ANTI-TUMOR ACTIVITY OF NOVEL RXR AGONISTS

By

Lyndsey Reich

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Pharmacology and Toxicology – Doctor of Philosophy

2022

ABSTRACT

Retinoid X receptor (RXR) agonists bind to and activate the nuclear receptor RXR, thereby regulating gene transcription relevant to cellular proliferation, apoptosis, cell cycle, immunity, and numerous other biological processes. RXR agonists have been explored as treatments for cancer, metabolic disorders, and neurodegenerative diseases, and one RXR agonist, bexarotene, has been FDA approved for the treatment of cutaneous T cell lymphoma (CTCL). Bexarotene has also been tested in clinical trials for lung and metastatic breast cancer, wherein subsets of patients responded despite advanced disease. By modifying structures of known rexinoids, we can improve potency and toxicity. We have previously developed an *in vitro* screening paradigm that predicts activity *in vivo* and tested a series of novel rexinoids using this system of assays. This thesis project tested newly synthesized RXR agonists in preclinical mouse models of breast and lung cancer, diseases that cause significant morbidity and economic burden in the United States and worldwide. These novel compounds, V-125 and MSU 42011, are efficacious and well tolerated in the MMTV-Neu model of HER2+ breast cancer and the A/J model of non-small cell lung cancer. To generate mechanistic hypotheses for the anti-tumor efficacy of these novel compounds, RNA sequencing data from treated MMTV-Neu tumors is contained herein. Enrichment analyses of several RXR agonists depicts unique gene expression profiles, with unifying themes of immunomodulatory activity and modification of adhesion molecules and extracellular matrix components. This data and validation data provided direction for future mechanistic studies on the biological, immune modulatory, and anti-tumor effects of these compounds. By studying

the mechanisms of these compounds, we may gain insight into how best to design future RXR agonists and how to utilize these compounds in human disease.

This thesis is dedicated to my father, Ralph Reich.
I know you would have read every page.

ACKNOWLEDGEMENTS

The challenges and needs of a dual degree student are unique, and I would not have made it this far without the support of several individuals. First and foremost, I am grateful to have had the opportunity to work with Dr. Karen Liby. I have appreciated her scientific and personal mentorship, especially some of the less conventional scientific wisdom she brings to the table. I have learned a great deal about thinking practically about answering scientific questions under her guidance. Her personal support was critical not only for the completion of my PhD, but for making it through the first couple of years of medical school as well. Her kindness will not be easily forgotten.

Other members of the Liby lab have provided important scientific expertise and morale. Special thanks to Dr. Ana Leal, who has been an excellent model of an early career scientist. I am constantly impressed by her scientific abilities and reasoning and her hands-on skills. I appreciate the time she has frequently taken out of her schedule to answer my questions.

When I first joined, Dr. Di Zhang was the only other graduate student in the Liby lab. She is an extremely driven and capable scientist with endless patience for me. Jessica Moerland, who joined the lab as an undergraduate, quickly became a peer and has surpassed many of my abilities. She has also been quick to offer her assistance with experiments, her company, and her sense of humor. Between Jess, Chris Occhiuto, and Peggy Hung, I can’t imagine a better team of graduate students to whom I will hand off my experiments. Thanks for making the lab an enjoyable and collegial place to be.

To Beth Lockwood, thank you for being a friend and confidant. In addition to your vast laboratory knowledge, you are a delight to be around.

Thank you to my committee members, Dr. Kathy Gallo, Dr. Cheryl Rockwell, Dr. Jamie Bernard and Dr. Andrea Doseff. Your input has been invaluable during experimental planning and grant writing. I have truly enjoyed working with all of you – interactions with each of you has shaped me as a young scientist.

To Dr. Justin McCormick and Bethany Heinlen, thank you for seeing potential in me when you accepted me into the DO-PhD program back in 2016. To Brian Schutte and Michelle Volker, thank you for advocating for me – I always feel like someone has my back should problems arise.

On a more personal note, I have friends and family who have always believed in my ability to achieve my goals. Katrina Robinson and Nina Bihani, Cindy and Mark Fischer, Jessica Bibbee, and my mother Lynne Reich, to name a few. Thank you to my fiancé Dr. Pierson Guthrey, who constantly encourages me to excel professionally and personally. Finally, I dedicate this dissertation to my father Ralph Reich, whose passion for learning and contributing to the advancement of science through enrollment in clinical trials inspired me to seek out improvements in cancer therapy. I know you would have liked to have been at my defense, to ask me some questions and hold my feet to the fire. We'll just have to leave it to the committee. I hope I've made you proud.

TABLE OF CONTENTS

KEY TO ABBREVIATIONS

RXR: Retinoid X Receptor

DC: Dendritic Cell

FXR: Farnesoid X receptor

IBD: Inflammatory Bowel Disease

PAMP: Pathogen-Associated Molecular Pattern

RA: Retinoic Acid

RAR: Retinoic Acid Receptor

RARE: Retinoic Acid Response Element

TNF: Tumor Necrosis Factor

TR: Thyroid Hormone Receptor

HER2: Human Epidermal Growth Factor Receptor 2

NSCLC: Non-Small Cell Lung Cancer

FDA: Food and Drug Administration

CTCL: Cutaneous T Cell Lymphoma

NMU: *N*-nitro-*N*-methylurea

ER: Estrogen Receptor

SERM: Selective Estrogen Receptor Modulator

MMTV: Mouse Mammary Tumor Virus

SREBP: Sterol Regulatory Element Binding Protein

PCA: Principal Component Analysis

NO: Nitric Oxide

iNOS: inducible Nitric Oxide Synthase

WT: Wild Type

KO: Knockout

LXR: Liver X Receptor

LPS: Lipopolysaccharide

CC3: Cleaved Caspase 3

PCNA: Proliferating Cell Nuclear Antigen

PD-L1: Programmed Death-Ligand 1

CD: Cluster of Differentiation

ANOVA: Analysis of Variance

DAB: 3, 3'-diaminobenzidine

HSD: Honestly Significant Difference

IL: Interleukin

COX-2: Cyclooxygenase-2

PPAR: Peroxisome Proliferator-Activated Receptor

VDR: Vitamin D Receptor

IPA: Ingenuity Pathway Analysis

ECM: Extracellular Matrix

MHC: Major Histocompatibility Complex

TLR: Toll-Like Receptor

IRF: Interferon Regulatory Factor

IFN: Interferon

AGE: Advanced Glycation End-Products

RAGE: Receptor for Advanced Glycation End-Products

TAM: Tumor-Associated Macrophage

KEGG: Kyoto Encyclopedia of Genes and Genomes

GEO: Gene Expression Omnibus

CHAPTER 1

Potential therapeutic uses of rexinoids

Reprinted with permission from *Advances in Pharmacology* 91, 141-183

Ana S. Leal, Lyndsey A. Reich, Jessica A. Moerland, Di Zhang, Karen T. Liby

LR Contributions: Conceptualization, writing the manuscript (Section 1.6 Clinical Studies), review and editing the manuscript

1.1: Abstract

The discovery of nuclear receptors, particularly retinoid X receptors (RXR), and their involvement in numerous pathways related to development sparked interest in their immunomodulatory properties. Genetic models using deletion or overexpression of RXR and the subsequent development of several small molecules that are agonists or antagonists of this receptor support a promising therapeutic role for these receptors in immunology. Bexarotene was approved in 1999 for the treatment of cutaneous T cell lymphoma. Several other small molecule RXR agonists have since been synthesized with limited preclinical development, but none have yet achieved FDA approval. Cancer treatment has recently been revolutionized with the introduction of immune checkpoint inhibitors, but their success has been restricted to a minority of patients. This review showcases the emerging immunomodulatory effects of RXR and the potential of small molecules that target this receptor as therapies for cancer and other diseases. Here we describe the essential roles that RXR and partner receptors play in T cells, dendritic cells, macrophages and epithelial cells, especially within the tumor microenvironment. Most of these effects are site and cancer type dependent but skew immune cells towards an anti-inflammatory and anti-tumor effect. This beneficial effect on immune cells supports the promise of combining rexinoids with approved checkpoint blockade therapies in order to enhance efficacy of the latter and to delay or potentially eliminate drug resistance. The data compiled in this review strongly suggest that targeting RXR nuclear receptors is a promising new avenue in immunomodulation for cancer and other chronic inflammatory diseases.

1.2: Introduction

1.2.1: RXR nuclear receptor signaling

The retinoid X receptor (RXR) is an essential member of the nuclear hormone receptor superfamily and is a key player in numerous physiological functions. Three isoforms of RXR (α, β, and γ) are expressed at various levels in different cell types [3]. RXRs share a canonical domain structure with other nuclear receptors, which includes a DNA-binding domain, ligand-binding domain, and regulatory molecule (co-activators and co-repressors) binding domain [4]. Besides forming homodimers, RXRs can heterodimerize with many other nuclear receptors, such as retinoic acid receptor (RAR), thyroid hormone receptor (TR), vitamin D receptor (VDR), liver X receptor (LXR), and the peroxisome proliferator-activated receptor (PPAR) [5]. In the absence of ligands, the heterodimers are normally associated with transcription corepressors that induce histone deacetylation and suppression of transcription. Upon ligand binding, corepressors dissociate from the nuclear receptors and the receptor binds with coactivators leading to transcriptional activation [6]. As transcription factors, RXRs regulate a wide variety of cellular processes, including inflammation, development, differentiation, apoptosis, and metabolism. Thus, RXR has been a promising therapeutic target in many diseases. In this chapter, the main goal is to focus on the effects of RXR signaling on the immune system and review RXR ligands (rexinoids) as immunomodulatory therapies in diverse diseases.

RXR is a unique therapeutic target in terms of the complexity of its signaling regulation. First, the dose response of rexinoids is more sophisticated than many receptor agonists. The partners of RXR can be classified as permissive (PPARs, LXR,

and FXR) or non-permissive (VDR, TR) [7-10]. Permissive binding partners can be activated by either RXR or its partner's specific ligands. Nonpermissive partners, in contrast, can only be activated by ligands specific for the partner, but not RXR ligands. Therefore, permissive dimers (e.g. RXR:LXR or RXR:PPARγ) require only one ligand, although simultaneous ligation of both RXR and a partner results in additive or synergistic activation. Nonpermissive heterodimers (e.g. RXR:VDR or RXR:TR) respond only to the partner ligand, as they function as hormone receptors and maintain an activation level that is proportional to hormone concentration for maintaining endocrine balance [11]. Moreover, various tissues maintain different expression levels and regulatory mechanisms (Brabender et al., 2005; Davies et al., 2001; Szanto et al., 2004). Increasing concentration of rexinoids may skew the activity from one dimer to another, inducing completely different downstream functions.

Second, ligand binding induces major conformational changes on RXRs, thus altering their receptor functions [12]. These structural changes affect the stability of dimeric receptor complexes, activity of self-association, and binding with other cofactors [12-14]. Structurally different ligands may recruit distinct coactivators or corepressors, leading to various downstream effects [8,15,16]. Some of these effects are common ligand-induced structural changes shared with other nuclear receptors, while some are unique to RXR [3], which could potentially result in different pharmacological consequences of targeting RXR compared to targeting its partners.

Third, although RXRs mainly function as transcription factors, they can mediate cytoplasmic functions independent of transcription regulation. Heterodimerization of RXRα with Nur77, an orphan receptor, induces nuclear export and mitochondrial

targeting of Nur77, where it interacts with Bcl-2 to induce apoptosis [17]. RXRα ligands actually inhibit this mitochondrial targeting and suppress the induction of apoptosis. In contrast, when RXRα facilitates the nucleocytoplasmic translocation of the orphan receptor TR3, the presence of a RXRα ligand is required [18].

1.2.2: Physiological functions of RXR

Mounting evidence supports an essential role for nuclear hormone receptors as coordinators of gene expression during development. Both RXR homodimers and heterodimers of RXR with other nuclear receptors regulate several steps during embryogenesis and subsequent essential physiological functions in several organ systems. For example, RAR:RXR dimers are required during the first stages of embryonic development (including placenta development), specifically in the central nervous system for neuronal differentiation [19-23]. LXR:RXR heterodimers regulate cholesterol homeostasis, lipogenesis and inflammation. The high concentration of cholesterol in the brain must be carefully balanced, and these receptors regulate cholesterol metabolism [24,25].

By using genetic deletion techniques, the following studies highlight the roles of RXR in both prenatal and postnatal development:

Prenatal development

Deletion/mutation of one of the isoforms of RXR (α, β or γ) leads to different physiologic outcomes but deletion and/or mutation of RXRα generates the most deleterious effects. Loss of RXRα in the mouse germ line resulted in embryonic lethality (E13.5–16.5) due to defects in the cardiac ventricles and placenta, as well as ocular abnormalities [22,26,27]. Deletion of just one allele of RXRα produced viable mice, but the

mice developed cardiac abnormalities and lung, muscle, skeletal and liver dysfunctions [28]. The cardiac deficiency was due to a lack of differentiation and metabolic perturbances in the ventricular cardiomyocytes. Similar defects in cardiac physiology were observed in RXRβ−/−, RXRα−/−, RXRγ−/−, RARα−/−, RARβ−/− and vitamin A-deficient mouse embryos [29-31]. Other physiologic abnormalities developed when RXR null mice were combined with RAR null mice that were not apparent in the single receptor knockout mice. These results suggested an interdependency of RAR:RXR gene regulation, as separate sets of genes were regulated by homodimers of both RXR and RAR [32]. RXRα null mice also showed deformities in the formation of the placenta, specifically in the maturation of trophoblasts. Interestingly, the deficiencies observed in the placenta of RXRα null mice resemble those of vitamin A deficiency, suggesting a role for RXR:RAR dimers in placental function [23]. Placenta from RXRα null mice were depleted of lipid droplets, reflecting the importance of RXR:PPARγ dimers [33]. Many of the defects in lipid metabolism are similar in both RXRα and LXR knockout mice, as RXR:LXR dimers regulate lipid metabolism in the liver [3].

RXRβ and RXRγ also play important roles in development, but their functions have not been studied as extensively as RXRα deficiencies. Most RXRβ null mutants died before or at birth; the surviving mice appeared normal, although the males were sterile [34]. RXRγ null mice developed normally and were indistinguishable from the heterozygous and wild-type animals [34]. Double RXRβ−/− RXRγ−/− mutants exhibited locomotor deficiencies due to a dysfunction in the dopamine signaling pathway [35].

Postnatal roles of RXR

To bypass the lethality of total body knockouts, conditional RXR knockout mice were generated, which allowed the study of these receptors in adult animals [36]. The selective ablation of RXRα in adipose tissue resulted in alterations in adipocyte differentiation and resistance to obesity induced by diet or chemicals [37]. PPAR is highly expressed in adipocytes, and PPAR/RXR heterodimers are essential for adipocyte survival and lipogenesis [33,38].

Nuclear receptors also regulate a series of genes and proteins involved in the regulation of lipid metabolism [39]. Both PPAR and LXR require dimerization with RXR for regulation of metabolic pathways, specifically lipid metabolism in the liver. Knockdown of RXR in hepatocytes revealed additional differences in lipid metabolism than the knockdown of PPAR or LXR alone, suggesting that RXR plays a unique role in lipid metabolism in the liver [40,41]. RXR also plays an important function in cell-autonomous mechanisms required for liver regeneration [37].

The deletion of RXR in skin and prostate led to abnormal cell growth [42-44]. In the skin, loss of RXRα resulted in epidermal interfollicular hyperplasia, keratinocyte hyperproliferation and aberrant terminal differentiation. These changes ultimately culminated in hair loss, hair follicle degeneration and utriculi, and dermal cysts. In prostate epithelial cells, loss of RXRα caused disturbances of secretory proteins and eventually the development of preneoplastic lesions.

The study of knockout RXR mice and the depletion of other nuclear receptors over time has elucidated the importance of these receptors in development and their roles in physiological processes. However, a gap in knowledge remains, mostly due to the

complications arising from the various receptor partners present in specific tissues and the lack of understanding of the specific roles of each of these heterodimers. Nevertheless, it is clear that RXR and its partners PPAR and LXR play crucial roles in adult physiology including metabolism, morphogenesis and cell differentiation.

1.3: RXR and the immune system in vitro

The immune system protects our body from invading foreign pathogens and, to an extent, against cancer [45]. The immune responses can be divided in two main categories: 1) hard wired responses to general foreign molecules not present in the host through innate immunity and 2) exquisite responses to a very specific protein (antigen) by binding to specific receptors through adaptive immunity [46]. Macrophages, dendritic cells (DC) and natural killer (NK) cells are the main cellular components of innate immunity, acting as first responders to remove pathogens and/or damage cells, therefore containing inflammation. The innate system processes and presents danger signals (antigen) to the adaptive immune system [47]. T cells are major components of the adaptive immune system that act as orchestrators and effectors of immunity. Depending on the immunological context, T cells can acquire functional and effector phenotypes whose activity has direct inflammatory or anti-inflammatory consequences [48].

Elucidation of the role of RXR in the immune system was aided by generating mice with a hypomorphic allele of RXRα [49,50]. These mice express a RXRα receptor with an altered ligand binding and heterodimerization domain that was poorly responsive to all-*trans* retinoic acid (RA), resulting in widespread defects in the immune system despite normal development of the thymus, spleen and lymph nodes. The generation of floxed RXRα mice allowed the restricted deletion of RXR to tissues and cell type, further

confirming the role of RXR in the immune system [51]. These mice developed an unbalanced ratio of effector versus memory T cells and decreased numbers of B cells after immunization [49,51]. In myeloid cells, RXR depletion reduced recruitment of leukocytes and generation of essential cytokines and chemokines required for function [52,53].

1.3.1: Innate immune system

Components of the innate immune system serve as the body's first line of defense against invading pathogens. Physical barriers such as the skin, complement peptides, and cellular components which recognize pathogen-associated molecular patterns (PAMPs) provide rapid, non-specific resistance to pathogen invasion and quickly opsonize, neutralize, or destroy pathogens as well as activate adaptive immune functions [54]. Macrophages are a highly heterogenous effector cell of the innate immune system. In addition to macrophage progenitors circulating in the blood, specialized subsets of resident macrophages exist in tissues. RXRα is highly expressed in macrophages; RXRβ is also expressed in macrophages but at lower levels than RXRα. Fifteen other nuclear receptors which form heterodimers with RXR are also expressed in these cells [55].

RXR and its heterodimeric partner PPARγ play an important role in myeloid progenitor cell fate and are necessary for the differentiation of macrophages during hematopoiesis [56]. The expression of RXRα increases as human blood monocytes differentiate into macrophages [57]. Pharmacological activation of RXR/PPARγ heterodimers with the PPARγ agonist rosiglitazone promoted hematopoietic stem cell differentiation to the myeloid lineage [58].

Highly plastic macrophages exist on a spectrum between two polarized subtypes commonly designated M1 and M2. The classically activated M1 phenotype is pro-inflammatory and is driven by cytokines such as IFNγ, while the anti-inflammatory alternatively activated M2 phenotype functions in wound healing and is driven by cytokines such as IL-4 [59]. To date, little is known about RXR expression or function in these different phenotypes. However, PPARγ acts in naïve macrophages to promote polarization to alternatively activated M2 phenotype [60]. The M2-polarizing cytokine IL-4 can upregulate the expression of PPARγ [61], and eicosanoids produced during inflammatory responses can activate RXR/PPAR heterodimers to promote M2 polarization and regulate cessation of the inflammatory response [62]. Repeated stimulation of macrophages by IL-4 drives chromatin remodeling by RXR/PPARγ heterodimers in a ligand-independent manner that facilitates M2 polarization through the establishment of transcriptional memory [60], contributing to tissue-specific subsets of resident macrophages throughout the body.

RXR/LXR heterodimers are critical for managing macrophage cholesterol homeostasis and play important roles in the regulation of the inflammatory response by inhibiting the expression of pro-inflammatory genes including iNOS, COX-2, and IL-6 [63]. RXR/LXR heterodimers also help prevent pathogen-induced macrophage apoptosis, which is an important immune evasion strategy employed by pathogens. Activation of RXR/LXR heterodimers in mouse macrophages upregulated the expression of anti-apoptotic genes while simultaneously inhibiting the expression of pro-apoptotic genes [64].

Macrophages control the inflammatory response by regulating chemokine expression, which controls leukocyte infiltration and migration around sites of inflammation. The chemokines CCL6 and CCL9 are RXR target genes in macrophages, and treatment with RXR agonists increases their expression and their chemoattractant potential in mouse macrophages. Inversely, lack of RXRα expression in macrophages extinguishes the expression of these chemokines, impairing the recruitment of leukocytes to inflammatory sites [52].

