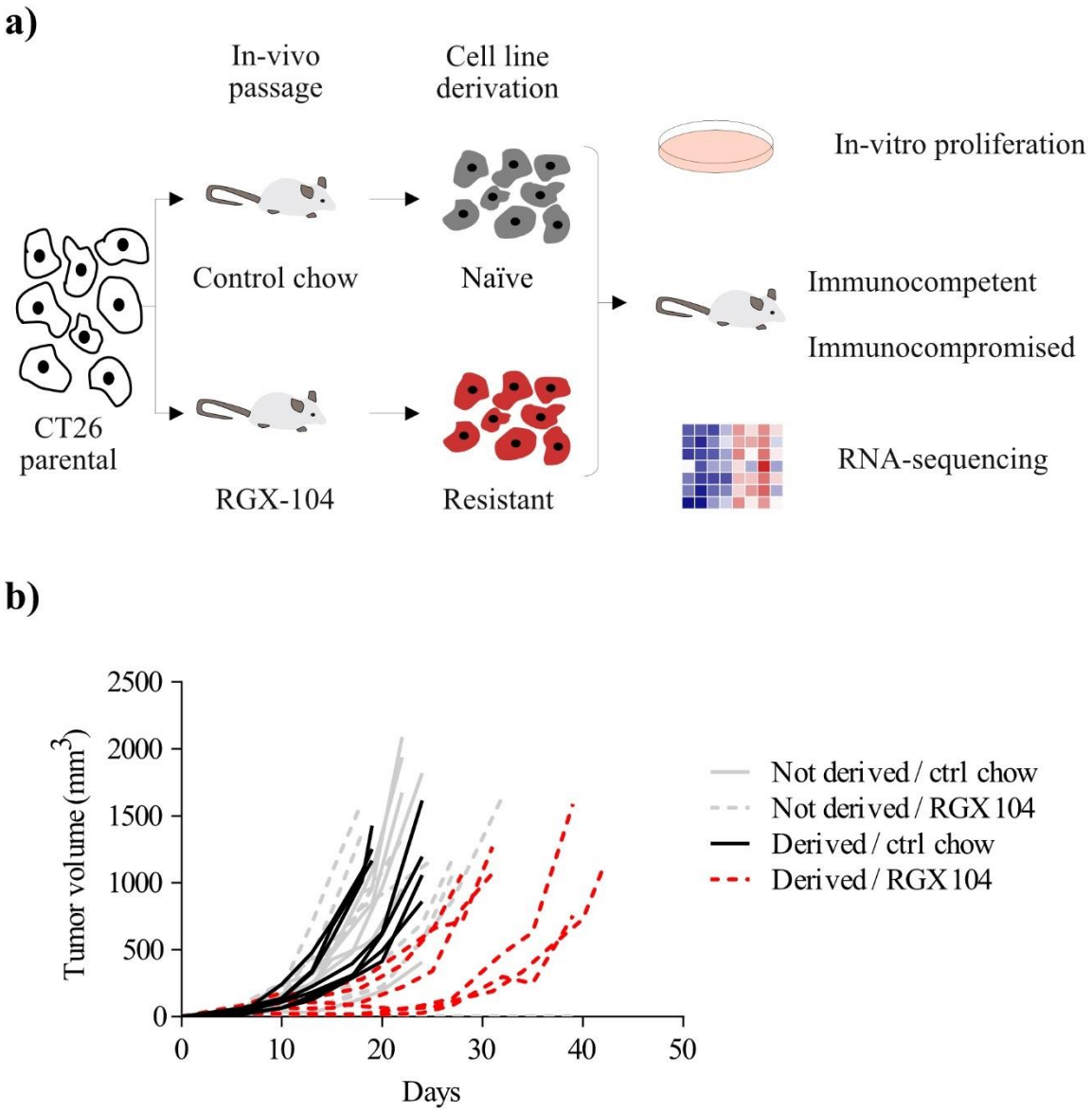

Figure 1 | In-vivo selection of syngeneic CT26 colon cancer cells under LXR agonistic treatment. a) Schematic of in-vivo selection of naïve and resistant CT26 colon cancer cells and downstream analysis. b) Volume of individual tumors during selection after injection of 200,000 CT26 cells into BALB/c mice. Cell lines were derived from black (untreated) and red (RGX-104 treated) tumors upon reaching >1000 mm^3.