RXR is also critical for phagocytic clearance of apoptotic cells and other debris by macrophages, and mice lacking RXRα expression in macrophages developed an autoimmune phenotype resulting from the impaired uptake of cellular debris by macrophages. In contrast, activation of RXR by its endogenous ligand 9-*cis* RA or by the synthetic agonist LG100268 increased phagocytic activity and increased transcription of phagocytosis-related genes in mouse macrophages [65]. The synthetic rexinoid bexarotene also increased microglia-mediated clearance of β-amyloid plaques in a mouse model of Alzheimer's disease [66]. LG100268 suppressed the secretion of inflammatory mediators including IL-6, NO, G-CSF, IL-1β, and CXCL2 and altered the transcription of several toll-like receptor target genes in RAW264.7 cells stimulated with LPS or TNFα [67-69]. Similar effects in RAW264.7 cells were observed with the rexinoids IRX194204, LG101506 and pyrimidine derivatives of bexarotene or LG100268 [1,69,70]. Because of the importance of RXR signaling in macrophages, RXR and its heterodimeric partners are being investigated as potential pharmacological targets for several immune disorders involving macrophages, including rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosus [63].

1.3.2: Adaptive immune system

T cells, the main component of the adaptive immune system, and their activation and differentiation are tightly controlled. T cells are divided into CD4 and CD8 cells, with both populations having several subtypes. CD4 T cells are involved in B cell differentiation, macrophage activation and stimulate inflammation in the presence of antigens [71]. CD4-FOXP3 cells are a subpopulation of CD4 T cells involved in the regulation of immune responses by suppressing other T cells, maintaining self-tolerance [72,73]. CD8 T cells are the main drivers for elimination of bacteria or viral infected cells and dying cells [74].

The immune system is not only activated by pathogens and cancers but is also influenced by hormones and nutrients. The role of vitamin A is well established in the immune system, since individuals with vitamin A deficiency have impaired immunity [75,76]. The absence of vitamin A leads to the subsequent depletion of its downstream derivatives retinol, all-*trans*-retinoic acid (RA) and 9-*cis* RA. Upon encounter with all-*trans* RA, RAR heterodimerizes with RXR and binds retinoic acid response elements (RARE) in the genome, and 9-*cis* RA induces homodimerization of RXR [77]. The role of RA in activating RAR has been thoroughly explored in studies on vitamin A. Although the presence of vitamin A and further conversion to RA is known to activate the heterodimer RXR-RAR in T cells, most research has focused on RAR activation and its effects on T cell activation and function [reviews for the role of RA in T cell activity include [76,78-80]] instead of RXR.

T cell and B cell function are critical to maintain host resistance to infectious diseases. Naive CD4 cells have to undergo positive selection prior to their exit from the

thymus to the periphery [81]. Naive CD4 T cells can follow at least two differentiation pathways: they can differentiate into either Th1 cells producing the signature cytokine IFN-γ or Th2 cells producing the signature cytokines IL-4, IL-5 and IL-13. The balance of Th1 and Th2 cell populations is of great importance for the generation of specific immune responses. Th1 cells are responsible for protection against intracellular pathogens and are usually considered anti-tumor. Th2 cells are protective against extracellular parasites and are considered pro-tumorigenic [81].

CD4 naive T cells collected from mice with a hypomorphic allele of RXRα [49,50] and stimulated under Th1 polarizing or mixed Th1/Th2 conditions produced considerably higher amounts of IFN-γ compared to IL-4. No differences between mutant and wild type IL-4 producing cells were observed when Th2 polarizing conditions were used for the differentiation of naive cells. These observations suggest that RXRα signaling normally suppresses the differentiation of naive CD4 T cells into Th1 cells and, secondly, RXRα indirectly permits Th2 differentiation of naive cells but is not directly required for this process [49,50]. The role of RXR in naïve T lymphocytes was also linked to increased Bcl2a1 promoter activity upon activation with agonist 9-*cis* RA and AGN194204 [82]. Bcl2a1 is an anti-apoptotic member of the Bcl2 family member. The stimulation of Bcl2a1 is independent of Th1/Th2 cytokines such as IL4, IL-12 and IFNγ, suggesting that RXR activation leads to an extended survival of naïve T lymphocytes after encounter with an antigen [82]. Other pathways implicated in T lymphocyte activation and cycling through regulation of RXRα are c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and Ser/Thr protein phosphatases (PPs) [83,84]. Understanding RXR activation and subsequent binding to RXR responsive elements in

T cells is still in its infancy. However, the data reported to date reveals that multiple pathways are involved and that activation of these pathways is highly dependent on context.

Th17 cells, which produce IL-17A (IL-17), IL-17F, IL-21, and IL-22 to control bacterial and fungal infections at mucosal sites, are another subtype of CD4 cells. Th17 cells provide an important layer of protection at mucosal interfaces [85]. FOXP3 expressing T cells (Treg) are essential for maintaining immune tolerance and homeostasis in the immune system [73]. RA enhances TGF-β–dependent differentiation of Foxp3 in Tregs and inhibits Th17 differentiation by binding RAR/RXR heterodimers. The RXR agonists PA024 and tributyltin augmented the ability of all-*trans* RA or the RAR agonist Am80 to enhance CD4 T expression of Foxp3. The effect of RXR agonists occurred largely through the suppression of cytokine production by bystander effector T cells [86,87]. However, direct suppression of Th17 differentiation by RXR agonists appeared to be largely independent of RAR-mediated signals [86,87].

A retinoic acid response element half-site was discovered in the promoter region of CCR9, which permits RARα/RXR heterodimer binding, suggesting that regulation of RAR/RXR establishes a link between T cell activation, effector function, and homing properties [86,88].The regulation of CCR9 induction by a RARα/RXR dimer is mediated by NFATc2 [88]. The NFAT family of transcription factors regulates an array of functions in multiple cell types; in T cells these include production of IL-2 and the acquisition of effector properties [89].

Dendritic cells (DCs) and other antigen presenting cells (APCs) are primary sentinel cells that initiate adaptive responses by providing antigen and costimulatory signals to T

cells [90]. Thus, RXR effects in DC and other APCs have implications for functionality and homing of T cells to a specific tissue. The role of RXR in DCs has mainly been explored through the use of RA [91] or all-*trans* RA [92]. In the presence of inflammatory stimuli, RXR homodimers or RXR/RAR heterodimer agonists, such as SR11237 and BMS749 or RA, BMS614, and BMS753, respectively, induced immature DC activation through enhancement of antigen presentation, expression of class II molecules and expression of NF-κB proteins [91]. The RXR antagonist HX603 prevented the maturation of DCs, but these effects were more pronounced when combined with the PPARγ inhibitor GW9662, suggesting that the activation of PPARγ-RXR is important in the maturation process [93].

The regulation of immunity and immunological pathways by nuclear receptors has been under scrutiny for several decades. However, because of the complexity of dimerization and regulation by other factors, the role of RXR in immunological pathways has not been fully explored. Further understanding will be essential to enhance the efficacy of therapeutic modalities targeting RXR.

1.4: RXR agonists (rexinoids)

Selective agonists for the nuclear receptor RXR are known as rexinoids; representative examples are shown in Figure 1.1. Endogenous rexinoids include 9-*cis* retinoic acid; synthetic rexinoids include bexarotene (or LGD1069), LG100268, LG101506, UAB30, IRX194204 and others [94-100]. Bexarotene is the only FDA-approved

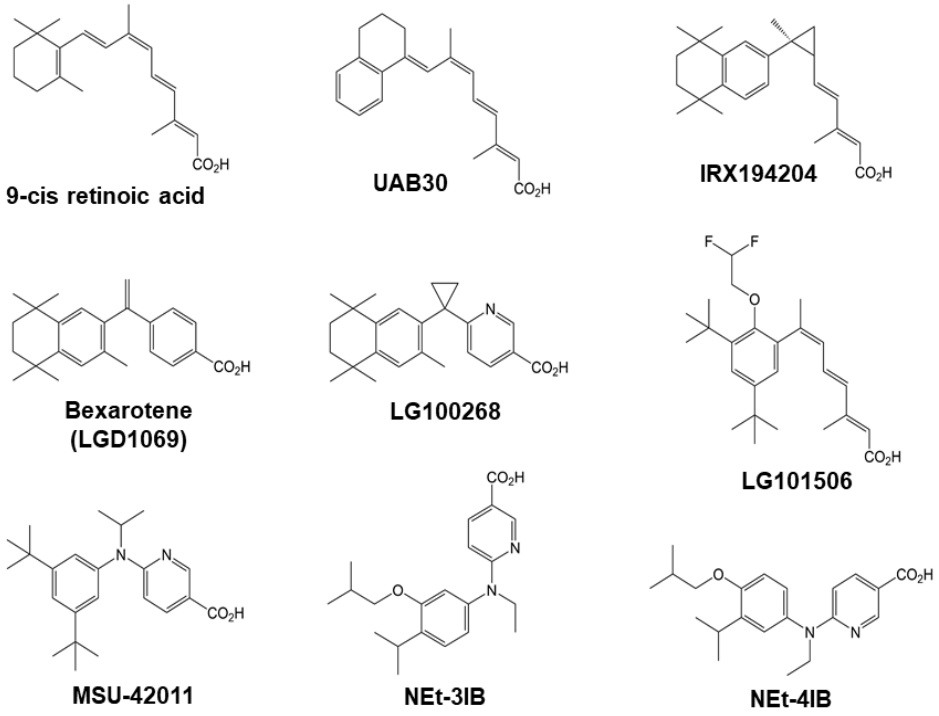

Figure 1.1. Chemical structures of representative retinoid X receptor agonists (rexinoids).

rexinoid, although this compound retains some binding to RAR [101]. Other synthetic rexinoids have been developed that are more selective for RXR, with EC50s for RXR activation at nanomolar concentrations [95,96].

These molecules interact with the orthosteric ligand binding domain of RXR, which is a single protein domain organized in an anti-parallel alpha-helical sandwich [102]. Binding of an agonist induces a conformational change involving sealing of the binding pocket by the repositioning of helix 12, generating an activated ligand-binding domain

which can interact with coactivators [12]. Ligation of RXR is sufficient to activate permissive heterodimers such as PPARγ and LXR, but not nonpermissive heterodimers such as RAR, as these partners are constitutively bound to corepressors and require the binding of their own ligand to release the corepressors and recruit coactivators [103]. Binding is restricted to elongated, flexible molecules that can adapt to the kinked shape and hydrophobic nature of the ligand binding pocket of RXR, as shown in Fig 1.1. Bulkier molecules which interfere with the repositioning of helix 12 result in antagonism of RXR and prevent coactivator recruitment [102].

Therefore, the endogenous ligand 9-*cis* RA has been used as a template for the design of synthetic rexinoids [94,104]. The decalin ring system which serves as a scaffold for rexinoids such as bexarotene, LG100268, UAB30, and IRX194204, can be replaced with alternate scaffolds enabling greater flexibility, as evidenced by LG101506, MSU-42011, NEt-3IB, and NEt-4IB (Fig 1.1). Additionally, the core vinyl or cyclopropyl groups of bexarotene and LG100268, respectively, can be replaced with a nitrogen in order to bridge the aromatic rings of these derivatives, a substitution which retains potent RXRα activity [105]. Structure-activity relationship studies have enabled the synthesis of RXR partial agonists as well, and these compounds may avoid toxicities by sub-maximally activating RXR [106-108]. Rotating the bridge between the aromatic rings of these compounds results in either full (NEt-3IB) or partial (NEt-4IB) agonism of RXRα [106], demonstrating how subtle structural changes can result in significant changes in biological activity (Fig 1.1). Indeed, derivatives with structural differences limited to a single molecule exhibit significant differences in biological activity in an RXR reporter assay [107]. By exploiting the physiochemical differences in the binding pockets of RXR

subtypes α, β, and γ, potent RXR subtype-selective agonists have also been developed with various degrees of lipophilicity [109].

Although rexinoids were originally developed for the treatment of metabolic disorders, the role of RXR in the modulation of cell differentiation, proliferation, and survival has made RXR an attractive pharmacological target for anti-cancer therapy [100]. Bexarotene is approved to treat refractory cutaneous T-cell lymphoma [110], but synthetic rexinoids have been developed and tested in preclinical models for diseases beyond cancer, including Alzheimer's disease [111], Parkinson's disease [112], diabetes [106], inflammatory bowel disease [113], and autoimmune diseases [63]. Rexinoids have also been proposed as preventative therapies in women at high risk of developing breast cancer. However, increased triglyceride levels and off-target signaling through RAR make bexarotene an unlikely candidate for chemoprevention. Safer rexinoids with improved selectivity, such as UAB30, are being investigated for this clinical application [114]. The limited and adverse side effects of the current rexinoids illustrate the need for novel synthetic rexinoids, which will also help elucidate the value and limitations of RXR heterodimer formation.

In addition to RXR, several agonists targeting permissive heterodimeric partners of RXR have been synthesized, a strategy which may help to reduce some of the toxicities associated with direct RXR activation. LXR is a permissive heterodimer of RXR which is involved in several inflammatory processes, and many synthetic LXR ligands have been developed for the purpose of treating inflammatory disorders. The LXR agonist GW3965 decreased pathological severity in a preclinical model of collagen-induced arthritis [115] and improved clearance of apoptotic debris in a preclinical model of lupus [85]. Similarly,

PPARs are permissive heterodimers of RXR with governance of numerous inflammatory processes. PPAR agonists, called thiazolidinediones, have been used for the treatment of several inflammatory or infectious diseases including malaria [116], ulcerative colitis [117], asthma, psoriasis [118], and others [119]. Although RXR plays an important role in inflammation and immunity [49,52,55], no rexinoids have been synthesized specifically for the use in treating inflammatory disorders. Recent observations [120] of the immunomodulatory effects of rexinoids may lead to the development or repurposing of novel rexinoids for this purpose.

1.5: Preclinical studies with rexinoids

1.5.1: Metabolic disorders

Acquired metabolic disorders pose a significant risk to human health and thus understanding the pathogenesis of these diseases is an intensive area of study. RXR and its partners, particularly PPARs, play important roles in the maintenance of normal metabolism and therefore are attractive targets for pharmacological intervention in these disorders [121]. PPARs are activated by dietary fatty acids and their derivatives and therefore perform surveillance of fatty acids and related factors to maintain energy homeostasis and redirect metabolism pertaining to carbohydrates, lipids and protein [122]. Overabundance of dietary fatty acids can lead to obesity, a chronic metabolic disturbance that contributes to several metabolic comorbidities including metabolic syndrome, type II diabetes, and non-alcoholic fatty liver disease [123]. The prevalence of nuclear receptor signaling in these metabolic conditions has led to a multitude of preclinical studies targeting these receptors.

The glucose-lowering, insulin-sensitizing, and anti-obesity effects of rexinoids have warranted investigation into the efficacy of bexarotene, LG100268 and LG101506 for the treatment of metabolic syndrome. Bexarotene and LG100268 lowered insulin and glucose levels in the ob/ob mouse model of obesity, and LG100268 markedly improved insulin resistance [124]. LG101506, a rexinoid that selectively activates RXR/PPAR heterodimers without raising triglycerides, reduced insulin and glucose levels in the Zucker fa/fa rat model of obesity with similar efficacy as LG100268 [125]. Additionally, LG100268 showed significant anti-obesity effects in the Zucker fa/fa rat [126]. Cevoglitazar, a PPAR agonist which selectively activates PPAR-α/γ, is a potent anti-obesity agent in mouse models and non-human primate models of obesity [127]. These studies suggest that rexinoids and other small molecules that target RXR heterodimeric partners may be useful therapeutic agents for the management of metabolic syndrome.

Selective agonists for PPARγ known as thiazolidinediones, such as rosiglitazone, are widely used for the treatment of type II diabetes, but adverse cardiovascular effects have warranted the development of safer drugs [121]. Rexinoids have also demonstrated promising effects in preclinical models of diabetes. The rexinoids bexarotene and LG100268 both decreased hyperglycemia and hyperinsulinemia in mouse models of diabetes, and a glucose tolerance test showed that LG100268 reduced insulin resistance by increasing glucose disposal [124]. The rexinoid LG100754, which selectively activates RXR/PPARγ heterodimers, decreased glucose levels and improved insulin resistance in the db/db mouse model of diabetes [128] and sensitized cells to PPARγ agonists [129], suggesting that this compound may be useful both as a single agent and in combination with other anti-diabetic agents. Additionally, LG100268 improved glycemic

control and decreased risk of cardiovascular dysfunction and cachexia in db/db diabetic mice with beta cell failure [130]. Toxicities associated with rexinoids do raise concerns regarding the potential therapeutic use of rexinoids, particularly for maintenance therapies. However, submaximal activation of RXR using partial agonists retains the glucose-lowering activity while minimizing adverse side effects [106,107], and additional studies are being conducted to synthesize full agonists with increased potency and decreased toxicity.

Activation of the farnesoid X receptor (FXR), a permissive heterodimeric partner of RXR, inhibited hepatic inflammation and lowered triglyceride levels in hepatocytes, making this an attractive target for the treatment of non-alcoholic fatty liver disease [131]. *Fxr*$^{-/-}$ mice fed a high fat diet exhibited increased triglyceride accumulation in hepatocytes compared to wild-type mice, which contributed to the development of non-alcoholic fatty liver disease [132]. Pharmacological activation of FXR by the agonist WAY-362450 in a mouse model of non-alcoholic fatty liver disease attenuated hepatic fibrosis and inflammation, a response that was not seen in *Fxr*$^{-/-}$ mice [133]. GFT505, a PPAR agonist selective for PPAR-α/δ, decreased hepatic lipid accumulation and exhibited protective effects shielding the liver from inflammation, fibrosis and steatosis in multiple mouse models of non-alcoholic fatty liver disease [134].

The complex roles of nuclear receptors in metabolic function have provoked extensive investigation into targeting RXR and its heterodimeric partners for the potential treatment of metabolic disorders. Preclinical studies targeting RXR and its partners with small molecules have shown promise in this field from the prevention of obesity to the treatment of late-stage type II diabetes with beta cell dysfunction. This

wide range of physiological response illustrates the diversity of the effects that can result from pharmacologically targeting nuclear receptors and the potential therapeutic applications of rexinoids and similar compounds. While the anti-inflammatory activity of PPAR activation has been described in the context of non-alcoholic fatty liver disease [135], the inflammatory effects of activating RXR:PPAR heterodimers were not elucidated in preclinical studies of type II diabetes. Inflammatory dysregulation is an increasingly important element of type II diabetes pathophysiology [136], and therefore future studies should evaluate these inflammatory processes in response to rexinoid treatment.

1.5.2: Neurodegenerative diseases

In 2012, an unexpected paper reported that bexarotene rapidly cleared β-amyloid plaques and reversed cognitive deficits in an apolipoprotein E (ApoE)-dependent manner in three different mouse models of Alzheimer's disease [66]. ApoE, which is regulated by RXR in coordination with LXR and PPARγ, enables the removal of β-amyloid in the brain. Independent groups were unable to replicate all of these findings and thus these results remain controversial (reviewed by [137]), but this landmark study provided the justification for testing bexarotene in a proof-of-concept clinical trial in patients with Alzheimer's disease. Although the study did not meet the primary endpoint of reducing cortical amyloid, amyloid levels were significantly lower in the brains of patients who were not *APOE4* carriers [138]. However, serum triglycerides were elevated in the patients who were treated with bexarotene, a side effect likely to limit the utility of this drug for long-term use in neurodegenerative diseases. The initial studies of bexarotene in Alzheimer's disease did not evaluate potential anti-inflammatory effects of this rexinoid in the brain. However, the OAB-14 derivative of bexarotene enhanced

phagocytosis by microglia and thereby reducing the accumulation of β-amyloid and subsequent cognitive defects in the amyloid precursor protein/presenilin 1 transgenic mouse model of Alzheimer's disease [139]. Promising areas for further investigation include using rexinoids to overcome insulin resistance in the brain [140,141] or stimulate neurogenesis in the hippocampus [142,143] in order to effectively intervene in disease pathogenesis.

The effects of rexinoids in the brain are likely pleiotropic [137,144]. As evidence, the rexinoid HX600 not only reduced the production of inflammatory cytokines by microglia and subsequent neuronal cell death in co-culture experiments but also prevented neuronal cell death and lymphocyte infiltration in a model of ischemic stroke induced by occlusion of the cerebral artery [145]. Rexinoids also suppressed inflammation in models of subarachnoid or intracerebral hemorrhage [146-148], activated phagocytosis by microglia in the brain [149,150], inhibited the production of proinflammatory cytokines in microglia [147,151,152] and/or activation of reactive microglia and astrogliosis [144,153], and increased axon sprouting [154]. Taken together, these known biological activities of rexinoids have implications in multiple CNS diseases beyond Alzheimer's disease including multiple sclerosis [151,155], stroke [146,147,150], depression [152] and traumatic brain injury [153,154,156].

1.5.3: Autoimmune diseases

The immunomodulatory effects of RAR and RXR agonism are supported by evidence of efficacy in preclinical models of autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease. In 2003, Beehler *et al.* documented that a novel RAR agonist inhibited matrix metalloproteinases in a rabbit model of arthritis and significantly stunted disease progression, as measured by swelling and joint

distortion/destruction [157]. Further, synthetic retinoids have demonstrated efficacy in both adjuvant arthritis and streptococcal cell wall-induced arthritic rat models [158,159]. More recently, bexarotene treatment of human fibroblast-like synoviocytes in a model of rheumatoid arthritis induced by TNFα downregulated the expression of pro-inflammatory cytokines and increased anti-inflammatory cytokines [160]. Following promising preclinical data, the retinoid N-[4-hydroxyphenyl] retinamide was tested in a small clinical trial of twelve patients with rheumatoid arthritis [161]. Although no beneficial clinical effect was observed, the patients in this trial had advanced disease and experienced dose-limiting toxicities which prevented this retinoid from being tested at higher doses. Despite this trial, there is unexplored potential for nuclear receptor agonists which regulate the expression of genes involved in inflammatory pathways for the treatment of rheumatoid arthritis and other immune disorders [162]. Future investigation into the use of RXR agonists in disease models of arthritis is warranted, as these drugs provide the potential for immune modulation without the dose-limiting toxicities observed with retinoid treatment.

Further evidence of the utility of RXR agonists in autoimmune diseases exists in models of inflammatory bowel disease and asthma. The activation of PPARγ, a heterodimeric partner of RXR, is protective in colitis [163]. When Desreumaux *et al.* administered the RXR agonist LG101305 in a mouse model of colitis, they reported increased survival, improved histological scoring, decreased levels of pro-inflammatory cytokines, and altered signaling through downstream inflammatory pathways. In a mouse model of asthma, the RXR partial agonist NEt-4IB suppressed airway hyperresponsiveness in a methacholine challenge test [164]. Hematoxylin and eosin

staining of lung tissue revealed reduced inflammatory cell accumulation in the lungs of NEt-4IB-treated mice, and bronchoalveolar lavage fluid from treated mice showed significantly reduced levels of the proinflammatory cytokines IL-5, IL-13, TNF-α and nitric oxide. This study provides further evidence of the role of RXR activation for the treatment of autoimmune disease in which immune dysfunction predominates [165-168].

Finally, an RXR-selective agonist effectively modulated the immune system in mouse models of multiple sclerosis [169]. Chandraratna *et al.* demonstrated that the rexinoid IRX194204 enhanced the differentiation of inducible T regulatory cells and inhibited the expansion of a Th17 population *in vitro.* In an experimental autoimmune encephalomyelitis model, IRX194204 reduced active disease by inhibiting pro-inflammatory CD4+ T cells and upregulating immune checkpoints on these cells.

1.5.4: Cancer

Most studies investigating the role of RXR in cancer have explored the ability of RXR agonists to induce differentiation or apoptosis [170,171]. Rexinoids have shown promising activity in many preclinical models of cancer [172]. Bexarotene induced regression of tumors in the NMU rat model of ER-positive breast cancer both as a single agent and in combination with other agents [173-177]. Similar anti-cancer activity was observed in the same NMU rat model with LG100268, which is more potent than bexarotene [178,179], and in preclinical models of ER-negative breast cancer [180-184]. Rexinoids have been also been used for both prevention and treatment of NSCLC [1,68-70,185]. However, these studies largely failed to evaluate the effects on the immune cells present in the tumor microenvironment. One recent study did report that a rexinoid decreased tumor-

associated macrophages and myeloid derived suppressor cells in a lung cancer model and skewed macrophages toward an anti-tumor M1 phenotype [1].

Cytotoxic T cell activation and reduction of tumor promoting T regulatory cells in the tumor microenvironment (TME) is a paradigm that supports combining drugs with immunotherapy in cancer treatment [186], especially the use of small molecules to enhance immunotherapy [187]. The use of RXR agonists and/or antagonists as TME immunomodulators has been reported. Bexarotene activated type 1 antigen presenting cells and increased cytotoxic CD8 T cells when combined with paclitaxel in a model of TgMMTV-neu breast cancer [188]. LG100268 decreased the presence of tumor promoting FOXP3 CD4 T cells in the MMTV-Neu mouse model and in an *in vitro* co-culture system, leading to an increase in the activation of CD8 cytotoxic T cells and consequently a decrease in tumor burden [120]. The combination of LG100268 and anti-PDL1 antibodies also increased the infiltration of cytotoxic CD8 T cells in a mouse model of triple negative breast cancer [120]. Notably, bexarotene did not alter CD4, CD25, FOXP3 positive cells in samples of patients treated for cutaneous T cell lymphoma [189], suggesting the effects on CD4 T cells in cancer is dependent on the cancer type and/or the rexinoid used.

The myeloid compartment plays important roles in cancer, with tumor associated macrophages and myeloid derived suppressor cells contributing to tumor progression and metastasis [190]. LG100268 reduced the infiltration of myeloid derived suppressor cells; reduced the expression of CD206, a marker of tumor-promoting macrophages; and increased PD-L1 expression in tumor associated macrophages in a HER2 positive mouse model of breast cancer [120]. While treatment with the rexinoid MSU-42011 did not

alter the total number of immune cells in the clinically relevant A/J model of NSCLC, MSU-42011 in combination with carboplatin and paclitaxel significantly reduced the number of alveolar macrophages in and decreased expression of CD206 in the lung [105]. When Lewis lung carcinoma cells were implanted in the lungs of mice in which RXR was selectively deleted from myeloid cells, the formation of metastasis was enhanced [191]. Metastatic growth was correlated with increased inflammatory mediators (*C1qa*, *Il1a*, *Il1b*, *Tnf*, *Lcn2*) that promoted the colonization of the pre-metastatic niche, but no effects were observed in the primary cancer cells. The deletion of RXR from serous macrophages (resident macrophages) in a murine model of ovarian cancer reduced tumor growth [53], as a result of the dysregulation of lipid accumulation and consequently apoptosis of tumor promoting macrophages.

Most studies testing rexinoids in cancer models did not examine or report the immunological effects of these small molecules. Future studies should critically examine immune populations within the tumor microenvironment following rexinoid treatment, and studies are needed to determine if the immunomodulatory activities of rexinoids are required for their anti-cancer activities. As immunotherapy has rapidly become standard of care treatment for many cancers, combinations of immune checkpoint inhibitors with rexinoids should also be explored in order to improve the success rate of current immunotherapies or avoid resistance [192].

1.6: Clinical studies

1.6.1: Cutaneous T cell lymphoma (CTCL)

In 1990, the RXR nuclear receptors were first described [193]. At this time, retinoids had already been tested in the clinic for treatment of squamous cell carcinoma of the skin and cervix, acute promyelocytic leukemia, and certain premalignant lesions such as oral leukoplakia [194-196]. Based on the clinical activity of various retinoids and the discovery that agonists of the RXR nuclear receptor enhanced the binding of RAR to DNA, investigations into the anti-proliferative activity of early rexinoids began [197]. In 1997, Miller et al. published the findings of an initial clinical trial with LGD1069 (bexarotene), wherein 52 patients with a variety of malignancies (including NSCLC and cutaneous T cell lymphoma) were treated with bexarotene to investigate the safety and pharmacokinetics of the compound. Bexarotene had a favorable therapeutic index in comparison to retinoids, reaching plasma concentrations up to tenfold higher without significant or dose-limiting toxicity [198]. Observed side effects of bexarotene included hyperlipidemia, elevation of liver enzymes, leukopenia and neutropenia. The dose-limiting toxicities seen in retinoid clinical trials, such as headache, hypercalcemia and mucocutaneous toxicity, were not dose-limiting for patients on bexarotene. This trial demonstrated the safety and tolerability of bexarotene and established a recommended dose for further single-agent trials.

In CTCL, an inflammatory dermatosis in which cytokines are released and then stimulate T cell proliferation and recruitment, bexarotene blocked T cell proliferation and the secretion of cytokines such as IL-2, leading to enhanced apoptosis of neoplastic cells [199]. In malignant T cells isolated from CTCL patients, bexarotene induced

apoptosis of the neoplastic cells in a dose-dependent manner at biologically relevant concentrations. Interestingly, the induction of apoptosis noted in a proportion of patient peripheral blood samples was comparable to the response rate observed in a CTCL clinical trial. Bexarotene inhibited IL-4 production by patient peripheral blood mononuclear cells, which correlated with sensitivity to apoptosis, suggesting that the efficacy of bexarotene treatment in CTCL is dependent on immune modulation [199]. In 2001, the findings of two multi-institutional Phase II-III clinical trials of bexarotene for the treatment of refractory CTCL were published. In the first trial, 58 CTCL patients refractory to two or more prior therapies received randomized doses of bexarotene. The response rate reached 67% in patients treated with doses over 300 mg/m^2/day [200]. In the second expanded trial of 94 CTCL patients, 45% of patients treated with 300 mg/m^2/day bexarotene achieved a complete or partial response, and 55% of patients treated with doses above 300 mg/m^2/day achieved the same responses [201]. These trials demonstrated that bexarotene was effective for the treatment of refractory CTCL and had an acceptable safety profile. Based on these clinical trials, bexarotene was approved by the FDA in 1999 for the treatment of cutaneous manifestations of CTCL in patients who had failed at least one systemic treatment [202].

The immunomodulatory effects of rexinoids in human patients has been examined by only a few groups. An investigation into the effects of bexarotene treatment on the regulatory T cell compartment in patients with CTCL followed studies demonstrating the effects of retinoids on regulatory T cell expansion [203]. In ten patients treated with bexarotene for six months, no significant increase in the regulatory T cell population was demonstrated by either immunohistochemical analysis of biopsy specimens or by

flow cytometry of peripheral blood compared to pre-treatment [189]. However, populations of CD4+CD25^{high} regulatory T cells were elevated in patients before bexarotene treatment in comparison to healthy controls, and the Tregs did inhibit proliferation of CD4+CD25- T cells. These data provide further evidence that disease processes in which RXR activation are therapeutic involve immune cell dysregulation and that immunomodulation by rexinoids could provide potential therapeutic avenues.

Treatment with ligands that activate both RAR and RXR nuclear receptors led to synergistic increases in apoptosis and decreases in proliferation of CTCL cells *in vitro*, and activation of both of these nuclear receptors simultaneously provided increased clinical benefit compared to the activation of RAR alone [204]. As the activation of RAR leads to a multitude of adverse effects, such as teratogenicity and mucocutaneous toxicity, it would be beneficial to develop compounds which activate RXR and maximize RXR-mediated immunomodulatory effects, while minimizing the toxicity associated with RAR activation.

1.6.2: Breast cancer

In addition to its use in CTCL, bexarotene has also been tested in clinical trial for the treatment of metastatic breast cancer. In this trial, 145 patients were randomly assigned to receive either 200 mg/m^2/day or 500 mg/m^2/day of bexarotene. Twenty percent of the patients derived clinical benefit - defined as either a partial response or stable disease for 6 months or longer[205]. While the overall efficacy of bexarotene in these patients was limited, only two patients experienced serious adverse events, and other patients experienced minimal toxicity including hypertriglyceridemia, dry skin, asthenia, and headache.

Clinical studies of breast cancer prevention have also been conducted with bexarotene in high-risk individuals. In a Phase II study investigating biomarkers which may be utilized in the future to quantify patient response to rexinoid treatment, women with a greater than 10% chance of carrying a BRCA mutation were treated with bexarotene (200 mg/m^2) or placebo for a period of 4 weeks. While patients treated with bexarotene did not demonstrate a significant decrease in the proliferation marker Ki67 by either IHC or qRT-PCR, there was a significant reduction in cyclin D1 expression in post-menopausal patients treated with bexarotene as determined by qRT-PCR [206]. Furthermore, the use of bexarotene in a topical 1% gel has been investigated in a Phase I dose escalation study in healthy patients, with the goal of determining a dose for a Phase II efficacy study, monitoring toxicity, and establishing tissue markers [207]. Clinical studies evaluating UAB-30 for prevention of breast cancer are ongoing[114]

1.6.3: Lung cancer

Bexarotene has shown even greater promise for the treatment of non-small cell lung cancer (NSCLC). Proof-of-principal studies have confirmed bioavailability of oral bexarotene in humans, with a good correlation between plasma bexarotene levels and intratumoral bexarotene levels in the lung [208]. Bexarotene, given at 300-400 mg/m^2/day, repressed the expression of proliferative biomarkers in lung tumors of a subset of patients with stage I/II NSCLC [208] and significantly improved median survival in patients with refractory stage IIIB NSCLC [209]. The subset of patients who responded to bexarotene in these trials experienced bexarotene-induced hypertriglyceridemia and/or had significantly higher intratumoral bexarotene levels compared to the majority of patients who did not respond, suggesting that increased exposure to bexarotene in the

tumor results in improved clinical response. In a placebo-controlled trial, patients with refractory stage IIIB-IV NSCLC exhibited a dose-dependent estimated time to progression of 56 days for patients receiving placebo, 82 days for patients receiving 300 mg/m^2/day bexarotene, and 128 days for patients receiving 600 mg/m^2/day bexarotene [210]. Although this trial was terminated early due to slow accrual, it demonstrated an improvement in time to progression in patients treated with bexarotene and determined that bexarotene toxicity is medically manageable.

As cancer patients are rarely treated with one drug alone, bexarotene has also been tested in combination with other anti-cancer agents. Bexarotene was tested in combination with cisplatin and vinorelbine in 43 patients with advanced (stage IIIB with pleural effusion or stage IV) NSCLC [211]. This study determined the maximum tolerated dose of bexarotene to be 400 mg/m^2/day and observed a 25% response rate in the phase II portion of the trial. Treatment with bexarotene was associated with a better than expected survival rate of 61% at 1 year follow up and 32% at 2 year follow up. In comparison, a comparable phase III clinical trial of docetaxel in NSCLC patients yielded a 2-year survival rate of 12% [212]. This striking improvement in survival in patients with aggressive disease warranted further study. Stage IIIB-IV NSCLC patients were treated with the chemotherapeutic drugs cisplatin and vinorelbine, alone or in combination with bexarotene at 400 mg/m^2/day. While no significant difference was observed between the two groups, median survival was longer in a subset of patients receiving bexarotene (12.3 months) compared to patients receiving cisplatin and vinorelbine alone (9.9 months) [213]. Similar to previous clinical trials, these patients experienced bexarotene-induced hypertriglyceridemia. Interestingly, patients who benefited the most from

bexarotene were smokers and/or had stage IV disease. An important conclusion of this clinical trial was that increased triglyceride levels can be used as a biomarker to predict increased survival benefit in patients treated with bexarotene. A similar trial tested the chemotherapeutic drugs carboplatin and paclitaxel, alone or in combination with bexarotene, in patients with stage IIIB-IV NSCLC. While no overall significant difference was observed between the two treatment groups, a subset of patients who developed hypertriglyceridemia had significantly longer median survival compared to patients receiving only carboplatin and paclitaxel (12.4 vs. 9.2 months, respectively). Again, those patients who received the most substantial benefits from bexarotene were smokers and/or had stage IV disease [214].

Mutation profiles are an increasingly important factor in understanding cancer disease progression and treatment. Gain-of-function *KRAS* mutations are associated with aggressive disease and poor patient survival in NSCLC, and although the trials described above did not control for different mutation profiles, *KRAS* mutations are common drivers of NSCLC in smokers [215]. Patients with *KRAS*-mutant refractory stage IIIB-IV NSCLC were treated with bexarotene at 400 mg/m^2/day in combination with the tyrosine kinase inhibitor erlotinib. This combination increased median overall survival to 22 weeks in these heavily pretreated patients, with prolonged progression-free responses of 583-1460 days in three patients [216]. The possibility of rexinoids being effective in the treatment of *KRAS*-driven cancers is of significant clinical importance, as no currently approved therapies directly target this aggressive mutation.

Despite the promising evidence from subsets of patients in clinical trials, bexarotene has not been approved for the treatment of NSCLC due to a lack of response from the

majority of patients in “all-comers” clinical trials and an array of adverse side effects, including hypertriglyceridemia. However, subsets of patients in these trials derived significant clinical benefit. Future study of the characteristics of patient “responders” could provide guidance as to which patient populations would benefit most from rexinoid treatment. The responses within these subsets of patients, combined with a favorable toxicity profile, merit further clinical investigation, particularly with rexinoids that are more potent and selective than bexarotene. Therefore, the development of novel rexinoids with increased potency and specificity for RXR will be imperative in the efforts to expand the clinical use of rexinoids.

1.7: Future directions

1.7.1: Determine the dependence of rexinoid-mediated immunomodulation on RXR signaling

Pharmacological agents often do not directly illustrate the definitive mechanisms of action of a physiological target. After elucidating the immunomodulatory effects of rexinoids, the next question becomes which signaling pathway is mediating their effects. Considering the ability of rexinoids to heterodimerize with many other nuclear receptors, especially permissive ones, further research needs to be done to determine the partners. Indeed, many nuclear receptor signaling pathways are highly involved in regulating inflammation and immune responses, such as LXR/RXR and PPAR□/RXR pathways.

1.7.2: Synthesis of novel rexinoids

As multifunctional agents, rexinoids offer great potential in numerous therapeutic areas. However, rexinoids still play limited roles in the clinic with bexarotene as the only

FDA-approved drug. Limited efficacy, undesired toxicities and patent issues prevent existing rexinoids from being widely used. Continued synthetic chemistry efforts are needed to unleash the great potential of this class of drugs. We and others have been working on developing the next generation of rexinoids and elucidating the immunomodulatory effects of novel rexinoids.

1.7.3: Combination with other therapeutic agents

Although relatively few studies have focused on the immunopharmacology of rexinoids in the context of cancer, documentation of rexinoid- induced immunomodulatory effects combined with evidence of altered PD-L1 expression in tumors of mice upon treatment with a rexinoid [120] provides justification for evaluation of the combination of rexinoids and checkpoint inhibitors for the treatment of cancer. Elevation of PD-L1 enhances the response to checkpoint blockade in tumors [217] and elevated levels of PD-L1 in breast cancer patients is correlated with increased survival [218]. Many clinical trials testing rexinoids in cancer patients have concluded that rexinoids are an excellent candidate for combination therapy based on their safety profile, so this therapeutic combination has the potential to improve efficacy and is worthy of future investigations.

1.7.4: Anti-inflammatory activities and other diseases

Although limited, previous studies evaluating the anti-inflammatory effects of RXR and its partners, particularly PPAR and LXR, have identified RXR as a promising therapeutic target in the treatment of disorders involving aberrant inflammation. These potential applications are not just limited to the diseases discussed in this review but could provide benefit across a wide variety of pathologies, including infectious diseases.

Host antiviral responses can lead to the development of autoimmunity [219], and antiviral responses can lead to a serious hyperactive immune response known as a cytokine storm, which damages host tissues and contributes to a worse prognosis [220]. The SARS-CoV-2 virus, the pathogen responsible for the COVID-19 global pandemic which erupted in late 2019 [221], can induce a cytokine storm. The anti-inflammatory effects of rexinoids suggest that these agents could potentially be used to prevent or control cytokine storm in patients with COVID-19 or related viruses. Indeed, the synthetic retinoid derivative fenretinide has been proposed as a possible adjuvant therapy for the treatment of COVID-19 [222], and a robust two-tier screening of a library of over 1500 FDA-approved drugs identified bexarotene as a potential therapeutic for the treatment of COVID-19 [152]. Additionally, the ability of rexinoids to modulate T cell function makes them a possible adjuvant for vaccines [223], illustrating their utility in preventive medicine.