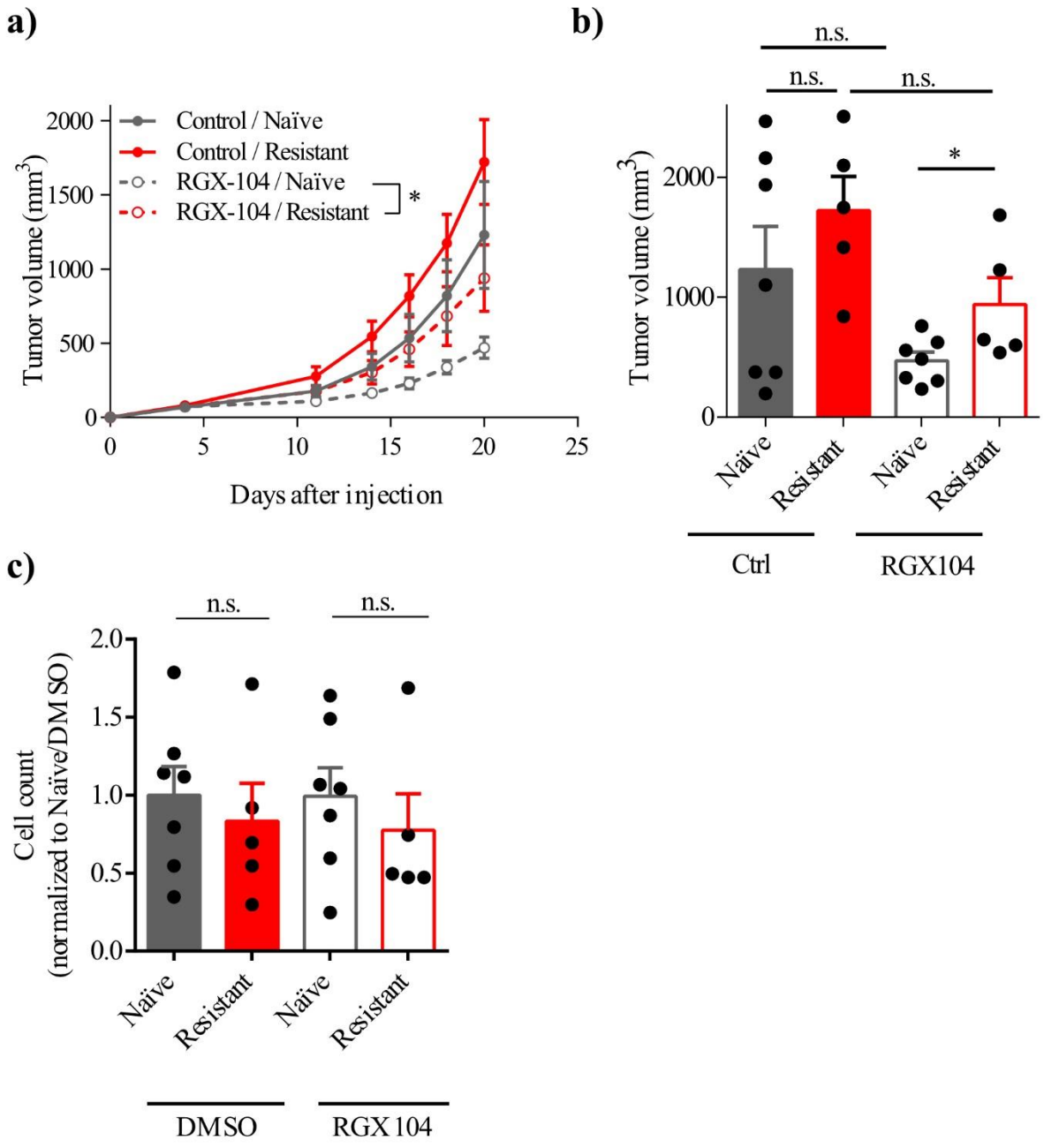

Figure 2 | CT26 resistant cell lines selected under LXR agonism grow faster than naïve cell lines in vivo but not in vitro. a) Mean Tumor volume on day 4, 11, 14, 16, 18, 20 after injection and b) Tumor volume on day 20 after injection of naïve versus resistant CT26 lines (P = 0.34 and P = 0.044 for control and RGX-104 treated cohorts, respectively, two-tailed t-test). c) In-vitro proliferation of resistant and naïve cell lines cultured in presence of DMSO or RGX-104 at 1uM in DMSO vehicle (two-tailed t-test; data points represent individually derived cell lines; one representative example of three experiments shown).

Tumors formed by resistant CT26 cell lines exhibit an immune desert phenotype

Previously, LXR-agonism was found to mediate anti-tumor effects partly in an immune-dependent manner (66). To explore whether immune evasion played a role in the observed growth benefit of the resistant CT26 cell lines, tumors derived from resistant and naïve CT26 cell lines were stained for CD8+ cells using immunofluorescence. Notably, infiltration of CD8+ cells was significantly decreased in resistant CT26 tumors in comparison to naïve tumors. (Fig. 3, P = 0.0079, two-tailed t-test; data points represent individually derived cell lines and are averaged for five fields of view per tumor). Thus, enhanced growth rates of resistant cell lines may partly be due to decreased recruitment of CD8+ cells to tumors, as a result of an immune evasive phenotype.

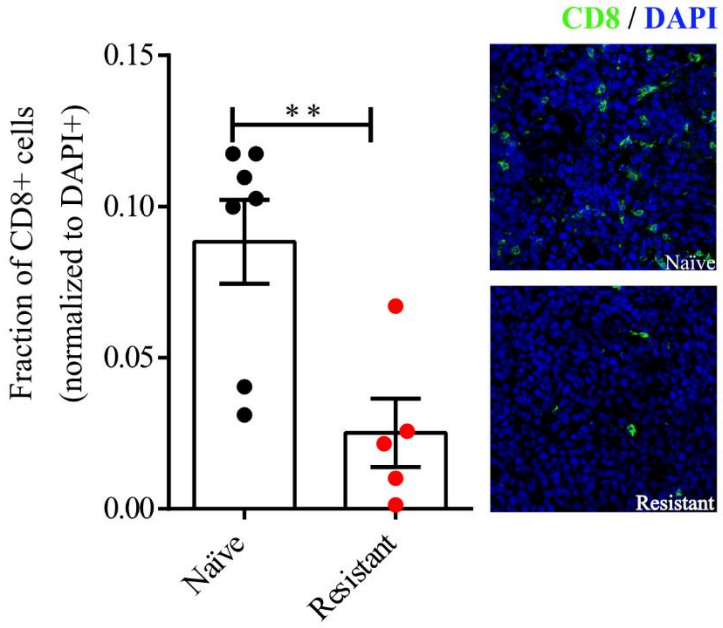

Figure 3 | Resistant CT26 cell lines exhibit an immune-desert phenotype. Fraction of CD8+ cells in tumors formed by naïve versus resistant CT26 lines under LXR therapy (P = 0.0079, two-tailed t-test; data points represent individually derived cell lines and are averaged for five fields of view per tumor).

Resistant CT26 cell lines exhibit enhanced tumor progression in immunocompetent but not in immunocompromised mice

In order to causally establish whether enhanced tumor progression of resistant CT26 cell lines depended on their interaction with adaptive immunity, tumor growth of resistant and naïve CT26 cell

lines was assessed in fully immunocompetent BALB/c mice versus in immunodeficient NSG mice under treatment with the LXRβ-agonist RGX-104. As shown above, resistant CT26 cell lines displayed a significant growth advantage in comparison with naïve CT26 cell lines in an immunocompetent context (Fig. 2 a-b). Remarkably, however, this growth advantage was completely abrogated in the context of the immunocompromised NSG mouse model. Interestingly, resistant clones not only lost their growth advantage but grew significantly more slowly in NSG mice compared to naïve CT26 cell lines (Fig. 4, $P = 0.005$ and $P = 0.018$ for BALB/c and NSG mice, respectively). In sum, resistant CT26 cell lines exhibited an immune evasive phenotype of resistance.

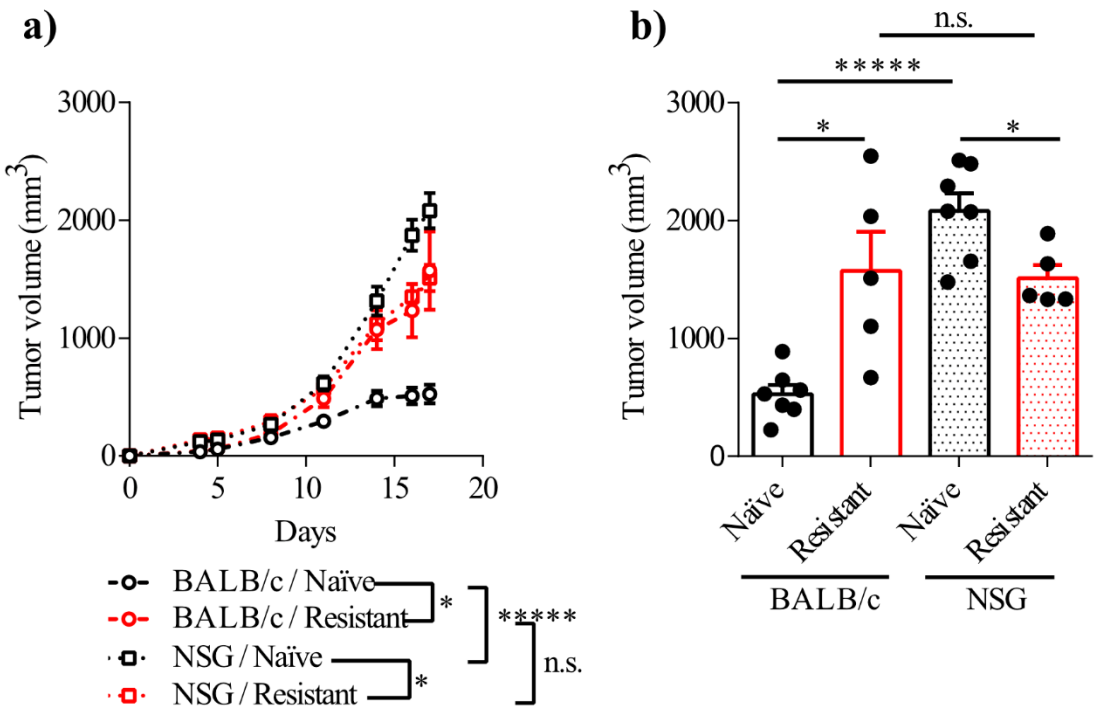

Figure 4 | Resistant CT26 cell lines grow faster in immunocompetent but slower in immunocompromised mice. a) Mean Tumor volume on day 4, 5, 8, 11, 14, 16, 17 and b) Tumor volume on day 17 after injection of naïve and resistant cell lines into immunocompetent BALB/c and immunodeficient NSG mice under LXR agonism (P = 0.005 and P = 0.018 for BALB/c and NSG mice, respectively, two-tailed t-test).

RNA-sequencing reveals known and novel candidate mediators of immune evasion

To investigate the mechanism driving immune evasion within resistant CT26 cell lines, the transcriptomic profile of resistant and naïve CT26 cell lines was compared by RNA-sequencing

(RNA-seq). Principal component analysis revealed one outlier in the control group (naïve cell line, data not shown). To increase the specificity of the results, this outlier was excluded, leading to 1382 differentially expressed genes, in which 621 were upregulated and 761 were downregulated.

The differentially expressed genes showed an upregulation of known mediators of immune evasion in the resistant cell lines, such as *Pdl1* (also known as Cd274), ligand of the programmed cell death protein 1 (PD-L1), and PD-L2 (Fig. 5a). The PD-1/PD-L1 axis is known to mediate the escape of tumor cells from the host immune system (78,79). To employ an unbiased approach to uncover possible novel mediators of tumor resistance, the top ten upregulated genes in the resistant CT26 cell lines were subsequently studied in more detail. These ten genes comprised: (1) *Abcg1*, encoding for an ATP-binding cassette transporter, (2) *Dpys*, encoding Dihydropyrimidinases, (3) *Hspb7*, encoding a heat shock protein, (4) Selectin L, encoded by *Sell*, a cell surface adhesion molecule, (5) *Tnfrsf9*, a member of the TNF-receptor superfamily, (6) *Aldh3a1*, encoding an aldehyde dehydrogenases, (7) *Ceacam1*, part of the carcinoembryonic antigen (CEA) gene family, (8) *Prkcb*, encoding a member of the protein kinase C (PKC) family, (9) *Sncg*, member of the synuclein family of proteins and (10) the protein coding gene of unknown function, *2010300C02Rik* (Fig. 5b)[5].

[5] Experiments and analysis conducted by Dr. med. Benjamin Ostendorf.