1.8: Conclusion

Despite the notable efficacy of rexinoids in preclinical models of multiple diseases and the promising results in clinical trials in patients treated with bexarotene, the mechanism of action for rexinoids is poorly understood. In this review, we summarized

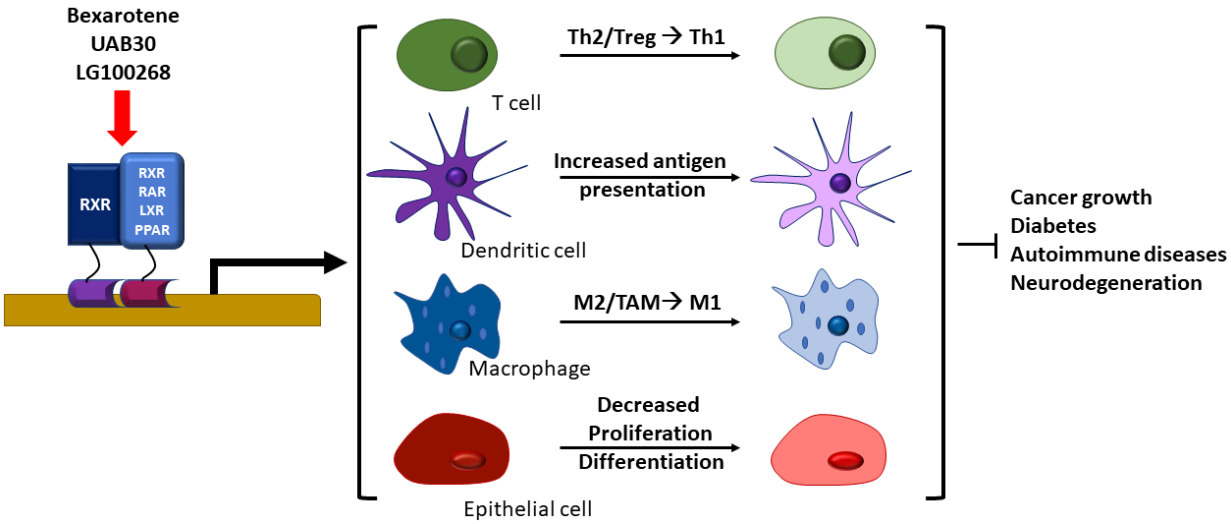

Figure 1.2. Immunomodulatory effects of RXR homodimer and heterodimer activation. Upon ligand (bexarotene, LG100268, UAB30, etc) binding, RXR dissociates from corepressors and partners with another RXR, forming a homodimer, or heterodimerizes with other nuclear receptors, such as retinoic acid receptor (RAR), liver X receptor (LXR), and the peroxisome proliferator-activated receptor (PPAR). The formation of the homodimer or heterodimer leads to transcriptional activation. Downstream transcription programs regulate the activation/maturation/and homing of T cells, dendritic cells, macrophages and epithelial cells. The different activation status of these immune cells upon RXR activation play important roles in cancer progression, autoimmune diseases, diabetes and neurodegenerative diseases.

the complex interactions between RXR and other nuclear receptors, which are understudied in the immune system. A greater understanding of the immunomodulatory biology of RXR and the development of novel and specific RXR agonists could provide effective novel therapies for diseases as diverse as cancer, autoimmune diseases, metabolic disorders and neurodegenerative diseases (Fig 1.2).

ACKNOWLEDGMENTS: We are grateful for funding from an MSU Molecular Discovery Group Pilot Grant, the Michigan Economic Development Corporation (Mi-Kickstart and Mi-Trac Awards), and the Breast Cancer Research Foundation (BCRF-20-096) to support our work on rexinoids.

CONFLICT OF INTEREST STATEMENT: ASL and KTL are named inventors on a patent filed on novel rexinoids and owned by MSU. LAR, JAM and DZ have no conflict of interest.

CHAPTER 2

The rexinoid V-125 reduces tumor growth in preclinical models of breast and lung cancer

Material from: Reich, The rexinoid V-125 reduces tumor growth in preclinical models of breast and lung cancer, *Scientific Reports* 12 (1), 1-12

Lyndsey A. Reich[1], Jessica A. Moerland[1], Ana S. Leal[1], Di Zhang[1], Sarah Carapellucci[1], Beth Lockwood[1], Peter W. Jurutka[2], Pamela A. Marshall[2], Carl E. Wagner[2], Karen T. Liby[1]

LR Contributions: Conceptualization, methodology, conducting experiments, writing the original manuscript, review and editing the manuscript

2.1: Abstract

Rexinoids are ligands which activate retinoid X receptors (RXRs), regulating transcription of genes involved in cancer-relevant processes. Rexinoids have anti-neoplastic activity in multiple preclinical studies. Bexarotene, used to treat cutaneous T cell lymphoma, is the only FDA-approved rexinoid. Bexarotene has also been evaluated in clinical trials for lung and metastatic breast cancer, wherein subsets of patients responded despite advanced disease. By modifying structures of known rexinoids, we can improve potency and toxicity. We previously screened a series of novel rexinoids and selected V-125 as the lead based on performance in optimized *in vitro* assays. To validate our screening paradigm, we tested V-125 in clinically relevant mouse models of breast and lung cancer. V-125 significantly ($p < 0.001$) increased time to tumor development in the MMTV-Neu breast cancer model. Treatment of established mammary tumors with V-125 significantly ($p < 0.05$) increased overall survival. In the A/J lung cancer model, V-125 significantly ($p < 0.01$) decreased number, size, and burden of lung tumors. Although bexarotene elevated triglycerides and cholesterol in these models, V-125 demonstrated an improved safety profile. These studies provide evidence that our screening paradigm predicts novel rexinoid efficacy and suggest that V-125 could be developed into a new cancer therapeutic.

2.2: Introduction

The landscape of cancer therapy has shifted significantly in recent years from standard of care cytotoxic chemotherapy alone to targeted therapies and immunotherapy[224]. However, the need for additional therapeutics still exists, particularly for patients with late-stage disease, aggressive molecular subtypes or who have failed existing treatments. While specific monoclonal antibodies (trastuzumab) or small molecule inhibitors (lapatinib, neratinib) of HER2 (human epidermal growth factor receptor 2) confer a survival benefit to patients with HER2+ breast cancer, subsets of patients do not benefit from these drugs, and others acquire resistance via multiple mechanisms[225]. Patients with non-small cell lung cancer (NSCLC) also acquire resistance to chemotherapy[226] or to targeted therapies[227], contributing to disease progression.

Rexinoids are synthetic agonists which selectively bind to Retinoid X Receptors (RXR). Upon activation, RXR acts as a transcription factor for genes involved in several cancer-related biological processes, such as inflammation, proliferation, and cell survival[11]. Rexinoids are effective in multiple preclinical models of cancer and have been tested as therapeutic options for neurodegenerative and autoimmune diseases[228]. The rexinoid bexarotene was FDA approved in 1999 for the treatment of cutaneous T cell lymphoma (CTCL)[202]. Bexarotene has also been evaluated in clinical trials for both metastatic breast cancer[205] and NSCLC[211], and subsets of patients derived significant clinical benefit despite late-stage, aggressive disease. Newly synthesized rexinoids have improved anti-tumor efficacy and reduced toxicity in comparison to

bexarotene[95,96], which has revitalized interest in developing and testing rexinoids for cancer treatment.

The first studies of bexarotene in animal models of breast cancer were in the NMU rat model of mammary carcinogenesis, wherein the carcinogen *N*-nitro-*N*-methylurea (NMU) initiates the formation of estrogen receptor positive (ER+) mammary tumors[229]. In these studies, bexarotene reduced tumor burden by 90% and was well-tolerated[230]. While retinoids are active in this model[231,232], they cause headaches and mucocutaneous toxicity in patients as a result of binding to the retinoic acid receptor (RAR)[233]. These side effects are not observed with the rexinoids that do not bind to RAR[198]. When the anti-tumor efficacy of bexarotene was compared directly to that of the selective estrogen receptor modulator (SERM) tamoxifen, bexarotene was superior to tamoxifen[230]. Tamoxifen is known to cause side effects in patients such as hot flashes and an increased risk of endometrial carcinoma[234]. SERMs are an effective treatment and prevention option for ER+ breast cancer, but fewer strategies exist for hormone receptor negative breast cancers, despite high morbidity and mortality. With their enhanced efficacy coupled with a favorable toxicity profile, rexinoids may be useful not only for cancer treatment but also for prevention. In 2002, Wu *et al*. tested bexarotene for prevention in MMTV-erbB2 transgenic mice. Bexarotene significantly increased tumor-free survival, even at doses as low as 10 mg/kg[235]. The more potent rexinoid LG100268 and a SERM were highly effective at delaying tumor development in a similar murine model of HER2+ breast cancer, while the combination of a SERM and LG100268 completely prevented tumor development over a 50 week period of treatment[182].

In addition to breast cancer, rexinoids are also effective in the A/J mouse model of lung cancer[105]. These mice develop *Kras* mutations in the lung after being challenged by carcinogens such as vinyl and ethyl carbamate, which form epoxides and DNA adducts, leading to the development of lung adenocarcinomas[236]. Activating mutations in *KRAS* are one of the most common oncogenic drivers in human lung adenocarcinomas[237]. Ethyl and vinyl carbamate are found in tobacco products[238], making the A/J model an appropriate model of NSCLC in smokers. Several rexinoids inhibit growth of lung tumors in this model, including LG100268, LG101506, and IRX194204[68,69].

Although rexinoids showed promising activity in several preclinical models of cancer[69,230,235], clinical trials of bexarotene in both metastatic breast cancer and NSCLC did not lead to FDA approval. To improve clinical responses, new rexinoids with greater potency are needed. As rexinoids are also effective in cancer prevention studies, the toxicity profile of rexinoids must be monitored, as patients on long-term treatment with bexarotene experienced cumulative side effects, particularly elevated triglycerides and hypothyroidism[170]. To address these challenges, we synthesized novel analogues of bexarotene using molecular modeling software to guide chemical substitutions and rational drug design. These new compounds activated RXR with nanomolar potency and stimulated RXR-regulated transcription with minimal RAR activation[10,239].

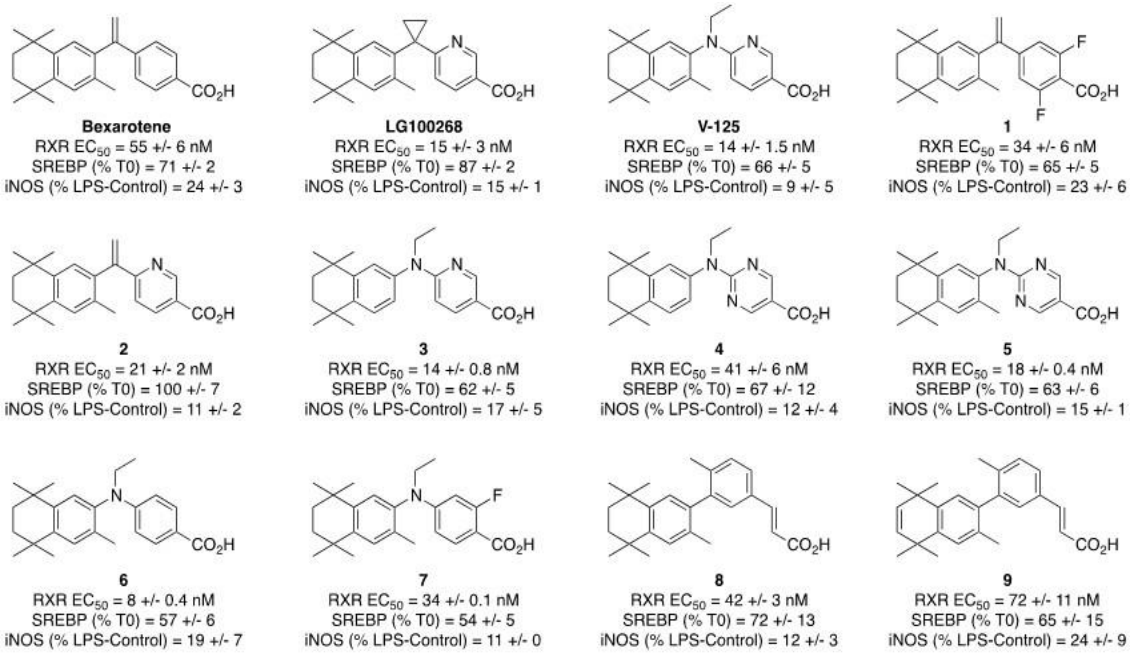

Figure 2.1. Structures and activities of bexarotene, LG100268, V-125 and nine rexinoids evaluated by PCA. Bexarotene is approved to treat cutaneous T-cell lymphoma, and V-125 was identified as a promising novel rexinoid based on its activity in published *in vitro* assays[1]. The iNOS suppression in RAW 264.7 cells was determined by cell treatment with 100 nM rexinoid followed by challenge with 1 ng/mL LPS over 24 h. Griess assay measured NO production normalized to LPS-stimulated control. SREBP and RXR activation were assessed as reported in prior work[1]. T0 is T0901317, a known LXR agonist and activator of SREPB.

To identify rexinoids with potential *in vivo* efficacy, we have developed a series of *in vitro* screening assays[1]. Of ten new rexinoids screened in these assays, V-125 (Fig. 2.1) was selected as the lead compound, as it was the most potent in the iNOS suppression assay, an assay which correlates with efficacy in the A/J model of lung cancer[1]. The EC_{50} value for RXRα activation for V-125 was 14 nm vs. 55 nm for bexarotene. Activation of sterol regulatory element-binding protein (SREBP), a biomarker of triglyceride elevation was lower with V-125 than either bexarotene or

LG100268[1]. To further validate our *in vitro* screening paradigm, we tested the efficacy and safety of V-125 in preclinical models of breast and lung cancer.

2.3: Materials and Methods

Drugs: Synthesis of V-125 was previously described[240]. Bexarotene was purchased from LC Laboratories. Rexinoids were dissolved in a vehicle (50 ml vehicle/kg diet) of 1 part ethanol:3 parts highly purified coconut oil (Neobee oil, Thermo Fisher) and then mixed into 1 kg of powdered diet (AIN-93M, BioServ for A/J mice or 5002 rodent chow, PMI Nutrition International for MMTV-neu mice) using a commercial KitchenAid mixer. The same vehicle was used in the control diets. In the MMTV-Neu model, doses were 30 mg per kg of diet (~7.5 mg per kg body weight) for prevention studies and 100 mg per kg of diet (~25 mg per kg body weight) for treatment studies, as utilized in previous publications[120,182]. In the A/J model, doses were 40 and 80 mg per kg of diet (~10 and 20 mg per kg body weight), doses used in previous publications[68]. Rexinoids remain stable in diet at 4°C for up to 6 weeks, as confirmed by liquid chromatography-mass spectrometry.

***In vivo* experiments:** All animal studies were approved by the Institutional Animal Care and Use Committee at Michigan State University (IACUC protocol number 201800050). All protocols were carried out ethically in accordance with the Regulations for the Management of Laboratory Animals at Michigan State University and in compliance with the ARRIVE guidelines. Every effort was made to minimize suffering throughout these studies. Mice were euthanized by inhalation of carbon dioxide followed by cervical dislocation.

Lung carcinogenesis studies. Female A/J (Jackson Laboratory) mice at 7 and 8 weeks of age were injected intraperitoneally with vinyl carbamate (16 mg/kg, Toronto Research Chemicals) in a vehicle of isotonic saline. One week after the last injection of vinyl carbamate, rexinoids were fed in diet as described above. After 16 weeks of treatment, mice were euthanized and tissues collected. Lungs were inflated with PBS, and tumor number on the surface of the lungs was evaluated. The left lung was then fixed in 10% neutral buffered formalin; samples were blinded and randomized. The lung was sectioned and stained with H&E for evaluation of the histopathology on multiple sections, using previously established criteria[68].

Breast cancer studies. For prevention studies, 10-week-old female MMTV-Neu mice[241] (Jackson Laboratory) were fed control diet or 30 mg rexinoid per kg diet. Littermate-matched controls were used whenever possible. Mice were palpated twice weekly for the development of new tumors. For treatment studies, mice were fed standard diet and palpated twice weekly until tumors reached 5 mm in diameter, at which time mice were randomized and started on control diet or 100 mg V-125 per kg of diet. Mice were fed until tumors reached IACUC-defined endpoints (overall survival studies) or for 10 days (biomarker studies). At study conclusion, mice were euthanized, tissues were harvested, and tumors and livers weighed.

Triglyceride quantification: Plasma was harvested from mice at necropsy. Triglyceride and cholesterol levels were measured using the Triglyceride Quantification Assay Kit (Abcam) or the Cholesterol Quantification Kit (Sigma-Aldrich) as per the manufacturers' recommended protocols.

Immunohistochemistry: Formalin-fixed tissues were embedded in paraffin and sectioned. Antigen retrieval was performed by boiling in citrate buffer, and endogenous peroxidase activity was quenched using hydrogen peroxide. Tissue sections were stained with antibodies against CD206 (1:200, Abcam), cleaved caspase 3 (1:100, 5A1E, Cell Signaling), PCNA (1:200, sc-56, Santa Cruz Biotechnologies), PD-L1 (1:50, MIH6, Abcam), and CD8 (1:40, 53-6.7, Biolegend) as described[120]. Sections were then labeled with biotinylated secondary antibodies (anti-rabbit, Cell Signaling; anti-rat, Vector Labs), as previously described[120]. Signal detection was performed using a DAB (3, 3'-diaminobenzidine) substrate (Cell Signaling). Sections were counterstained with hematoxylin (Vector Labs).

Statistical analysis: Results were expressed as the means ± standard errors as indicated in figure legends. Kaplan-Meier survival curves from *in vivo* experiments were analyzed using the log rank test. Data from plasma triglyceride and cholesterol experiments were analyzed using one-way ANOVA, and significant differences between groups were determined by the Tukey HSD multiple comparison method (VassarStats.com). Data from lung experiments were analyzed by one-way ANOVA. When data fit a normal distribution, the Holm-Sidak test for multiple comparison was used. The Kruskal–Wallis one-way ANOVA on ranks followed by the Dunn test for multiple comparisons was used if the data did not pass the normality test. McNemar's Z test was used for analysis of histopathological grades of lung tumors. $p < 0.05$ was considered statistically significant throughout all experiments.

Principal component analysis (PCA) Plot: Clustvis[242] was used to analyze data and prepare PCA and heatmap plots as seen in Figure S.1-S.2 (Appendix) to

analyze compounds for biological activity motifs. Ellipses in the PCA indicate 95% confidence intervals[243,244].

PCR Array: Tumors from mice treated with V-125 (100 mg/kg diet) or control diet for 10 days were flash frozen, and total RNA was extracted using a RNeasy mini kit (Qiagen). DNA was eliminated with a RNase-free DNase kit (Qiagen). RNA purity and quality were assessed by Nanodrop and an Agilent 2100 Bioanalyzer. Quality of all samples exceeded the manufacturer's recommendations. cDNA was synthesized using RT2 First Strand Kit (Qiagen), and samples were analyzed with the RT2 Profiler PCR Array (Mouse Breast Cancer, Qiagen) as per the manufacturer's recommended protocols in the QuantStudio 6 Flex system with the following cycling conditions: 10 min at 95°C, 15 s at 95°C, 1 min 60°C for 40 cycles.

Quantitative PCR: qPCR was performed to validate mRNA expression of IL-6, Jun, and COX2 in tumors. mRNA was quantified using SYBR green, and values were normalized to the housekeeping gene GAPDH.

2.4: Results

V-125 clusters favorably in computational analyses of *in vitro* activity

We have found that applying principal component analysis (PCA) in our prior work[243,244] with rexinoids has helped to visualize particularly active rexinoids with improved therapeutic potential group, and to that end, we have conducted a PCA with V-125 and the nine other novel rexinoids (Fig. 2.1) to visualize where they group relative to each other as well as bexarotene and LG100268 (Fig. S.1-S.2, Appendix). The PCA analysis resulted in three groups—A, B, and C—in which rexinoids clustered according to their activities in *in vitro* assays. In Group A, containing bexarotene, we observed the

difluorobexarotene analog (**1**) as well as a rexinoid with a biphenyl structural motif (**9**). In Group B, containing LG100268, we observed a pyridine analog of bexarotene (**2**) as well as a pyrimidine containing rexinoid (**4**) and another biphenyl rexinoid (**8**). In group C, containing V-125, we observed the other rexinoids with structurally similar features—**3**, **5**, **6**, and **7**—though, V-125 was somewhat isolated whereas the other rexinoids in this group clustered more closely together. We envision PCA to be useful to quickly assess potential of novel rexinoids by seeing where they group according to their activities in these assays.