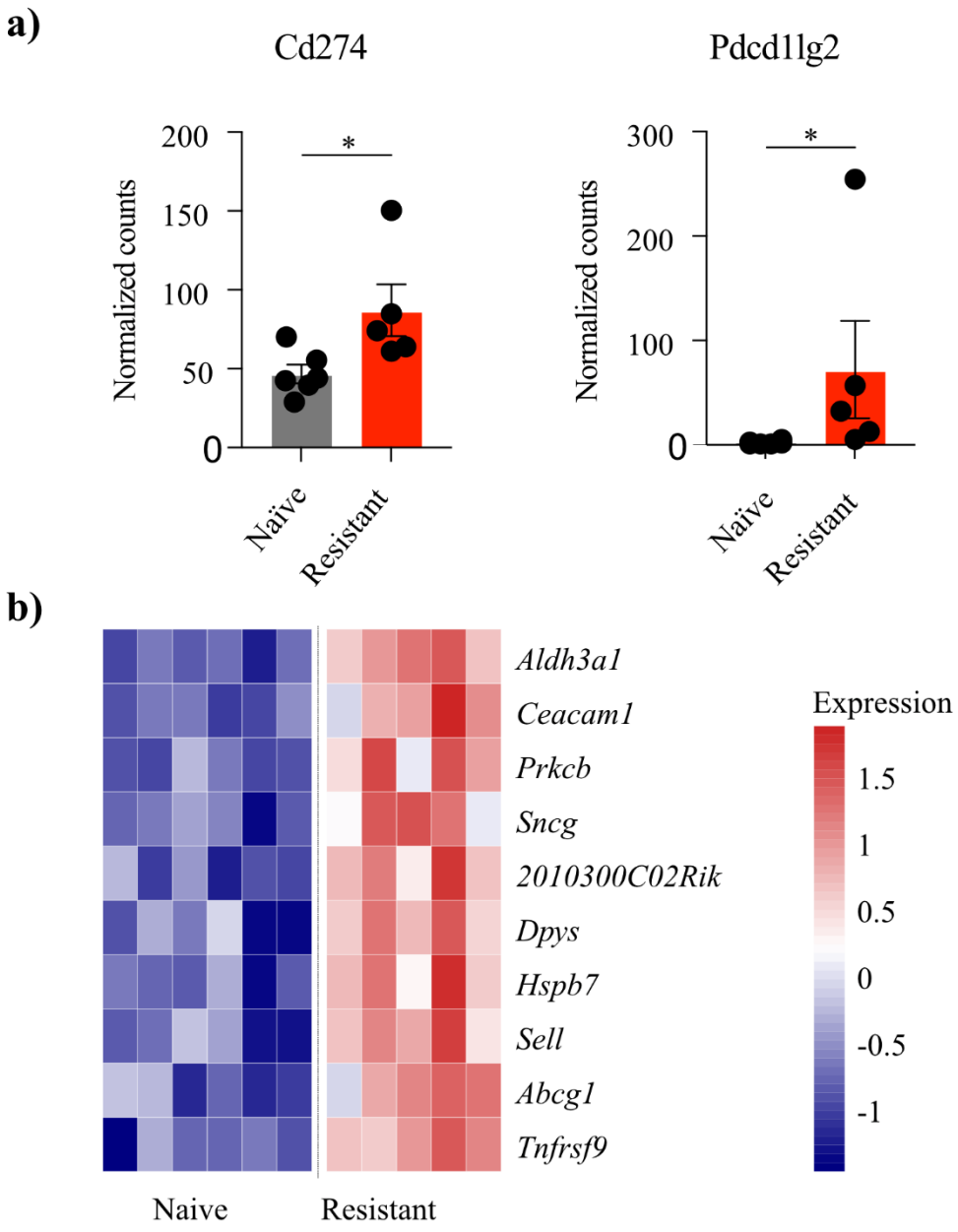

Figure 5 | In-vivo selected CT26 cell lines upregulate known and novel candidate mediators of immunoevasion. a) Resistant CT26 cell lines show higher expression of the immune checkpoint ligands Pd-l1 and Pd-l2. Normalized counts for Cd274 (Pd-l1) and Pdcd1lg2 (Pd-l2) as measured by RNA sequencing of ex-vivo cultured cell lines (P= 0.018 and P = 0.017 for CDd274 and Pdcd1lg2, respectively; Wald tests, adjusted for FDR). b) Heatmap of the expression of the top ten upregulated genes in resistant versus naïve CT26 cell lines.

Eight out of the top ten upregulated genes stay upregulated in second generation resistant clones

Assuming that genes relevant for the growth benefit of the tumor cells under LXR-agonistic pressure would stay upregulated in a second round of *in vivo* selection, resistant CT26 cell lines were re-injected into immunocompetent BALB/c mice and the mice were treated with RGX-104. Tumors were then resected, and tumor cells dissociated, generating second-generation resistant cell lines (Fig. 6a). To validate the RNA sequencing gene hits, real time quantitative PCR (RT-qPCR) was performed, confirming all 10 genes to be upregulated in the first-generation resistant CT26 cell lines (Fig. 6b). Subsequently, gene expression levels of the ten genes described above were determined in the second-generation resistant cell lines using RT-qPCR. Comparison of gene expression levels in second-generation resistant CT26 cell lines to first generation resistant and naïve CT26 cell lines showed that the upregulation of two of the target genes (*Tnfrsf9* and *Aldh3a1*) was lost in second-generation resistant cell lines. Therefore, these two genes were excluded from further analysis. (Fig. 6b).

Knockdown of *Hspb7*, *Abcg1*, *Dpys* and *Sell* in the representative resistant cell line CT26-R41 leads to reduced tumor growth *in vivo*

In order to further determine the impact of each of the remaining eight target genes on the observed tumor growth benefit, genes were knocked down in a representative resistant CT26 cell line, CT26-R41. To this end, CT26-R41 cells were transduced with either a small hairpin RNA (shRNA) targeting one of the aforementioned genes or a non-targeting control shRNA (shCtrl), generating separate knockdown cell lines for each target. Knockdown levels were confirmed using RT-qPCR (Fig. 7a). Then *in vivo* tumor growth of the knockdown cell lines was determined in immunocompetent BALB/c mice treated with RGX-104, demonstrating that knockdown of *Abcg1*, *Hspb7* and *Sell* in the resistant cell line CT26-R41 led to significantly reduced tumor growth (Fig. 7b, c, d; $P = 0.0349$, $P = 0.0358$ and $P = 0.0264$, for *Sell*, *Hspb7* and *Abcg1*, respectively). Depletion of *Dpys* resulted in smaller tumors, but did not reach statistical significance (Fig. 7d, $P = 0.074$ on last day of measurement), compared to tumors of shControl CT26-R41 cells.