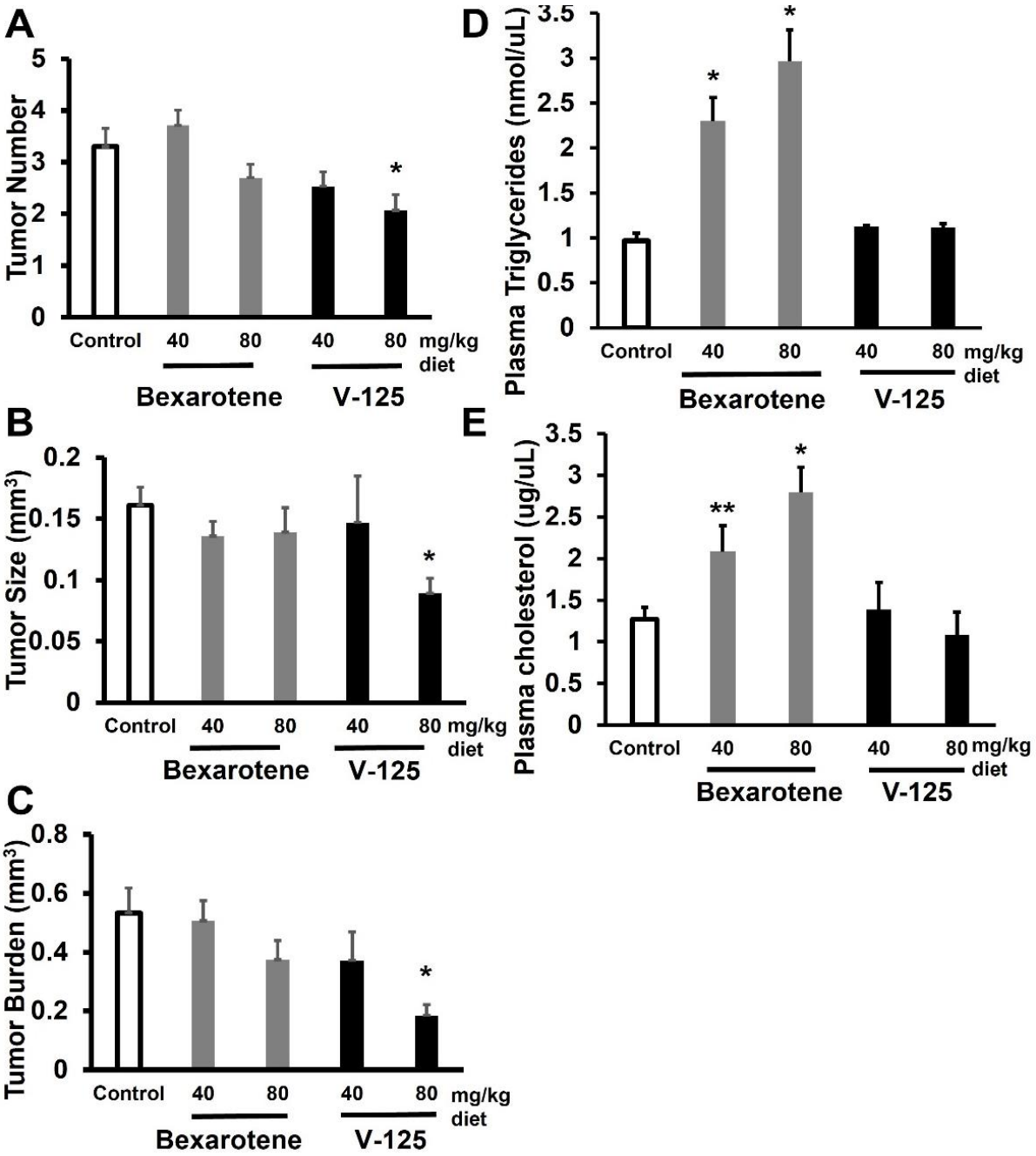

Figure 2.2. V-125 reduces lung tumor burden in A/J mice. Female A/J mice were injected with vinyl carbamate and starting 2 weeks later, fed control diet or V-125 in diet for 16 weeks. At the end of the study, mice were euthanized and lungs sectioned for analysis of histopathology. Average tumor number **(A)**, tumor size **(B),** and tumor burden **(C)** in control vs. drug treated groups. Results shown as means ± SE (*, $P < 0.05$ vs. control). Control, bexarotene 40 mg/kg, bexarotene 80 mg/kg, and V-125 40 mg/kg: N = 15 mice per group. V-125 80 mg/kg: N = 14 mice per group. Plasma triglycerides **(D)** and cholesterol **(E)** were measured with commercial kits. (*, $P < 0.01$ vs. control; **, $P < 0.001$ vs. control; n = 6 mice/group). Error bars represent standard deviations.

V-125 prevents lung tumor development in A/J mice

A/J mice were challenged with vinyl carbamate to induce *Kras* mutations and subsequent lung adenocarcinomas[245] and then treated with V-125. Mice were fed control diet or rexinoids in diet starting two weeks post-carcinogen for 16 weeks. At the conclusion of the study, evaluation of lung tumor number, size, and histopathology on slides (2 per mouse) was done in a randomized and blinded manner by two independent investigators. V-125 at 80 mg/kg of diet significantly ($p < 0.01$, Fig. 2.2A-C) reduced tumor size by 45% (0.09 ± 0.01 mm^3/tumor vs. 0.16 ± 0.01 mm^3/tumor in the control group), tumor number by 37% (2.07 ± 0.30 vs. control 3.3 ± 0.35), and tumor burden by 65% (0.18 ± 0.04 mm^3 vs. 0.53 ± 0.08 mm^3 in the control group). While there was a trend toward lower tumor parameters in mice treated with bexarotene (Table 1), these changes were not statistically significant at either the 40 or 80 mg/kg doses.

Although both V-125 and bexarotene were well-tolerated in this model based on animal weights (Figure S.3A, Appendix), bexarotene elevates triglycerides in animal models[105] and in human patients[201]. As shown in Fig. 2.2D, bexarotene significantly ($p < 0.01$) increased plasma triglyceride levels at both the 40 and 80 mg/kg doses (2.30 ± 0.26 nmol/μL and 2.97 ± 0.35 nmol/μL, respectively, vs. control 0.97 ± 0.09 nmol/μL) in the A/J mice. In contrast, V-125 had no effect on plasma triglycerides. Bexarotene also significantly ($p<0.001$) elevated plasma cholesterol (Fig. 2.2E) in a dose-dependent manner (2.08 ± 0.31 μg/μL in mice fed bexarotene at 40 mg/kg of diet and 2.80 ± 0.30 μg/μL in mice fed

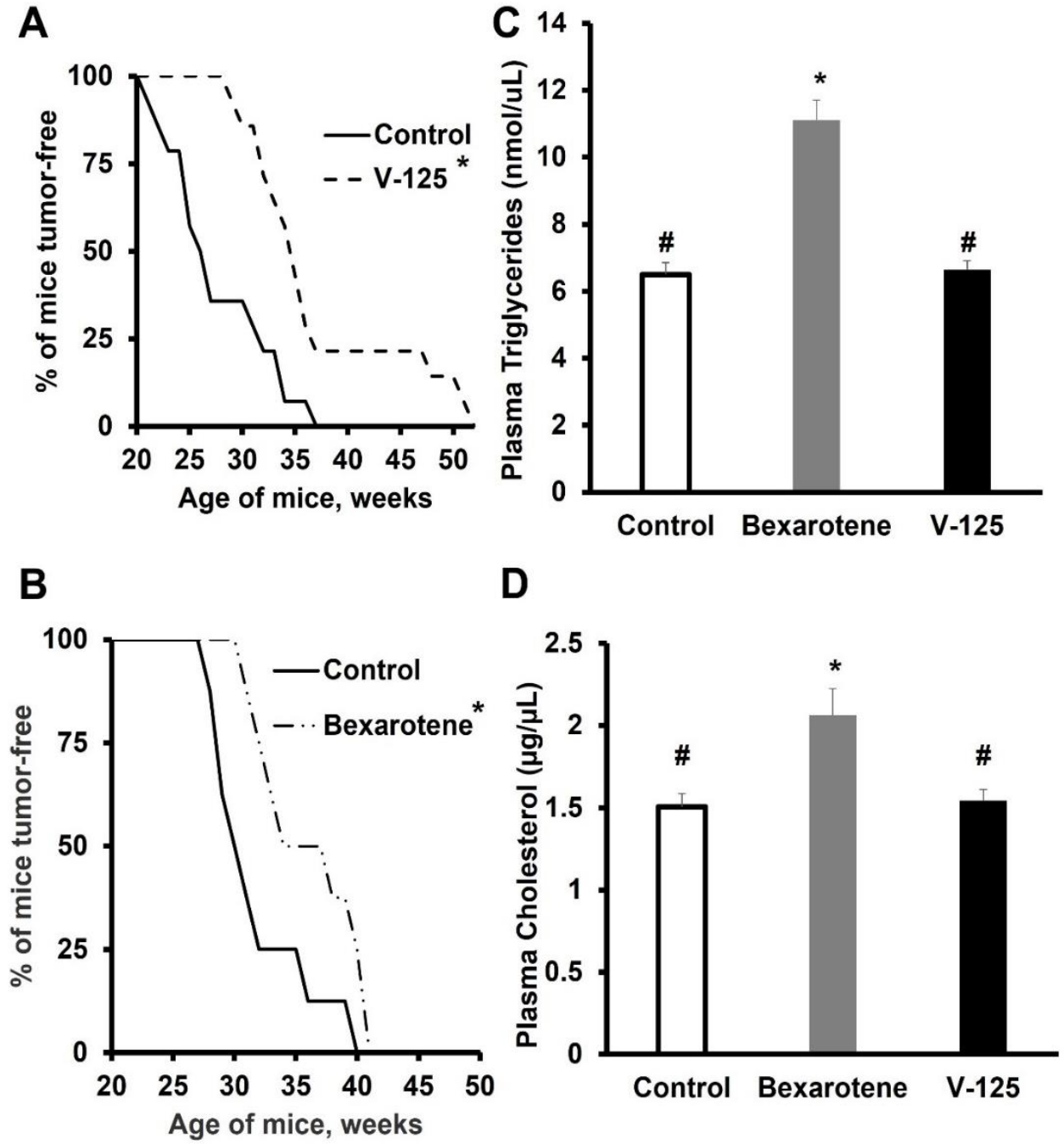

Figure 2.3. V-125 delays development of mammary tumor in MMTV-Neu mice. 10 week old female MMTV-Neu mice were fed control diet or 30 mg/kg diet of the rexinoids V-125 **(A)** or bexarotene **(B**). Mice were palpated twice weekly for the development of new tumors. **A.** *, $P < 0.001$ vs. control via log rank test; N = 14 mice per group. **B**. *, $P < 0.05$ vs. control via log rank test; N=8-9 mice per group. Plasma triglycerides **(C)** and cholesterol **(D)** levels in mice treated with control or rexinoids (30 mg/kg diet) were quantified using commercial kits (*, $P < 0.01$ vs. control; #, $P < 0.01$ vs. bexarotene; n = 8 mice/group).

bexarotene at 80 mg/kg of diet, vs control 1.27 ± 0.14 μg/μL), while V-125 did not significantly increase plasma cholesterol at either dose.

Lung tumors were classified as low, medium, or high grade based on previously established criteria[68] (Table 1). Treatment with 80 mg/kg V-125 significantly ($p < 0.05$) increased the proportion of tumors graded low/medium (66% vs. control 43%) and reduced ($p < 0.05$) the proportion of tumors classified as high grade (34% vs. control 57%). In comparison, bexarotene did not significantly change the proportions of tumors of any grade at either dose.

V-125 delays the development of HER2-positive mammary tumors in MMTV-Neu mice

MMTV-Neu mice express wild-type, unactivated Neu in mammary tissue under the control of the mouse mammary tumor virus (MMTV) promoter[241] and develop focal mammary adenocarcinomas by 25-35 weeks of age[246]. To investigate the chemopreventive effects of V-125 in this preclinical model of HER2+ breast cancer[247,248], MMTV-neu mice were fed control diet or V-125 in diet (30 mg/kg diet) starting at 10 weeks of age. V-125 significantly ($p < 0.001$) delayed initial tumor development compared to the control group (Fig. 2.3A), resulting in an approximately 10 week increase in mean time to tumor development (36.1 ± 7.8 weeks vs. control 26.5 ± 4.9 weeks). Treatment with V-125 was well tolerated in this study (Figure S.3B, Appendix). Bexarotene also significantly ($p < 0.05$) increased the time to initial tumor development in MMTV-neu mice (Fig. 2.3B) but was not as effective as V-125, delaying tumor development by only 4 weeks (35.9 ± 4.2 weeks vs. control 31.7 ± 4.1 weeks).

Plasma triglycerides and cholesterol levels were measured at the end of this study (Fig. 2.3C-D). Bexarotene significantly ($p < 0.01$) increased plasma triglyceride levels (11.1 ± 1.7 nmol/μL vs. control 6.5 ± 0.98 nmol/μL). In comparison, V-125 did not elevate plasma triglycerides (6.6 ± 0.73 nmol/μL). Bexarotene also significantly ($p < 0.01$) raised plasma cholesterol levels (Fig. 2.3D) (2.1 ± 0.16 μg/μL vs control 1.5 ± 0.08 μg/μL), while V-125 had no effect.

Treatment with V-125 extends overall survival in MMTV-Neu mice

To evaluate the anti-tumor efficacy of V-125, MMTV-Neu mice with established tumors measuring 5 mm in diameter were treated with control diet or V-125 at a dose of 100 mg/kg of diet. Tumors were measured twice weekly until they reached 10 mm in diameter, at which point they were euthanized per IACUC guidelines. Time from initiation of drug diet to euthanasia was compared between groups. Treatment with V-125 significantly ($p < 0.0172$) increased overall survival (51.6 ± 15.8 days vs. control 32.8 ± 13.6 days) (Fig. 2.4A). Average tumor volume over the first 14 days of treatment was calculated and normalized to the initial tumor volume when treatment diet was started. The average tumor volume of the V-125-treated group was significantly ($p < 0.01$) lower than the average tumor volume in the control group at day 4 (0.57 ± 0.10 vs. control 1.8 ± 0.22), day 11 (0.53 ± 0.14 vs. control 2.9 ± 0.33), and day 14 (0.89 ± 0.21 vs. control 3.6 ± 0.86) of treatment (Fig. 2.4B).

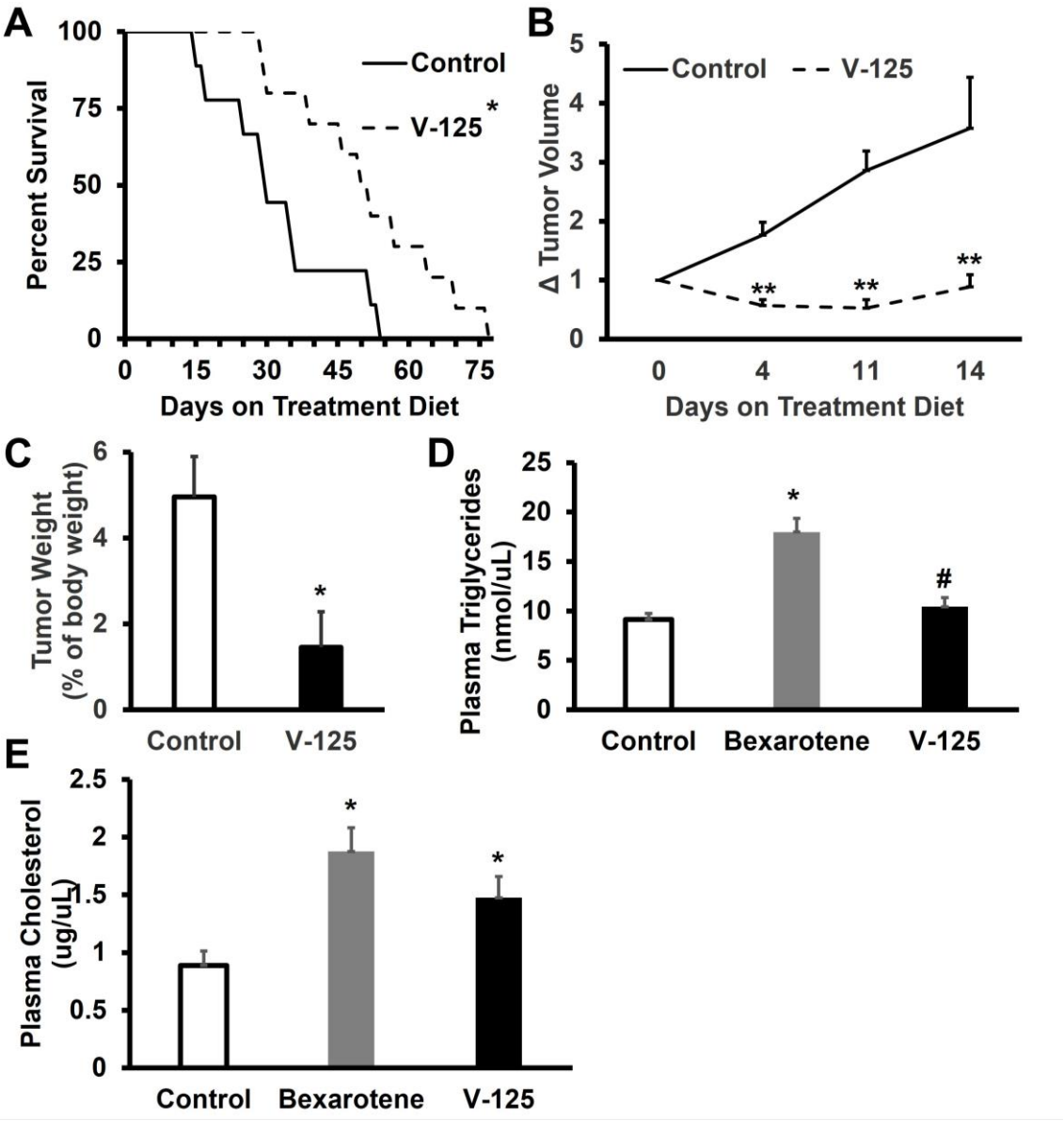

Figure 2.4. V-125 extends overall survival in MMTV-Neu mice. Female MMTV-Neu mice with established mammary tumors 5 mm in diameter were fed control diet or V-125 (100 mg/kg in diet) until tumors reached endpoint as defined by IACUC guidelines **(A,B)** or for 10 days **(C-E) A.** Overall survival is plotted for control and V-125-treated groups (*, $P < 0.0172$ vs. control via log rank test; N = 9-10 mice per group). **B**. Tumor volume over the first 14 days of treatment with V-125 was calculated and normalized to initial tumor volume (**, $P < 0.01$ vs. control). **C.** Tumor weight at the end of 10 days of treatment is presented as a percent of body weight (*, $P < 0.05$ vs. control; N=6-8 mice per group). Plasma triglycerides **(D)** and cholesterol **(E)** levels in mice treated with control or rexinoids (100 mg/kg diet) for 10 days were quantified using commercial kits (*, $P < 0.01$ vs. control; #, $P < 0.01$ vs. bexarotene; n = 6-11 mice/group). All error bars represent the standard errors.

To identify biomarkers of V-125 efficacy, MMTV-Neu mice with established tumors were treated with 100 mg V-125/kg diet for 10 days. A treatment duration of 10 days was selected based on the drastic decrease in tumor volume observed on days 10-11 of treatment (Fig. 2.4B). As shown in Fig. 2.4C, V-125 significantly ($p < 0.05$) decreased tumor weight, presented as a percentage of total body weight (1.46 ± 0.82% vs. control 4.95 ± 0.95%). Ten days of treatment with 100 mg/kg diet bexarotene (Figure S.4, Appendix) also significantly ($p < 0.05$) decreased tumor weight (2.52 ± 0.41% of total body weight vs. control 3.78 ± 0.49%), but only by 1.26%, nearly three times less than the effect observed with V-125.

When triglyceride levels in MMTV-Neu mice were evaluated, treatment with 100 mg/kg diet bexarotene for 10 days significantly ($p < 0.01$) elevated plasma triglyceride levels (18.0 ± 1.4 nmol/μL vs. control 9.1 ± 0.61 nmol/μL). In contrast, V-125 did not change plasma triglycerides (10.4 ± 2.3 nmol/μL vs. control 9.12 ± 0.61 nmol/μL), and the triglyceride levels observed in mice treated with V-125 were significantly ($p < 0.01$) lower than those in bexarotene-treated mice (Fig. 2.4D). Both bexarotene and V-125 significantly increased plasma cholesterol at this dose (1.87 ± 0.21 μg/μL and 1.47 ± 0.18 μg/μL respectively, vs. control 0.89 ± 0.12 μg/μL; Fig. 2.4E).

V-125 alters immune-related biomarkers in MMTV-Neu tumors

MMTV-Neu tumors were harvested from mammary glands after 10 days of treatment with V-125 and sectioned for immunohistochemistry (Fig. 2.5). In these tumors, V-125 did not change expression of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation. However, there was a marked increase in cleaved caspase 3, a marker of apoptosis, in tumors treated with V-125. As previous studies have

demonstrated the immunomodulatory effects of rexinoids[120], we also examined the expression of CD206 and programmed death-ligand 1 (PD-L1) in tumors. Tumors of mice treated with V-125 had a striking decrease in CD206, a cell surface marker expressed by immunosuppressive macrophages. V-125 also increased expression of PD-L1, an immune checkpoint molecule.

V-125 differentially regulates gene expression in tumors of MMTV-Neu mice

MMTV-Neu tumors were harvested after 10 days of treatment with V-125 (100 mg/kg) or control diet. A PCR array (Fig. 2.6) revealed upregulation of the following genes in the V-125 group with a log fold change > 2: *Adam23, Cdkn1c, Esr1, Gli1, Grb7, Hic1, Krt19, Mmp2, Slit2,* and *Twist1* (Fig. 2.6A). The following genes were downregulated by treatment with V-125, again with a log fold change > 2: *Abcg2, Cdkn2a, Igf1r, IL-6, Jun, Pten, Ptgs2, Serpine1, Sfrp1,* and *Thbs1* (Fig. 2.6B). To validate these results, qPCR for *IL-6* (Fig. 2.6C), *Jun* (Fig. 2.6D), and *COX2* (Fig. 2.6E) mRNA using tumor RNA from 2 mice per group confirmed the decreased expression of these genes in tumors from mice treated with V-125 in comparison to the controls.