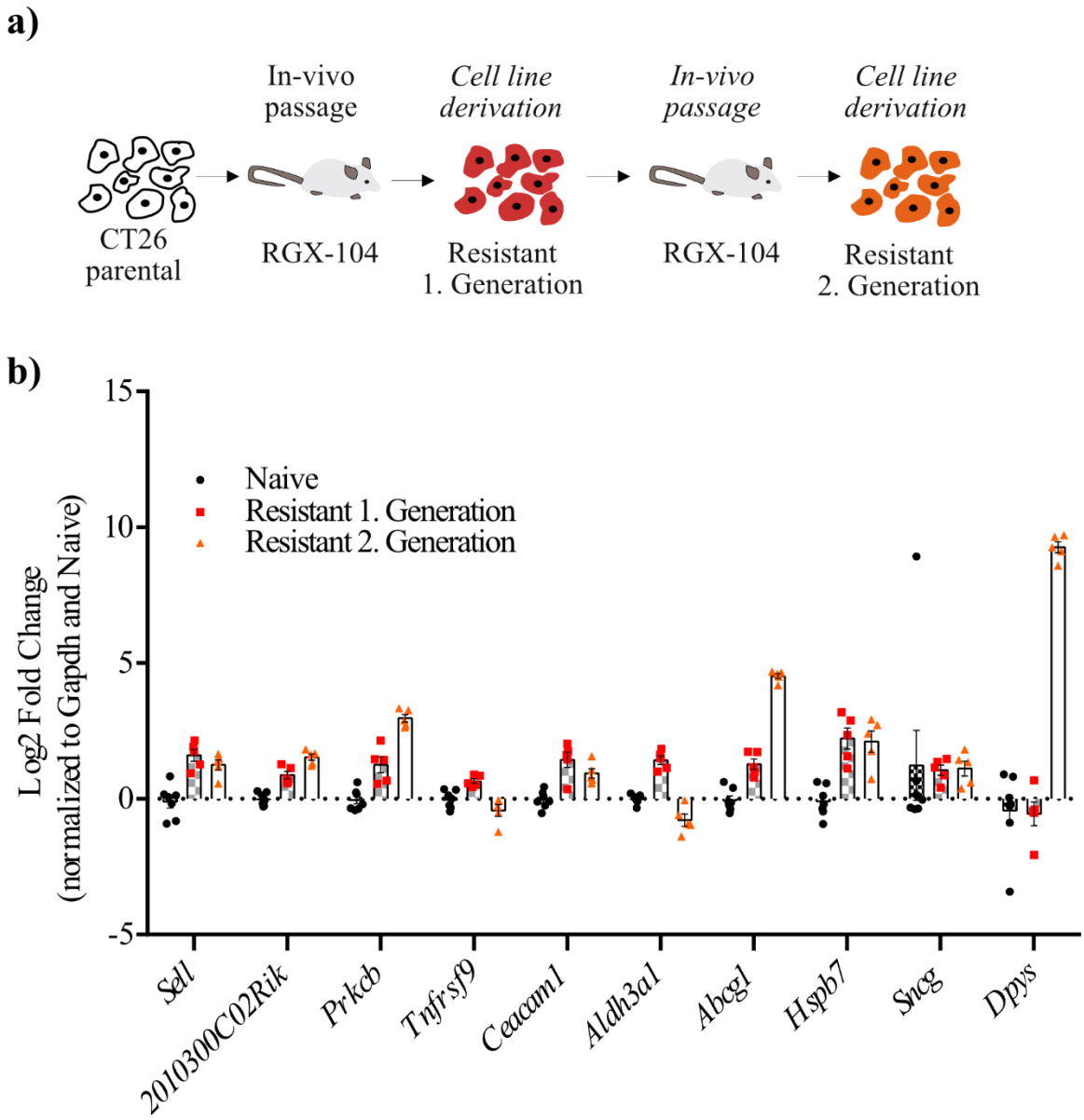

Figure 6 | Real time quantitative PCR validation of candidate mediators of resistance in resistant CT26 cell lines. a) Schematic of derivation of second generation resistant cell lines in a second in vivo passage of first generation resistant cells under LXR agonistic selection pressure. b) Expression levels of candidate mediators of immunoevasion in naive, first generation resistant and second generation resistant cell lines (Data points represent individually derived cell lines; one representative example of two experiments shown).