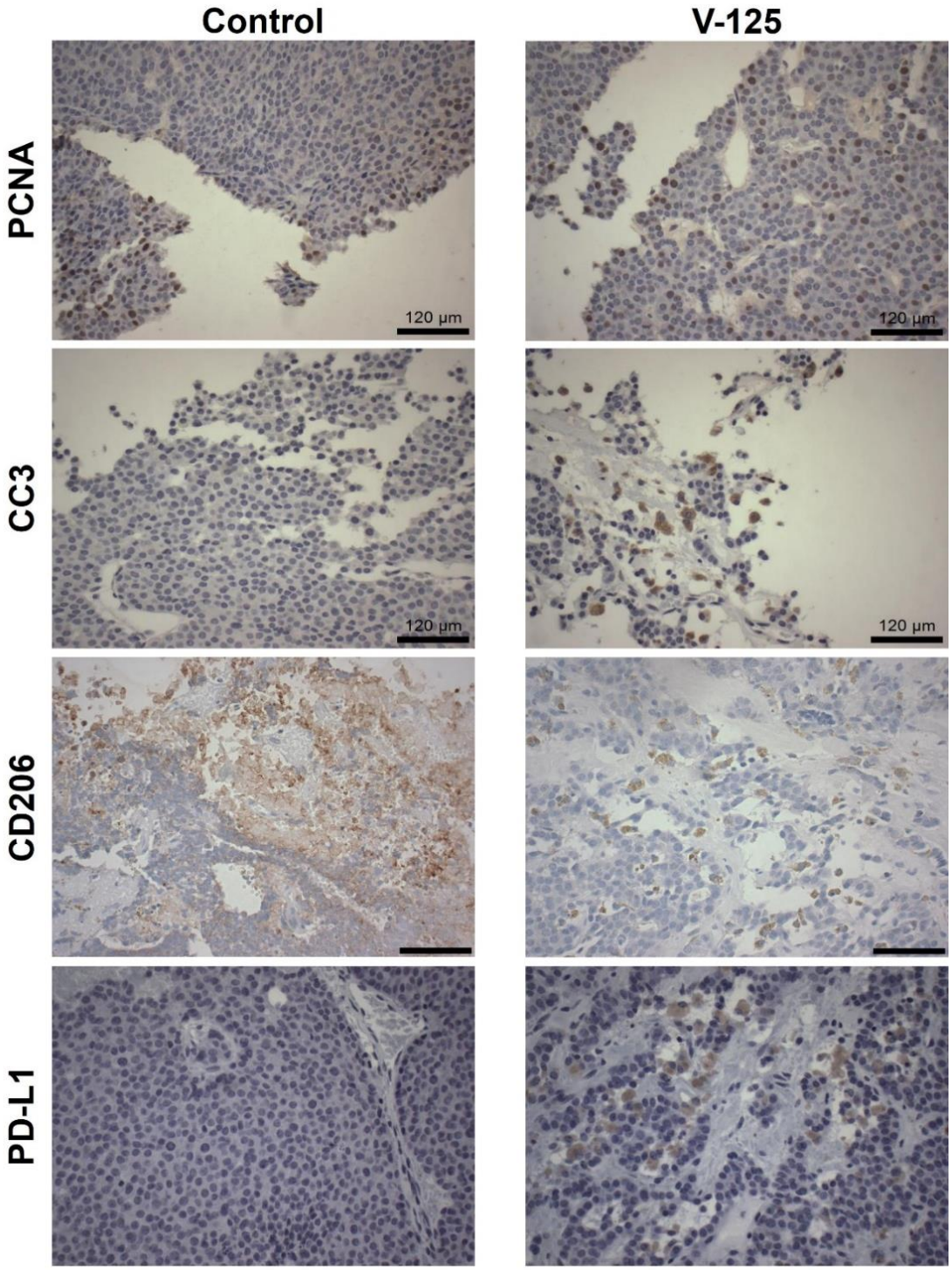

Figure 2.5. V-125 alters cell surface and intracellular markers in the MMTV-neu model of breast cancer. Mice with established mammary tumors 5 mm in diameter were treated with control or V-125 (100 mg/kg in diet) for 10 days. Immunohistochemical staining was performed for PCNA (proliferation), cleaved caspase-3 (CC3, apoptosis), CD206 (tumor-associated macrophages), and PD-L1 (immune checkpoint). Scale bar represents 120 µm.

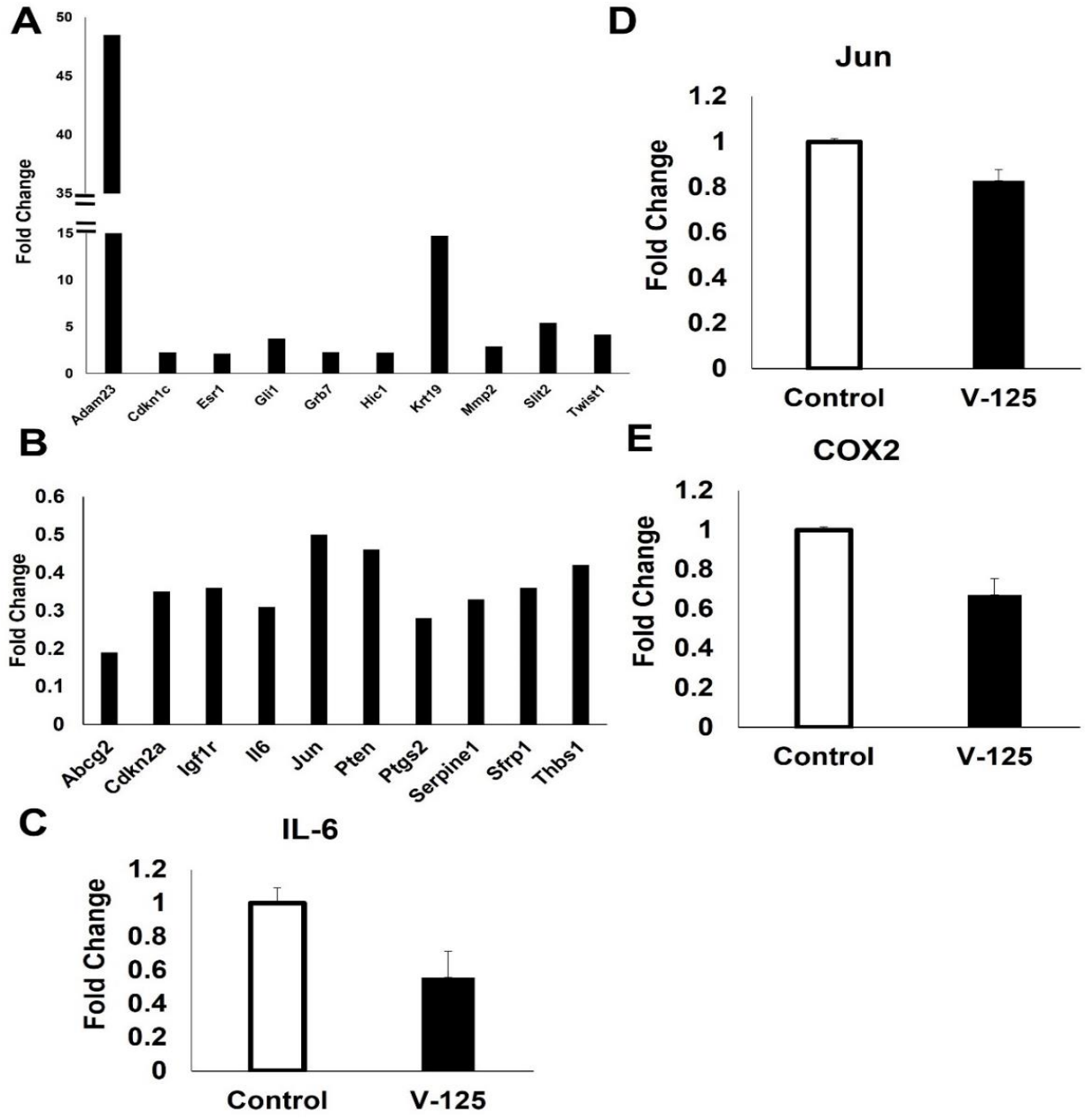

Figure 2.6. V-125 differentially regulates cancer-associated genes in tumors. Mice with established mammary tumors 5 mm in diameter were treated with control or V-125 (100 mg/kg in diet) for 10 days. Total RNA was extracted for PCR array. Analysis was performed using GeneGlobe software (Qiagen), revealing the upregulation of 10 genes with a log fold change > 2 (**A**), and the downregulation of 10 genes with a log fold change > 2 (**B**). Validation was performed on IL-6 (**C**), Jun (**D**), and COX2 (**E**) by qPCR. N = 2 mice/group. Data shown is representative of two independent experimental runs.

2.5: Discussion

Our results demonstrate significant efficacy of the novel rexinoid V-125 for prevention and treatment in the MMTV-Neu model of HER2+ breast cancer and prevention in the A/J model of Kras-driven NSCLC. Notably, V-125 was more efficacious than the clinically approved rexinoid bexarotene in both models. Unlike bexarotene, V-125 did not elevate plasma triglycerides, although it did elevate plasma cholesterol levels at its highest treatment dose (100 mg/kg diet) in the MMTV-Neu model. The combination of improved efficacy and reduced toxicity in two different clinically relevant animal models suggests that V-125 is a strong candidate for further development toward a new therapy for the treatment of patients with breast or lung cancer.

These studies also provide proof of principle that more effective and better tolerated rexinoids can be developed through structural modifications to existing rexinoids and further validate the *in vitro* screening paradigm we previously optimized. These *in vitro* assays demonstrated that V-125 does not elevate SREBP, a transcription factor which regulates genes responsible for lipid homeostasis (Fig. 2.1).

Elevation of SREBP *in vitro* predicts for increased plasma cholesterol and triglycerides *in vivo*[1,105]. Furthermore, V-125 has anti-inflammatory activity in the iNOS suppression assay at the nM level (Fig. 2.1), which correlates with efficacy for prevention in the A/J lung cancer model, and nM activity in a RXRα reporter assay[1]. As predicted by our *in vitro* screening assays, V-125 treatment resulted in superior anti-tumor efficacy without elevating triglycerides as observed with bexarotene. This screening paradigm was also used to select the compound MSU42011 which, as

predicted based on the *in vitro* screening assays, reduced lung tumor burden in A/J mice[105].

Rexinoids have been used clinically for both treatment (NCT00003752 and NCT04664829) and prevention (NCT00055991 and NCT03323658) of breast cancer. As expected, lower doses are needed for prevention studies (30 mg/kg diet in the MMTV-neu model and 40-80 mg/kg diet in the more aggressive A/J model of lung cancer) than treatment studies (100 mg/kg in the MMTV-Neu model), as early intervention is more amenable to pharmacological intervention than treating late-stage, advanced disease[249]. A compound must be very well-tolerated to warrant long-term prevention studies in patients, and lower doses will help reduce toxicity. In comparison, higher doses are used for treatment, as larger tumors have acquired multiple mutations and are more resistant to drugs. Importantly, with the combination of high anti-tumor efficacy and a favorable safety profile, rexinoids may be utilized in either clinical setting[170]. While recent strategies utilizing novel aerosolized delivery systems for administration of bexarotene have been effective in rodent models of lung cancer without inducing hypertriglyceridemia or hypercholesterolemia[74,250], it is prudent to continue to synthesize new RXR agonist with improved efficacy and toxicity profiles in addition to optimizing drug delivery strategies. A combination of these two strategies may yield the best results.

The mechanism of action of V-125 is complex. V-125 had a limited effect on proliferation of tumor cells, visualized by PCNA expression (Fig. 2.5). However, V-125 induced apoptosis in MMTV-Neu tumors as evidenced by the increased expression of cleaved caspase 3. Tumor size is dependent on the balance between cancer cell

growth and death[251], therefore despite a limited effect on cell proliferation, V-125 efficaciously reduced tumor size by inducing apoptosis. Rexinoids have limited ability to induce apoptosis of cancer cells *in* vitro[120], leading to the hypothesis that immune cells in the tumor microenvironment play a significant role in mediating the anti-tumor efficacy of rexinoids observed *in vivo*. Previous studies from Leal *et al*. investigated the immunomodulatory effects of the rexinoids LG100268 and bexarotene, and noted significant changes in T cell populations and activation markers only with LG100268[120]. Zhang *et al*. detected rexinoid-induced changes in the phenotype and function of macrophages in the tumor microenvironment[1], which may contribute to anti-tumor efficacy[252]. These data, combined with the iNOS screening data in RAW 264.7 macrophage-like cells[1], suggest that the effects of V-125 on macrophages may be necessary for anti-tumor activity. Indeed, the reduction in CD206 staining evident in MMTV-Neu tumors treated with V-125 (Fig. 2.5) confirms that this compound reduces populations of immunosuppressive macrophages associated with tumor progression. There are several possible explanations for this reduction: V-125 treatment may result in macrophage skewing, altering the polarization of these cells away from a tumor-promoting phenotype and towards a tumor-suppressive phenotype[253]. Alternatively, this decrease in CD206 staining in mice treated with V-125 could be the result of altered immune cell infiltration into the tumor, a change in the localization of immunosuppressive macrophages, or systemic effects on monocyte differentiation or circulation. Notably, we have previously demonstrated that novel pyrimidinyl derivatives of bexarotene and LG100268 decreased F4/80+ lung macrophage populations in A/J mice[1]. Further studies are needed to determine how V-125 exerts these

immunomodulatory effects in these models and how critical these effects are for anti-tumor efficacy.

The increased PD-L1 expression in tumors treated with V-125 provides an intriguing potential avenue for the combination of rexinoids with immunotherapy. The expression of PD-L1 is a positive prognostic marker in breast cancer, indicating a hot immune microenvironment[254]. In basal tumors, upregulation of PD-L1 correlates with strong local cytotoxic T cell responses and positive responses to neoadjuvant chemotherapy[254]. High PD-L1 expression and increased numbers of tumor-infiltrating lymphocytes may be predictive of patient populations that would benefit from immunotherapy[255]. As V-125 increased the expression of PD-L1 in tumors, this may bolster response to immunotherapies such as atezolizumab, a monoclonal antibody which selectively targets PD-L1[256]. Combination treatment with rexinoids and immunotherapy is a promising area for further investigation.

A breast cancer PCR array revealed differentially regulated genes in mammary tumors treated with V-125. *Adam23*, which is increased in V-125 tumors compared to controls, is a cellular adhesion molecule that enhances progression of breast cancer when downregulated[257]. *Slit2* expression in breast cancer inhibits migration[258] and is inactivated in both breast and lung cancer[259]. Importantly, *IL-6* expression was lower in V-125-treated tumors, suggesting that V-125 plays a role in modulating the tumor immune compartment. As IL-6 is known to promote tumor growth and resistance to therapy[260], decreasing IL-6 expression could be beneficial. This finding was confirmed by qPCR and recapitulates our previous studies with other rexinoids[67]. Future studies will explore the role of these gene products in mediating the anti-tumor activity of V-125.

In conclusion, the novel rexinoid V-125 is an effective treatment in murine models of both breast cancer and lung cancer. The favorable toxicity profile of this compound allows for use in either cancer treatment or prevention.

Acknowledgements

Sectioning of tissues for immunohistochemistry was done by the Investigative HistoPathology Laboratory at Michigan State University. RNA quality assessment on Agilent 2100 Bioanalyzer was performed by the RTSF Genomics Core at Michigan State University. These studies were supported by the Breast Cancer Research Foundation (BCRF-20-096), the National Institutes of Health (1R15CA139364-01A2, and 1R15CA249617-01), and a pilot grant from the Clinical and Translational Sciences Institute. Patent applications covering the novel materials described in this work have been applied for on behalf of the Arizona Board of Regents.

Author Contributions

Conceptualization: L.A.R., A.S.L., C.E.W. and K.T.L.; Methodology: L.A.R., A.S.L. and K.T.L.; Conducting experiments: L.A.R., J.A.M., A.S.L., D.Z., S.C. and B.L.; Writing the original manuscript: L.A.R.; Review & editing the manuscript, L.A.R., J.A.M., A.S.L., P.W.J., P.A.M., C.E.W. and K.T.L,; Funding acquisition, C.E.W. and K.T.L.; Supervision K.T.L.

Competing Interest Statement

Patent applications covering the novel materials described in this work have been applied for on behalf of the Arizona Board of Regents; Peter W. Jurutka, Pamela A. Marshall, and

Carl E. Wagner are named inventors. Lyndsey A. Reich, Jessica A. Moerland, Ana S. Leal, Di Zhang, Sarah Carapellucci, Beth Lockwood, and Karen T. Liby declare no competing interests.

Data Availability

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

Table 2.1. V-125 reduces lung carcinogenesis in A/J mice

	Control	Bexarotene		V-125	
mg/kg diet		40	80	40	80
# of slides/group	30	28	30	30	28
# of tumors/group	99	104	81	76	58
Total # tumors/slide	3.3 ± 0.35	3.71 ± 0.30	2.7 ± 0.26	2.53 ± 0.28	2.07 ± 0.30*
(% control)	(100%)	(112.6%)	(81.8%)	(76.8%)	(62.8%)
Total Tumor Volume, mm^3	15.98	14.17	11.247	11.16	5.17
Ave Tumor Size(mm^3)/tumor	0.16 ± 0.01	0.14 ± 0.01	0.14 ± 0.02	0.15 ± 0.04	0.09 ± 0.01*
(% control)	(100%)	(84.4%)	(84.4%)	(91.0%)	(55.2%)
Ave Tumor Burden (mm^3)	0.53 ± 0.08	0.51 ± 0.07	0.37 ± 0.06	0.37 ± 0.10	0.18 ± 0.04*
(% control)	(100%)	(95.0%)	(70.4%)	(69.8%)	(34.7%)
Total # L/M Grade (% total)	43 (43%)	50 (48%)	43 (53%)	38 (50%)	38 (66%)*
Total # HH Grade (% total)	56 (57%)	54 (52%)	38 (47%)	38 (50%)	20 (34%)*

Female A/J mice were injected with vinyl carbamate and fed control diet or V-125 in diet for 16 weeks, as described in Figure 2. L = low, M = medium, H = high. *, $P < 0.05$ vs. control.

CHAPTER 3

The novel RXR agonist MSU 42011 differentially regulates gene expression in mammary tumors of MMTV-Neu mice

Lyndsey A. Reich[1], Ana S. Leal[1], Edmund Ellsworth[1], Karen T. Liby[1]

LR contributions: Conceptualization, methodology, conducting experiments, writing the original manuscript, review and editing the manuscript

3.1: Abstract

Retinoid X receptor (RXR) agonists, which activate the RXR nuclear receptor, are effective in multiple preclinical cancer models for both treatment and prevention. While RXR is the target of these compounds, the downstream effects on gene expression differs between compounds. RNA sequencing was used to elucidate the effects of the novel RXR agonist MSU 42011 on the transcriptome in tumors of MMTV-Neu mice. For comparative analysis, RNA sequencing on tumors treated with the FDA approved RXR agonist bexarotene was also analyzed. Both treatments differentially regulated cancer-relevant gene categories, such as focal adhesion, extracellular matrix, and immune pathways. Many of the top genes differentially regulated by RXR agonists are positively correlated with survival in breast cancer patients. While MSU 42011 and bexarotene act on many common pathways, these experiments highlight the differences in regulation of gene expression between these compounds. Exploration of these unique effects on gene transcription may lead to an increased understanding of the complex biology behind RXR agonists and how the activities of this diverse class of compounds can be utilized to treat cancer.

3.2: Introduction

Retinoid X receptor (RXR) agonists are a class of compounds which bind to and activate the nuclear receptor RXR. RXR is a type II nuclear receptor, which is found in the nucleus bound to DNA and corepressor proteins[261,262]. Upon activation by a ligand, conformational changes in the structure of RXR promote dissociation of corepressor proteins and recruitment of coactivator proteins. Because of its flexible dimerization domain, RXR homodimerizes or heterodimerizes with another nuclear receptor, such as peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), or vitamin D receptor (VDR), to initiate transcription[11]. Upon activation, RXR regulates the transcription of target genes, which include genes related to proliferation, differentiation, survival, and immune cell function[263].

Bexarotene is an RXR agonist which is currently approved to treat cutaneous T cell lymphoma (CTCL)[202]. Bexarotene has been tested in clinical trials for breast cancer and non-small cell lung cancer but failed to attain FDA approval for these indications, despite promising responses in some patients and manageable side effects[205,211]. As a result, many investigators have aimed to improve the efficacy of bexarotene via novel drug delivery systems and formulations[74] or by synthesizing new RXR agonists[105,264 264,265]. One of these novel RXR agonists, V-125, is effective in prevention and treatment studies in the MMTV-Neu model of HER2+ breast cancer[264] - an established mouse model which recapitulates human disease well as determined by gene expression profiling[266,267]. This model expresses wild-type, unactivated Neu in mammary tissue under the mouse mammary tumor virus (MMTV) promoter[241]. Preliminary PCR arrays of tumors treated with V-125 revealed several differentially regulated genes, including

genes relevant to immune function such as *IL-6* and *Cox-2* and genes related to extracellular matrix function such as *Mmp2* and *Krt19*[264]. Another novel RXR agonist, MSU 42011, is also effective in the MMTV-Neu model[265] and the A/J model of carcinogen-induced lung cancer[105]. Interestingly, in the lungs of A/J mice, changes in immune cell population differed between MSU 42011 and bexarotene treatment groups[105], suggesting that these compounds have distinct patterns of immunomodulatory activity.

Nuclear receptor biology is complex, and gene transcription varies based on the nuclear receptor binding partner of RXR. For example, target pathways under the control of RXR:RAR heterodimers include genes which induce the enzymes phosphoenolpyruvate carboxykinase (PEPCK) and tissue transglutaminase 2 (TG2), immune-related genes such as B cell translocation gene 2 (*Btg2*), and retinoic acid response genes such as aberrant cellular retinol binding protein 1 (*Crbp1*) and cellular retinoic acid-binding protein 1 (*Crabp1*)[268]. Several genes involved in lipogenesis (*Agpat2, Acsl1, Gpat3*) and glucose metabolism (*Hk2, Taldo1*) are RXR:PPAR target genes in adipocytes[269]. VDR, another nuclear receptor for which RXR is an obligate heterodimer, regulates expression of an extensive list of genes which act as VDR response elements. In quiescent hepatic stellate cells, binding of calcipotriol to the VDR nuclear receptor initiates binding to a cistrome of 6,281 target sites, which expands to 24,984 sites when these cells are activated by lipopolysaccharide (LPS) or transforming growth factor beta (TGFβ)[270]. Because the network of nuclear receptor target genes is vast, the biological effects of RXR activation are numerous and diverse.

Others have previously investigated the effects of bexarotene on the transcriptional regulatory network in mammary glands of mouse models of breast cancer[271], but to date no one has comparatively analyzed gene expression data from tumors treated with different RXR agonists. To this end, we used RNA sequencing to compare pathways activated by treatment with MSU 42011 versus pathways activated by bexarotene and validated selected genes by qPCR and immunohistochemistry. These data provide additional information about the extensive network of cancer-relevant transcriptional regulation of RXR agonists, and the diversity of activities of these compounds.

3.3: Materials and Methods

Drugs: MSU 42011 was synthesized as previously described[105,264,265], and bexarotene was purchased from LC Laboratories. For *in vivo* studies, RXR agonists were dissolved in a vehicle of 1 part ethanol:3 parts highly purified coconut oil (Neobee oil, Thermo Fisher Scientific). 50 mL vehicle or drug dissolved in vehicle was mixed into 1 kg of powdered 5002 rodent chow (PMI Nutrition) using a KitchenAid mixer. RXR agonists remain stable in this diet when stored at 4°C for up to 6 weeks, as confirmed by LC-MS.

***In vitro* experiments:** Bone marrow-derived macrophages (BMDM) were isolated from femurs of adult C57BL/6 mice and differentiated using MCSF, as previously described[1]. Conditioned media was harvested from E18-14C-27 cells, derived from MMTV-Neu tumors, after 48 hours of culture. BMDMs were treated using conditioned media with or without 300 nM RXR agonists for 24 hours.

***In vivo* experiments:** All animal studies were approved by the Institutional Animal Care and Use Committee at Michigan State University (IACUC protocol number 202100188). MMTV-Neu mice[241] from our breeding colony (founders were purchased from Jackson Laboratory) were fed pelleted chow and palpated for tumors. Once tumors were detected, mice were switched to powder 5002 chow. Tumors were measured twice weekly with a caliper until reaching 4 mm in diameter, at which time mice were randomized and fed control diet or 100 mg per kg diet of RXR agonist diet (~25 mg per kg body weight) for 10 days. Tumors were harvested and sections were either flash frozen for RNA-seq/qPCR or saved in neutral buffered formalin for immunohistochemistry

RNA Sequencing: Frozen tumor sections (4 samples per treatment group) were weighed and homogenized. RNA was extracted using a RNeasy Mini Kit (Qiagen), and the quality of the RNA measured with an Agilent Bioanalyzer. RNA sequencing was completed by Novogene (Sacramento, CA) as described previously[272]. Raw read counts were analyzed using the DESeq2 package in R to generate differential expression profiles, and EnrichR and Ingenuity Pathway Analysis (Qiagen) were used for enrichment analysis. Raw and processed date were deposited in the Gene Expression Omnibus and are available through GSE211290.

qPCR: RNA harvested from frozen tumor sections was normalized across samples using Nanodrop, and 500 ng of RNA was used to synthesize cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR was run on QuantStudio 7 Flex using SYBR green fluorescence. PCR data was analyzed using the delta-delta CT method using GAPDH as a housekeeping control. Error bars represent

standard error of biological replicates, as indicated in figure legends. The following forward/reverse primers (Integrated DNA Technologies) were used: IL-18, 5' – TCCTTGAAGTTGACGCAAGA – 3' / 5' – TCCAGCATCAGGACAAAGAA – 3', Col6a3, 5' AAGGACCGTTTCCTGCTTGTT – 3' / 5' – GGTATGTGGGTTTCCGTTGAG – 3'. Map9, 5' – GAAGAGTGCTACAGCCAACAC – 3' / 5' – ACAACAAGGTTTTTCCCCTTCC – 3', H2-AA, 5' - TCAGTCGCAGACGGTGTTTAT – 3' / 5' - GGGGGCTGGAATCTCAGGT – 3'.

Immunohistochemistry: Formalin-fixed tissues were embedded in paraffin and sectioned by the Histology Core at Michigan State University. Boiling citrate buffer was used for antigen retrieval, and endogenous peroxidase activity was quenched using hydrogen peroxide. Tissue sections were stained with antibodies against IL-18 (1 µg/mL, PA5-79481, Thermo Fisher Scientific), Col6a3 (Thermo Fisher Scientific), Mat1a (Thermo Fisher Scientific), and Slit2 (Thermo Fisher Scientific) as described[120]. Sections were then labeled with biotinylated secondary antibodies (anti-rabbit, Cell Signaling; anti-rat, Vector Labs), as previously described.[120] A DAB substrate (Cell Signaling) was used for signal detection, as per manufacturer-provided protocols, and sections were counterstained with hematoxylin (Vector Labs). The Fiji ImageJ image processing package was used for quantification of intensity of DAB staining by the color deconvolution method[273] and mean gray value was used to calculate optical density by the formula OD = log (max intensity/mean intensity, with a maximum intensity of 255 for 8–bit images[274].

Statistical analysis: Results were expressed as the mean ± standard error. Data from tumor qPCR experiments were analyzed by one-tailed t test. $p < 0.05$ was

considered statistically significant throughout all experiments. For RNAseq, differential expression analysis was performed using DESeq2. Outliers are detected by Cook's distance and removed[275]. P values were adjusted to correct for multiple comparisons using the Benjamini and Hochberg method, and padj < 0.05 was considered statistically significant[276]. Data from *in vitro* experiments were analyzed using one-way ANOVA, and significant differences between groups were determined by the Tukey HSD multiple comparisons test.

3.4: Results

RXR agonists regulate pathways relevant in breast cancer

To characterize differential expression across the whole transcriptome, use of high-throughput techniques such as RNA sequencing (RNA-seq) allows us to parse differentially expressed genes into biological pathways for comprehensive analysis of RXR agonist response in tumors. For these studies, MMTV-neu mice with tumors 4 mm in diameter (4 mice/group) were fed control diet, MSU 42011 (100 mg/kg diet), or bexarotene (100 mg/kg diet) for 10 days. Tumors were harvested and RNA was analyzed by RNA-seq (Fig. 3.1A). In comparison to control tumors, tumors treated with RXR agonists had higher expression of canonical immune pathways such as binding of antigen presenting cells and proliferation of immune cells, mononuclear leukocytes, and lymphocytes (Fig. 3.1B). Causal network analysis[277], a means of identifying upstream regulators of differentially expressed genes from RNA-seq, identified SMAD4, IRF3, IRF7, and ZBTB10 as possible regulatory nodes.

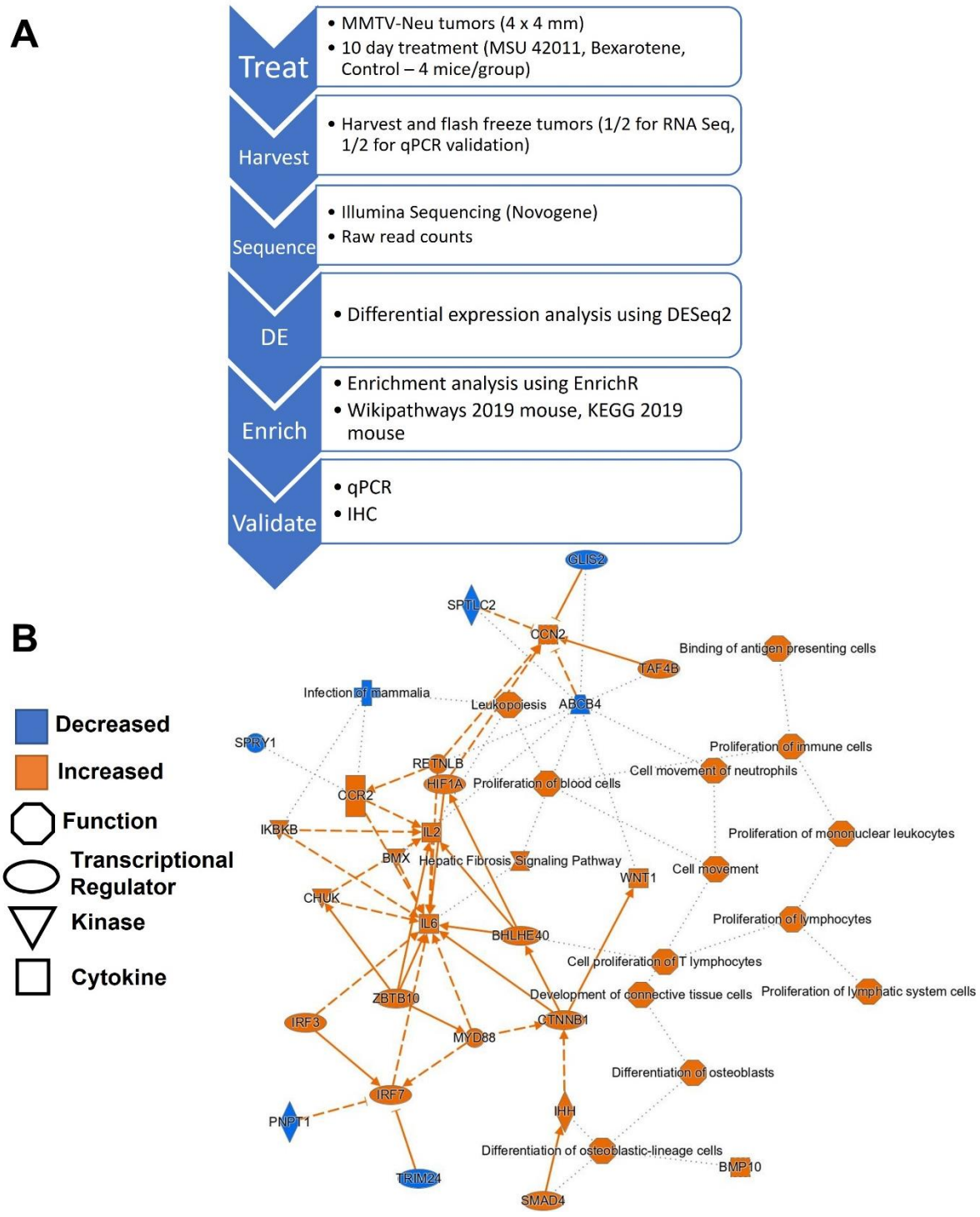

Figure 3.1. RXR agonists regulate pathways relevant in breast cancer. A. Experimental flow diagram of RNA Sequencing studies. **B**. Qiagen Ingenuity Pathway Analysis reveals a network of pathways differentially regulated by RXR agonists**.**

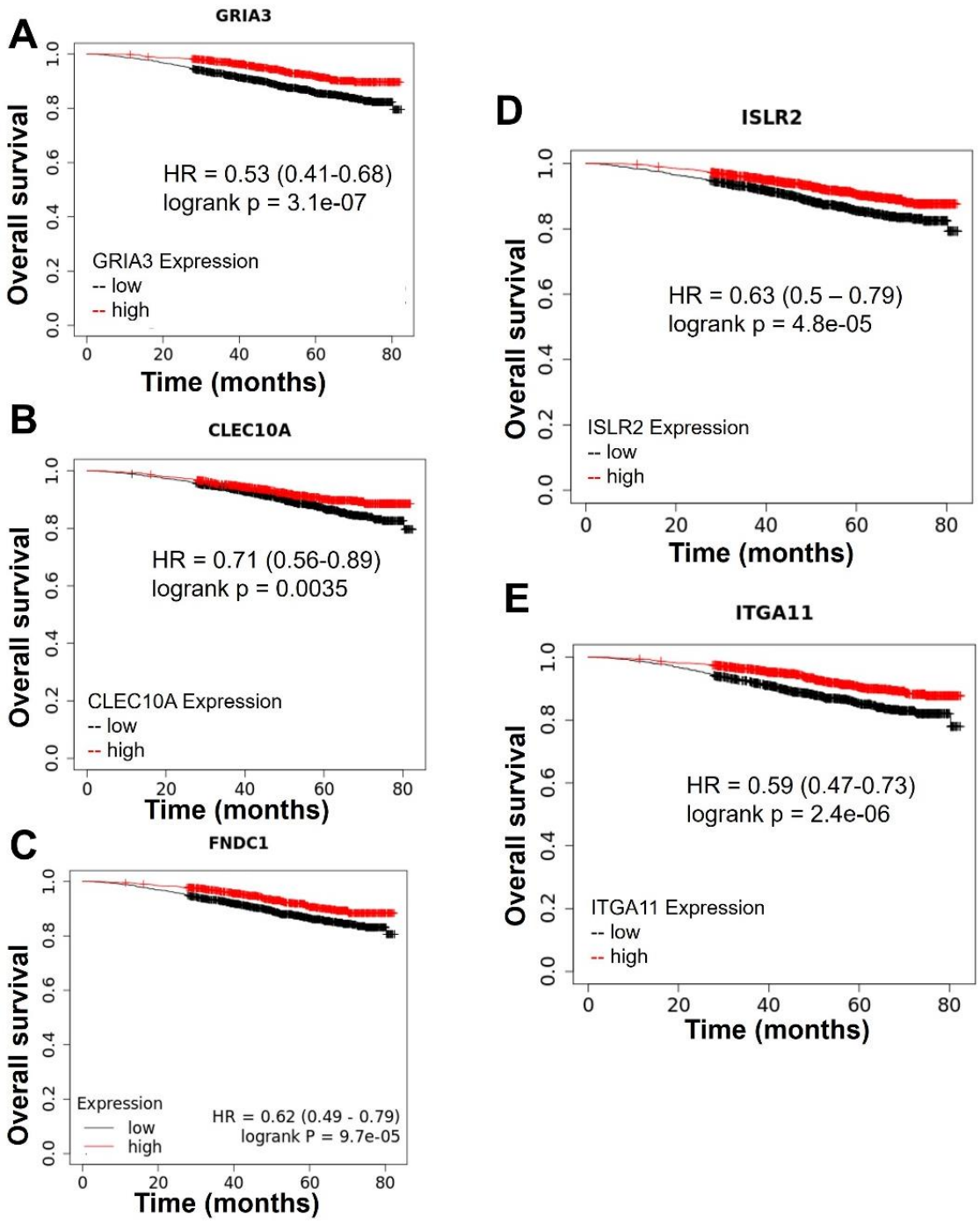

Figure 3.2. Top genes differentially expressed in tumors treated with MSU 42011 and bexarotene correlate with patient survival. Kaplan-Meier survival curves from the top 10 differentially expressed genes from MSU 42011 vs bexarotene vs control where expression is correlated with overall survival in breast cancer patients (n=2976; 1259 high 1717 low in **A**, 1220 high 1756 low in **B**, 1252 high 1724 low in **C**, 1588 high 1388 low in **D**, 1703 high 1273 low in **E**). Data generated using KMPlot (http://www.kmplot.com). No stratification strategies were used. Cutoffs for high and low expression were determined by auto-cutoff tool based on false discovery rate and p value[2].

Top genes differentially expressed in tumors treated with MSU 42011 and bexarotene correlate with patient survival

Differential expression analysis revealed a list of genes differentially expressed in control tumors vs. tumors treated with MSU 42011 vs tumors treated with bexarotene. This list of 289 significantly (padj < 0.05) upregulated or significantly downregulated genes was sorted by adjusted p value. Of the top ten most significant differentially expressed genes, high levels of expression of five genes significantly correlate with improved overall survival in breast cancer patients – *GRIA3* (logrank p = 3.1e-07) (Fig. 3.2A), *CLEC10* (logrank p = 0.0035) (Fig. 3.2B), *FNDC1* (logrank p = 9.7e-05) (Fig. 3.2C), *ISLR2* (logrank p = 4.8e-05) (Fig. 3.2D), and *ITGA11* (logrank p = 2.4e-06)[278] (Fig. 3.2E). These genes code for a glutamate receptor linked to migration and invasion (*GRIA3*)[279], a c-type lectin with a role in cellular adhesion, signaling, and inflammation which serves as a dendritic cell marker (*CLEC10*)[280], a fibronectin protein associated with invasion and chemoresistance (*FNDC1*)[281], a member of the immunoglobulin superfamily which participates in nervous system development (*ISLR2*)[282], and an alpha integrin which regulates adhesion to the extracellular matrix and the organization of collagen (*ITGA11*)[283]. Survival curves were generated using the Kaplan-Meier Plotter (KmPlot)[2], without further stratification of breast cancer patients.

RXR agonists regulate cancer-relevant biological pathways in MMTV-Neu tumors

Enrichment analysis on control vs MSU 42011 vs bexarotene differential expression data using EnrichR reveals a set of pathways enriched by treatment with RXR agonists (Fig. 3.3). These pathways include genes associated with ECM-receptor interaction, chemokine signaling, focal adhesion, PI3K-Akt signaling, complement and coagulation cascades, and the phagosome. Genes within these pathways code for macromolecules involved in cellular structure and function, cellular behavior such as adhesion and migration, and downstream signaling pathways. The KEGG 2019 mouse database was used for these analyses.