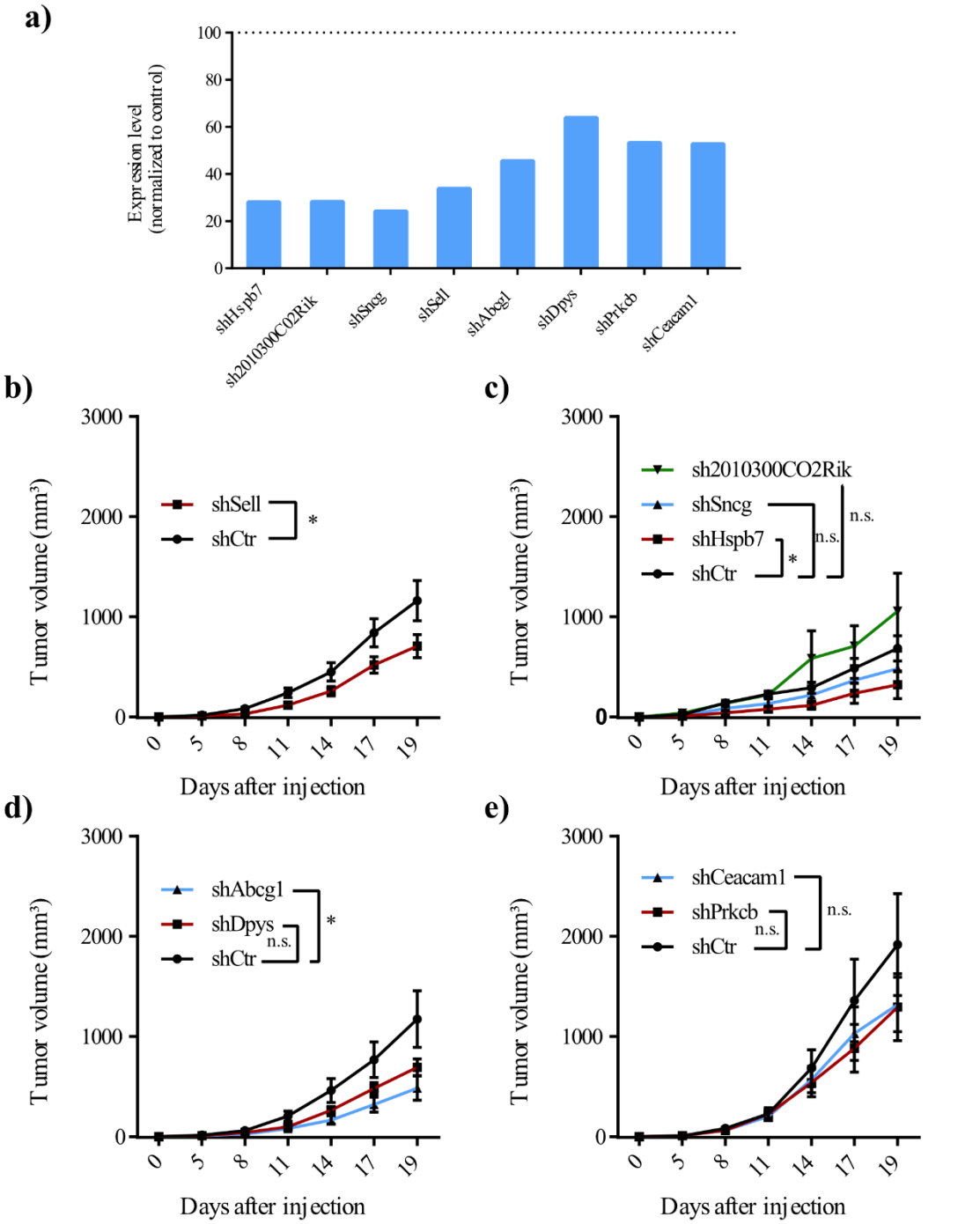

Figure 7 | Knockdown of *Abcg1*, *Sell* and *Hspb7* in representative resistant CT26 cell clone decreases tumor growth *in vivo*. a) Validation of knockdown of *Hspb7*, *2010300C02Rik*, *Sncg*, *Sell*, *Abcg1*, *Dpys*, *Prkcb* and *Ceacam1* in representative resistant cell clone R41, normalized to Control (R41 cells transduced with shCtr) b) Mean Tumor volume on day 5, 8, 11, 14, 17 and 19 after injection of shSell, c) shHspb7, sh2010300C02Rik, shSncg, d) shAbcg1, shDpys, e) shCeacam1, shPrkcb and control CT26 R41 cell clone into BALB/c mice treated with RGX-104 (P = 0.0349, P = 0.0358 and P = 0.0264, for shSell, shHspb7 and shAbcg1, respectively, one-tailed t-test, on last day of measurement)

4. DISCUSSION

Overview of major findings

Acquired drug resistance is one of the major problems in cancer therapy (80). In this study, a systematic approach was used to discover mediators of resistance to the LXR agonist RGX-104. This led to the identification of effector genes potentially involved in the development of drug resistance in murine colon carcinoma cells. In the present work, *Abcg1*, *Hspb7* and *Sell* were shown to be robustly upregulated in cell lines displaying resistance to the anti-tumor effects of RGX-104. Moreover, knockdown of each of these genes in a resistant cell line led to significantly reduced tumor growth in the context of RGX-104 therapy, suggesting either a direct impact on drug resistance or a drug independent mechanism for driving cancer progression. Additionally, the results of this study imply that the resistant cell lines exhibit an immune evasive phenotype.

Immune evasion as a mechanism of resistance in colon cancer cells treated with LXR-agonism

LXRs have previously been implicated to play a role in immune modulation (48,51) and have been associated with various immunological diseases in humans. Anderson and colleagues associated LXR-β variants with the risk of inflammatory bowel disease (81). Jeon and colleagues linked genetic polymorphism of the LXR gene to susceptibility and clinical manifestation of systemic lupus erythematosus (82). Tavazoie *et al.* showed that therapeutic LXR-agonism reduces immunosuppressive MDSC levels in cancer and, thus, promotes anti-tumor immunity (66).

The present study demonstrated that colon carcinoma cells that succeeded in growing out under LXR-agonistic pressure displayed a growth advantage specifically in mice with proficient immunity that were treated with the synthetic LXR agonist RGX-104. As LXR-agonistic therapy increases immune selective pressure on the tumor cells, it seems reasonable to assume that part of the mechanism of resistance to RGX-104 could be evasion from the immune system. Consistent with this hypothesis, resistant CT26 tumors exhibited reduced infiltration with CD8+ T-cells.

Increasing immune selective pressure frequently leads to the upregulation of certain genes in the tumor cells allowing them to survive in the hostile environment. As such, genetic approaches have been exploited to investigate the molecular underpinnings of tumoral immune evasion. Manguso *et al.* identified the phosphatase *Ptpn2* as a mediator of immune evasion, using an *in vivo* CRISPR screen on tumors under immune selective pressure (83). Similarly, Pan et al. employed CRISPR-based

screening in an *in vitro* based approach to identify genes that promote escape from CD8+ T cell attack (84). While these CRISPR-based approaches are powerful in uncovering novel mediators of tumoral resistance to immunotherapy and enable the screening of a broad set of different genes for their potential to promote cancer cell survival, *in vivo* selecting for tumor cells that independently become resistant to the therapeutic effects of LXR-agonism may enrich for genes that are upregulated in tumor cells as a mechanism of resistance to LXR-agonism, thus potentially being more relevant in humans. A combination of both methods, for example by *in vivo* selecting for tumor cells that exhibit an immune evasive phenotype, which would first help find possible mediators of resistance that tumor cells are able to endogenously exploit, and then using CRISPR-based approaches to further characterize the anti-tumoral effects of these genes, could maximize the identification of viable therapeutic target genes. Pan and colleagues as well as Manguso and colleagues used a combination of several immune activating methods to create immune selective pressure to find genes specifically mediating immune evasion. In the screen described here, tumor cells were selected for under LXR-agonistic therapy only, to search for genes able to mediate resistance to the therapeutic effects of LXR-agonism. Hence, it is possible that upregulation of *Abcg1, Hspb7* or *Sell* may be a general mechanism of immune evasion in tumor cells, which would make them promising targets in cancer therapy. To follow up on this, it will be important to explore whether upregulation of either of these genes will lead to increased tumor growth, also under the immune selective pressure of other drugs, for example, checkpoint inhibitors.

Characterization of *Abcg1, Hspb7* and *Sell* as potential mediators of cancer resistance to an LXR agonist

ABCG1

The ATP-binding cassette transporter G1 is a member of the superfamily of ABC-transporters, a group of proteins involved in the transmembrane transport of various molecules. Many members of this superfamily have been previously implicated in drug resistance in cancer, for example, the well-studied drug efflux transporter ABCB-1 (MDR-1), as well as ABCC1 (MRP-1) and ABCG-2 (BCRP) (85–88).

ABCG1 has been shown to play a role in cholesterol and phospholipid export process in macrophages and is also implicated in cholesterol efflux and intracellular cholesterol transport in other tissues (89–93).

Interestingly, it has been shown that ABCG1 expression in macrophages is inducible by LXRs (41). This might indicate that RGX-104 directly targets *Abcg1* in the CT26 cells, leading to the *Abcg1* upregulation observed in the present work. However, in the screen employed here, the cell lines were propagated *in vitro* for several passages without LXR treatment before sequencing, making it unlikely that Abcg1 was detected as a direct consequence of RGX-104 treatment. It constitutes an intriguing possibility that cancer cells exploit Abcg1, a cholesterol efflux transporter, in shuttling RGX-104 out of the cell.

ABCG1 has previously been described as a mediator of tumor progression by others. Sag and colleagues demonstrate in their work of 2015 that absence of Abcg1 in mice dramatically suppresses growth of MB49-bladder carcinoma and B16-melanoma and explain these effects by a modulation of macrophage function (94). In 2016 Tian *et al.* found ABCG1 to be upregulated in lung cancer tissue and showed that ABCG1 promotes proliferation, migration and invasion in HKULC4 lung cancer cells (95) . Thus, pro-tumoral phenotypes of Abcg1 in both the tumor and stromal compartments have been shown, indicating that systemic therapy targeting Abcg1 may be particularly effective.

HSPB7

Heat shock proteins are a ubiquitous group of proteins that are essentially involved in the process of folding, assembling and transporting various proteins in the cell (96). When cells are exposed to oxidative or thermal stress, to hypoxia, or to stress due to viral or bacterial infections, heat shock proteins become strongly induced, protecting the proteome from degradation and thereby promoting cell survival (97–99). Based on their molecular weight, human heat shock proteins are categorized into HSP90 (HSPC), HSP70 (HSPA), HSP40 (DNAJ) and small HSP (HSPB) (100).

HSPB genes encode for the family of small heat shock proteins, a group of ATP-independent chaperones with a molecular mass of 15-30 kDa, which are known to bind misfolded or unfolded proteins, preventing them from uncontrolled aggregation (101–103). The small heat shock protein HSPB7, also known as cardiovascular small heat shock protein, is particularly expressed in cardiovascular and insulin-sensitive tissue (104) and has been described to play a role in idiopathic cardiovascular diseases (105,106). Scarce data have linked HSPB7 expression to cancer progression: Lin *et al.* found HSPB7 to be downregulated in renal cell carcinoma and showed that introduction of HSPB7 leads to reduced colony formation of various RCC cell lines *in vitro* (107). Additionally, HSPB7 seems to be downregulated in a variety of cancer types in humans when compared to matching normal tissue (108). These discrepancies to the findings of the present work,

wherein downregulation of *Hspb7* impairs tumor progression, might be due to a dependency of the observed effect on treatment with RGX-104 or on differences in the studied tumor types. Importantly, the aforementioned studies only showed a correlation between HSPB7 and tumor outcome, while the study presented here used loss-of-function assays to imply a causal role of HSPB7 in mediating resistance to an immune-activating anti-cancer therapy.

Dysregulated expression of heat shock proteins is known to play a role in cancer development and cancer progression. Unstable malignant cells and their oncoproteins depend on the supportive function of many HSPs, turning them into interesting targets in cancer therapy. By reducing the cell death of unstable malignant cells, HSPs could reduce the activation of immune cells by cell debris, which might be the role played by Hspb7 in the resistant CT26 cell lines. Additionally, Hspb7 might be secreted by CT26 tumor cells and, subsequently, mediate anti-inflammatory responses as it has been previously reported for other secreted small heat shock proteins. Reddy and colleagues described an extracellular role for the small heat shock proteins Hsp27, αBC and Hsp20 that implicates them in immunological, signaling, and inflammatory pathways (109). The well-studied heat shock protein Hsp27 has been additionally shown to target and inhibit p53-signaling, rendering cancer cells resistant to induced cell death or senescence (110). Inhibition of Hsp27 by the antisense oligonucleotide OGX-427 has been shown to successfully reduce pancreatic cancer progression *in vivo* (111). With the exception of Hsp27, the role of small heat shock proteins in cancer progression and drug resistance remains unexplored and Hspb7 constitutes an interesting new candidate for further studies.

Selectin L

Selectin L, previously known as lymphocyte homing receptor, belongs to the family of selectins, a group of cell adhesion molecules (112–114). This family consists of three members: P-selectin, E-selectin and L-selectin. P-selectin (GMP-140, PADGEM) is expressed by activated endothelial cells and activated platelets, E-selectin (ELAM-1) is also expressed by activated endothelial cells. L-selectin (LECAM-1, LAM-l) is found on the surface of most leukocytes (115). The molecular structure of all three selectins is similar and consists of “an amino terminal C-type lectin domain, a single epidermal growth factor (EGF)-like domain, from two to nine short concensus repeat (SCR) domains, a single mem- brane spanning region, and a cytoplasmic tail” (116). Selectins mediate interactions between blood cells and endothelial cells and play an essential role in leukocyte recruitment to inflammatory sites or lymph nodes (116). Additionally, selectin - selectin ligand interactions play an important role in initiating intracellular signaling pathways. P-selectin

glycoprotein ligand-1 (PSGL-1) is one of the best characterized selectin ligands and selectin binding to PSGL-1 on leukocytes has been shown to mediate activation of β2-integrin, which then slows rolling on intracellular adhesion molecule 1 (ICAM-1) (117). Tinoco and colleagues described PSGL-1 as a checkpoint regulator of T cells and showed that PSGL-1 ligation on CD8+ cells inhibits IL-2 signaling and upregulates PD-1 (118). Although L-selectin is known to bind PSGL-1 only with low affinity, it seems possible that increased expression of L-selectin on CT26 tumor cells dampens the T-cell mediated immune response to the tumor by binding to PSGL-1 on CD8+ cells. Additionally, cross-linking of activated L-selectin on leukocytes leads to receptor tyrosine and serine phosphorylation and can induce leukocyte degranulation, L-selectin shedding and activation of integrins and the Ras pathway (119). The Ras proteins have been shown to be constitutively activated in many cancers and are known to drive tumor cell proliferation and survival (120). Thus, cross-linking of activated L-selectin on CT26 tumor cells might mediate tumor progression through upregulation of this pathway.

Selectins and selectin ligands have been previously indicated to play a role in cancer progression. It has been described many times that enhanced expression of selectin ligands on cancer cells is associated with enhanced cancer progression and increased metastatic potential (121,122). Moreover, tumoral expression of L-selectin has been linked to tumor development. Lafouresse *et al*. showed that L-selectin is expressed on tumor cells in chronic lymphocytic leukemia (CLL), mediating binding of tumor cells to high endothelial venules (HEVs) and, thus, trafficking of CLL cells to lymph nodes (123). Choudhary and colleagues saw increased L-selectin expression in high-grade bladder cancer in comparison to low-grade bladder cancer and found correlation between L-selectin expression and metastatic spread of tumor cells (124). In conclusion, L-selectins mediate a variety of important intra- and extracellular processes, and their enhanced expression on tumor cells and impact on cancer progression should be further explored.

Limitations and perspective

The results of this study suggest a role of *Abcg1, Hspb7* and *Sell* in promoting resistance to the LXR agonist RGX-104 in colon carcinoma cells. One major question arising from this work concerns the mechanism through which the overexpression of the genes identified here leads to increased tumor growth under LXR agonistic treatment. In the following section, possible approaches for addressing this question will be discussed.

According to the present study, downregulation of *Abcg1*, *Hspb7* and *Sell* leads to significantly

reduced tumor growth in mice treated with RGX-104. Future work will have to study to what extent this effect relies on treatment with RGX-104 and examine the impact of the TME and the tumor type on this effect.

To study whether the increased tumor growth mediated by *Abcg1, Hspb7* and *Sell* is due to direct inhibition of RGX-104 and its effects, it will be interesting to see whether knockdown of these genes also leads to impaired tumor growth in mice that are not treated with RGX-104. As the results of this study imply a dependency of the observed effect on the immune system, it is possible that tumor cells benefit from *Abcg1, Hspb7* and *Sell* upregulation only under immune-selective pressure. Therefore, tumor growth of knockdown cell lines should be additionally studied in mice that are treated with different immune-stimulating drugs, such as granulocyte macrophage colony-stimulating factor (GM-CSF), irradiated tumor cell vaccine (GVAX) and antibody mediated immune checkpoint inhibition.

Moreover, it will be important to further explore the phenotype of the knockdown cell lines. Lentiviral knockdown of *Abcg1, Hspb7* and *Sell* could lead to reduced proliferation through targeted and off-target effects. To investigate proliferative capacity, *in vitro* growth of knockdown cells should be compared to the control cell line. Overexpressing *Abcg1, Hspb7* and *Sell* in knockdown and parental CT26 cells will help to identify gene-specific effects. As a first step to determine whether increased expression of *Abcg1, Hspb7* and *Sell* can help cancer cells to evade the immune system, tumor growth of knockdown and control cell lines in immunocompetent mice should be compared to growth in NSG mice.

Furthermore, all experiments have been conducted in the CT26 cell line. To determine the impact of *Abcg1, Hspb7* and *Sell* on tumor progression and drug resistance in other cancer types, it will be important to study their role in different murine and human carcinomas.

5. CONCLUSION

In this work a systematic *in vivo* screen was used to identify mediators of resistance to the LXR agonist RGX-104. Multiple genes involved in different biological pathways were discovered to be significantly upregulated in CT26 colon carcinoma cells that were able to grow out under LXR-agonistic treatment. Knockdown of three of these upregulated genes, *Abcg1, Hspb7* and *Sell,* led to reduced tumor growth in mice treated with RGX-104, indicating that they play a direct or indirect role in the development of tumor resistance to LXR-agonistic treatment. ABC transporter G1 plays an important role in cholesterol and phospholipid export and has been previously implicated in promoting tumor progression. The small heat shock protein HSPB7 is known to play a role in the development of cardiovascular disease, but its role in cancer has not yet been conclusively studied. Selectin L, a main mediator of leucocyte rolling, is expressed on the surface of most leukocytes and has been shown to be overexpressed in some cancer types. In conclusion, the screen uncovered three interesting potential mediators of tumor progression and drug resistance. Future research should be aimed at elucidating the mechanisms underlying the resistance-promoting phenotype of these genes as well as their role in other cancer types.

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STATUTORY DECLARATION / EIDESSTATTLICHE VERSICHERUNG

„Ich, Kimia Nathalie Tafreshian, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema *‚A screen to uncover mediators of resistance to liver X receptor agonistic cancer therapy – Ermittlung potenzieller Vermittler von Resistenz gegen die Liver-X Rezeptor agonistische Krebstherapie‘* selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autor*Innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe. Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit der Erstbetreuerin, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

LIST OF PUBLICATIONS

Tavazoie MF, Pollack I, Tanqueco R, Ostendorf BN, Reis BS, Gonsalves FC, Kurth I, Andreu-Agullo C, Derbyshire ML, Posada J, Takeda S, Tafreshian KN, Rowinsky E, Szarek M, Waltzman RJ, Mcmillan EA, Zhao C, Mita M, Mita A, Chmielowski B, Postow MA, Ribas A, Mucida D, Tavazoie SF. *LXR/ApoE Activation Restricts Innate Immune Suppression in Cancer.* Cell [Internet]. 2018;172(4):825-840.e18. Available from: https://doi.org/10.1016/j.cell.2017.12.026

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