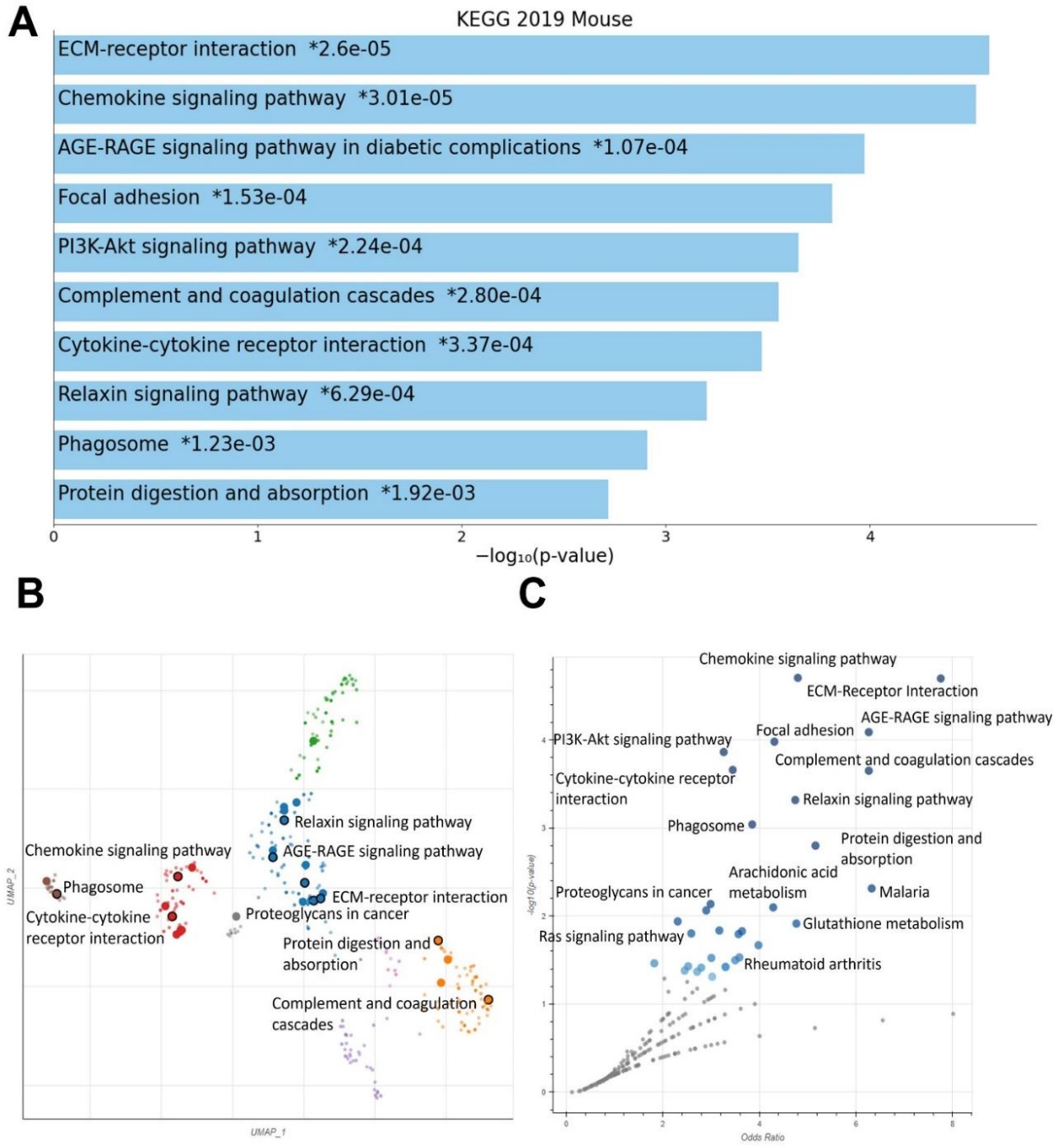

Figure 3.3. **RXR agonists regulate cancer-relevant biological pathways in MMTV-Neu tumors.** Female MMTV-Neu mice with established mammary tumors 4 mm in diameter were fed control diet, bexarotene (100 mg/kg in diet), or MSU 42011 (100 mg/kg in diet) for 10 days. KEGG 2019 mouse was used as a database for enrichment analysis. **A**. Bar graph of top differentially expressed pathways. Scatterplot (**B**) and volcano plot (**C**) of top differentially expressed pathways.

MSU 42011 and bexarotene induce unique gene expression profiles with some unifying characteristics in treated tumors

Enrichment analysis was used to compare differentially expressed genes in control vs. MSU 42011 and control vs. bexarotene groups. Bar charts of these analyses reveal enrichment of shared pathways (focal adhesion, ECM-receptor interaction), as well as pathways unique to MSU 42011 (rheumatoid arthritis, ribosome) and pathways unique to bexarotene (PI3K-Akt signaling pathway, Rap1 signaling pathway) (Fig. 3.4A, B). These unique pathways include genes which code for critical components related to cellular proliferation, immunity, and cellular migration and invasion. Scatterplot depictions of pathways regulated by MSU 42011 (Fig. 3.4C) and by bexarotene (Fig. 3.4D) highlight the similarities and differences in pathway enrichment within a particular cluster across different drug treatments. Volcano plot depictions of pathways regulated by MSU 42011 (Fig. 3.4E) and bexarotene (Fig. 3.4F) highlight the pathways unique to MSU 42011, especially the ribosome pathway. This pathway contains genes which code for components necessary for rapid cellular turnover, which is particularly relevant to tumor biology[284,285]. KEGG 2019 was used as a database for these analyses.

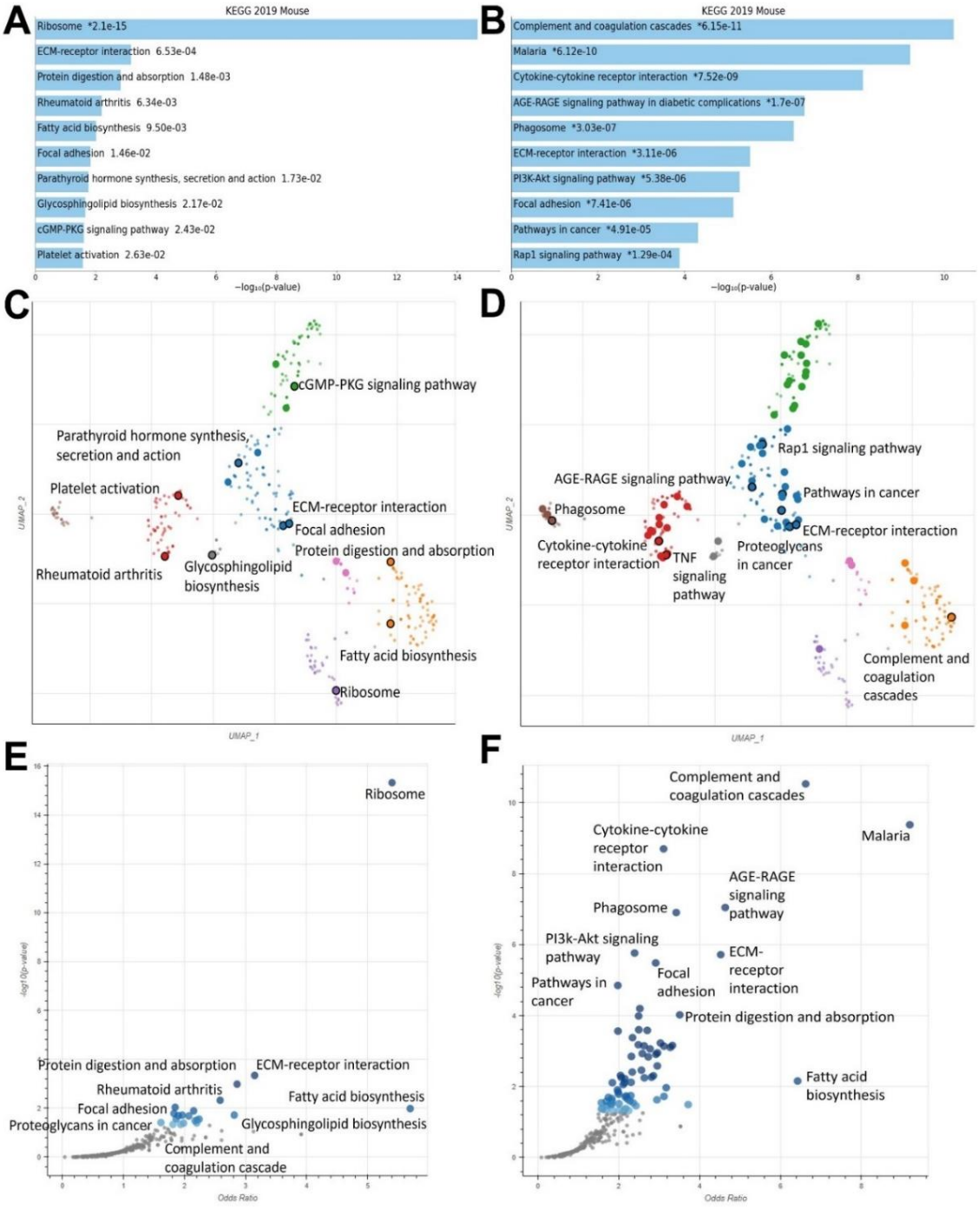

Figure 3.4. MSU 42011 and bexarotene induce unique gene expression profiles with some unifying characteristics in treated tumors. A-B. Bar graph of differentially regulated pathways in MSU 42011-treated vs control tumors (**A**) and bexarotene-treated vs control tumors (**B**) using the KEGG 2019 Mouse database in Enrichr. **C-F**. Scatterplot (**C,D**) and volcano plot (**E,F**) depictions of differentially regulated pathways in MSU 42011-treated vs control tumors (**C,E**) and bexarotene-treated vs control tumors (**D,F**).

MSU 42011 increases Col6a3 and Map9 expression in mouse mammary tumors

A series of genes was selected from the differential expression analysis for validation of mRNA expression by qPCR and protein levels by IHC. Collagen type VI a3 chain (COL6A3) is an extracellular matrix protein which is altered in several types of cancer[286]. *Col6a3* mRNA expression (Fig. 3.5A) is increased in tumors treated with MSU 42011 ($p = 0.0425$) but not in tumors treated with bexarotene (Figure S.7 – Appendix). IHC (Fig. 3.5B) demonstrates a striking increase in Col6a3 protein levels in tumors treated with MSU 42011, and no apparent increase in Col6a3 in bexarotene-treated tumors. Kmplot was used to investigate the relevance of *Col6a3* expression in human breast tumors (Fig. 3.5C). High expression of *COL6A3* is correlated with increased survival ($p = 0.031$) in HER2+ breast cancer patients. qPCR (Fig. 3.5D) also confirms a significant ($p = 0.0026$) increase in *Map9* mRNA in MSU 42011-treated tumors, while there was no significant increase observed in bexarotene-treated tumors. MAP9 is a microtubule-associated protein which plays a role in cell cycle regulation and DNA damage response[287]. High expression of *MAP9* is positively correlated with survival (Fig. 3.5E) in breast cancer patients ($p = 0.0023$).

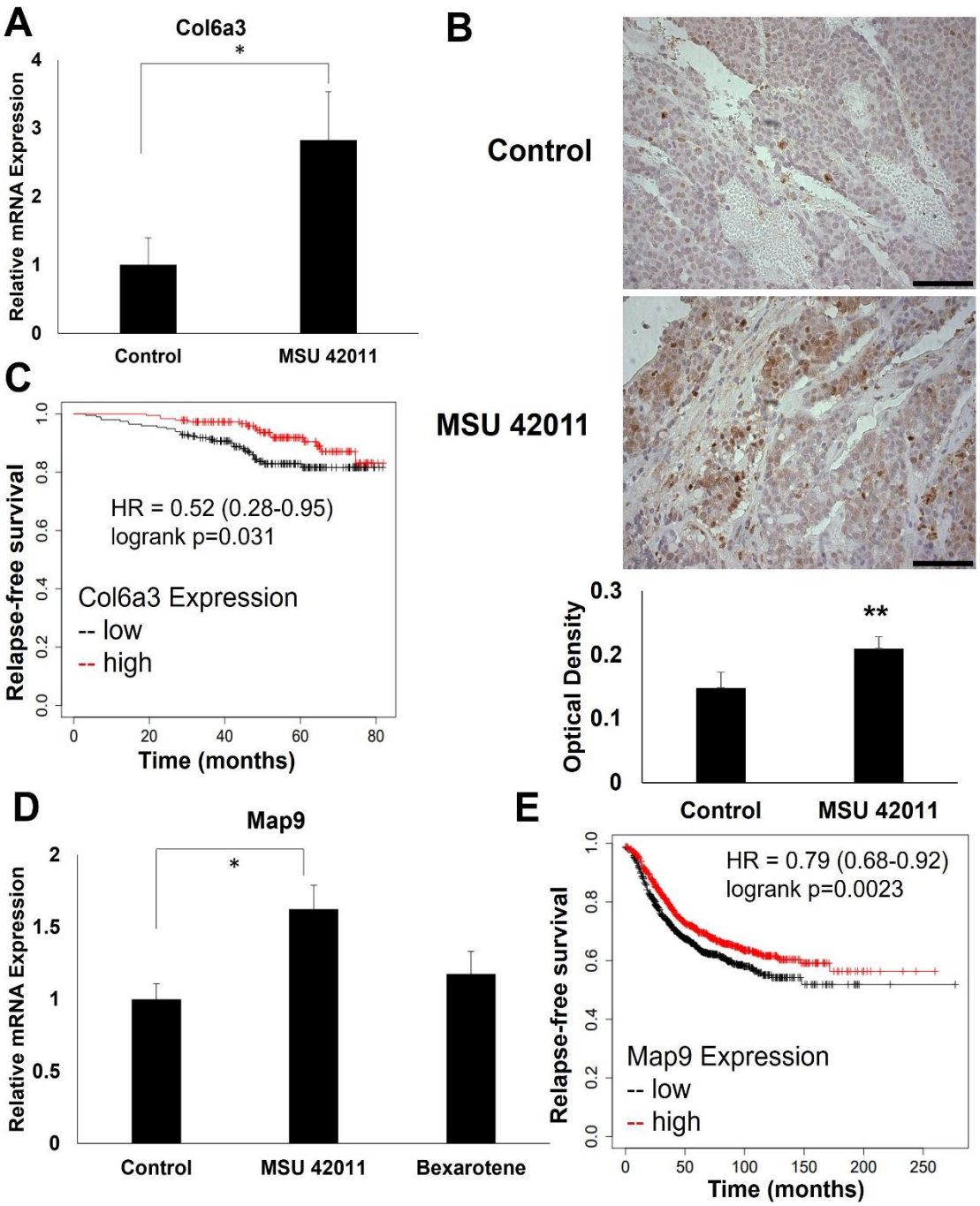

Figure 3.5. MSU 42011 increases Col6a3 and Map9 expression in mouse mammary tumors. Tumors treated with MSU 42011 (100 mg/kg diet) or control were harvested and flash-frozen. Tumors were homogenized and RNA extracted. **A, D**. mRNA expression was detected by qPCR on QuantStudio 7 Flex. N=5 mice/group. Error bars represent standard error. * $p < 0.05$ **B**. Immunohistochemical staining for Col6a3, representative of 4 mice per group and quantified using ImageJ. **C, E**. Kmplot survival curves correlate expression of Col6a3 (**C)** with survival in HER2+ breast cancer patients (n = 379; 183 high, 196 low) and Map9 (**E**) with survival in breast cancer patients (n = 2032; 1015 high, 1017 low).

MSU 42011 increases IL-18 and H2-AA expression in mouse mammary tumors

As shown in Figure 3.4, the rheumatoid arthritis pathway is differentially regulated by MSU 42011, but not by bexarotene. The genes within this pathway include immune response genes which may contribute to MSU 42011's anti-tumor immunomodulatory activity and are good candidates for validation. As previous studies from our lab have investigated the immunomodulatory effects of RXR agonists[120], the cytokine *IL-18* was selected from the rheumatoid arthritis pathway for validation (Fig. 3.6A). mRNA expression increased in tumors treated with MSU 42011 ($p = 0.0116$) but not in tumors treated with bexarotene. IHC (Fig. 3.6B) revealed a marked increase in IL-18 in MSU 42011-treated tumors. Interestingly, bexarotene-treated tumors display an apparent paucity of IL-18, even in comparison to control tumors (Figure S.7 – Appendix). Importantly, Kmplot analysis reveals that high IL-18 expression is correlated with increased relapse-free survival in breast cancer patients ($p = 0.00022$) (Fig. 3.6C). MSU 42011-treated tumors also demonstrate a significant ($p = 0.040822$) upregulation of the gene coding for major histocompatibility complex (MHC) component H2-AA by qPCR (Fig. 3.6D).

MSU 42011 differentially regulates genes in bone marrow-derived macrophages (BMDMs) relevant to polarization towards an anti-tumor phenotype

RXR agonists regulate pathways relevant to the function of the immune system, such as rheumatoid arthritis, complement and coagulation cascade, and cytokine-cytokine receptor interaction. To validate and further characterize the immunomodulatory activity of these compounds, BMDMs treated with RXR agonists were evaluated for expression of cancer-relevant genes within these pathways. BMDMs

were harvested on Day 0 from WT C57B/6 mice and differentiated with MCSF. On Day 5, BMDMs were treated with conditioned media from E18-14C-27 cells, derived from MMTV-Neu mammary tumors, to induce a tumor-educated macrophage phenotype. BMDMs were treated with conditioned media alone, or with 300 nM of either MSU 42011 or bexarotene. After 24 hours of treatment, the expression of genes associated with macrophage phenotype was evaluated. Treatment with 300 nM of either RXR agonist significantly ($p = 0.0016$) decreased IL-13, an immunosuppressive cytokine (Fig. 3.7A). BMDMs treated with RXR agonists show a trend of increasing TLR9 and IRF1 mRNA expression, associated with a pro-inflammatory, anti-tumor phenotype (Fig. 3.7B, C). Rexinoids also induce a significant ($p = 0.00015$) increase in expression of CCL6, a pro-inflammatory cytokine (Fig. 3.7D).

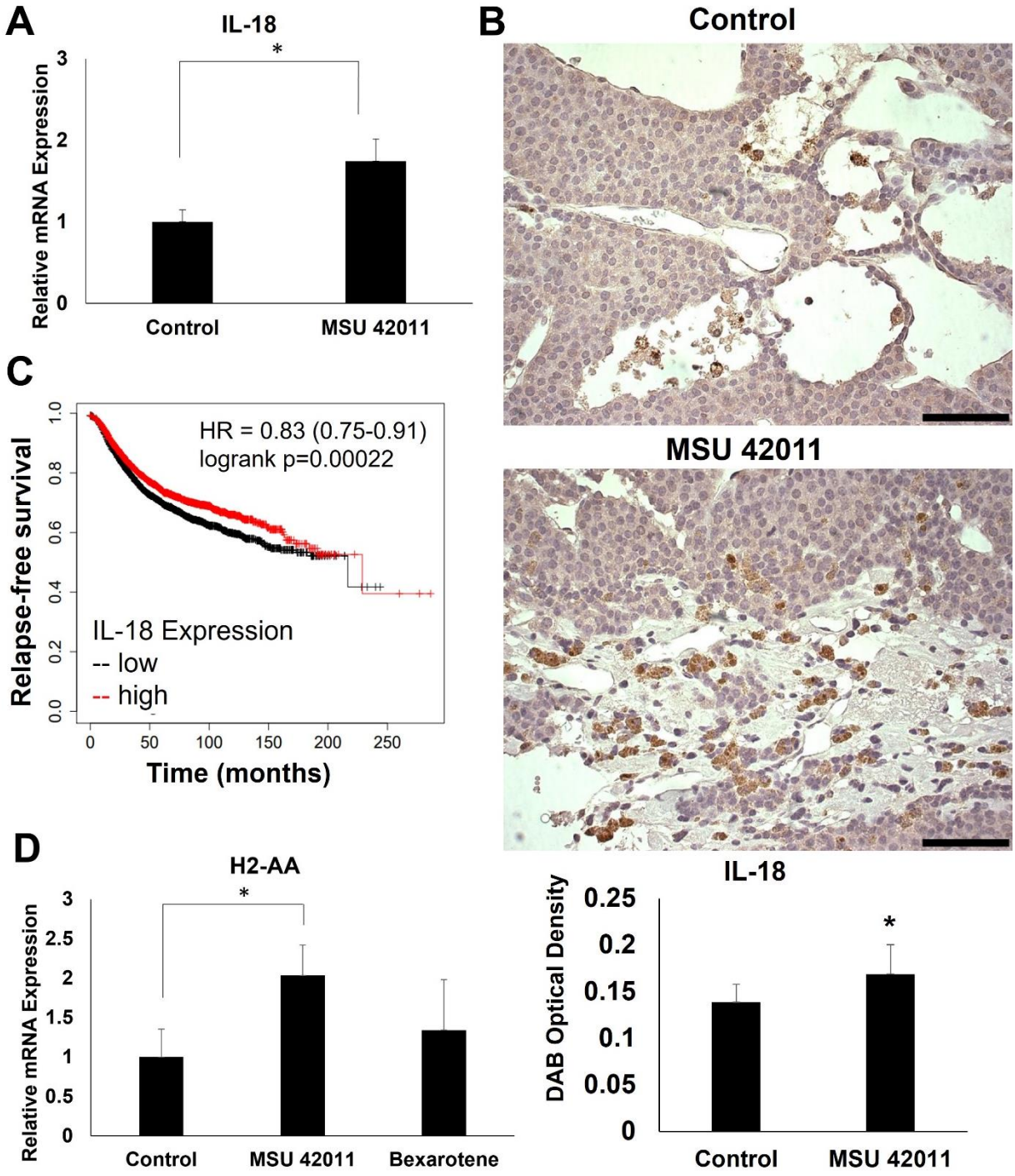

Figure 3.6. MSU 42011 increases IL-18 and H2-AA expression in mouse mammary tumors. Tumors treated with MSU 42011 (100 mg/kg diet) or control were harvested and flash-frozen. Tumors were homogenized and RNA extracted. **A, D**. Gene expression was detected by qPCR on QuantStudio 7 Flex. N=5 mice/group. Error bars represent standard error. * $p < 0.05$ **B**. Immunohistochemical staining for IL-18, representative of 4 mice per group and quantified using ImageJ. **C**. Kmplot survival curve correlates expression of Il-18 with survival in breast cancer patients (n = 4929; 2456 high, 2473 low).

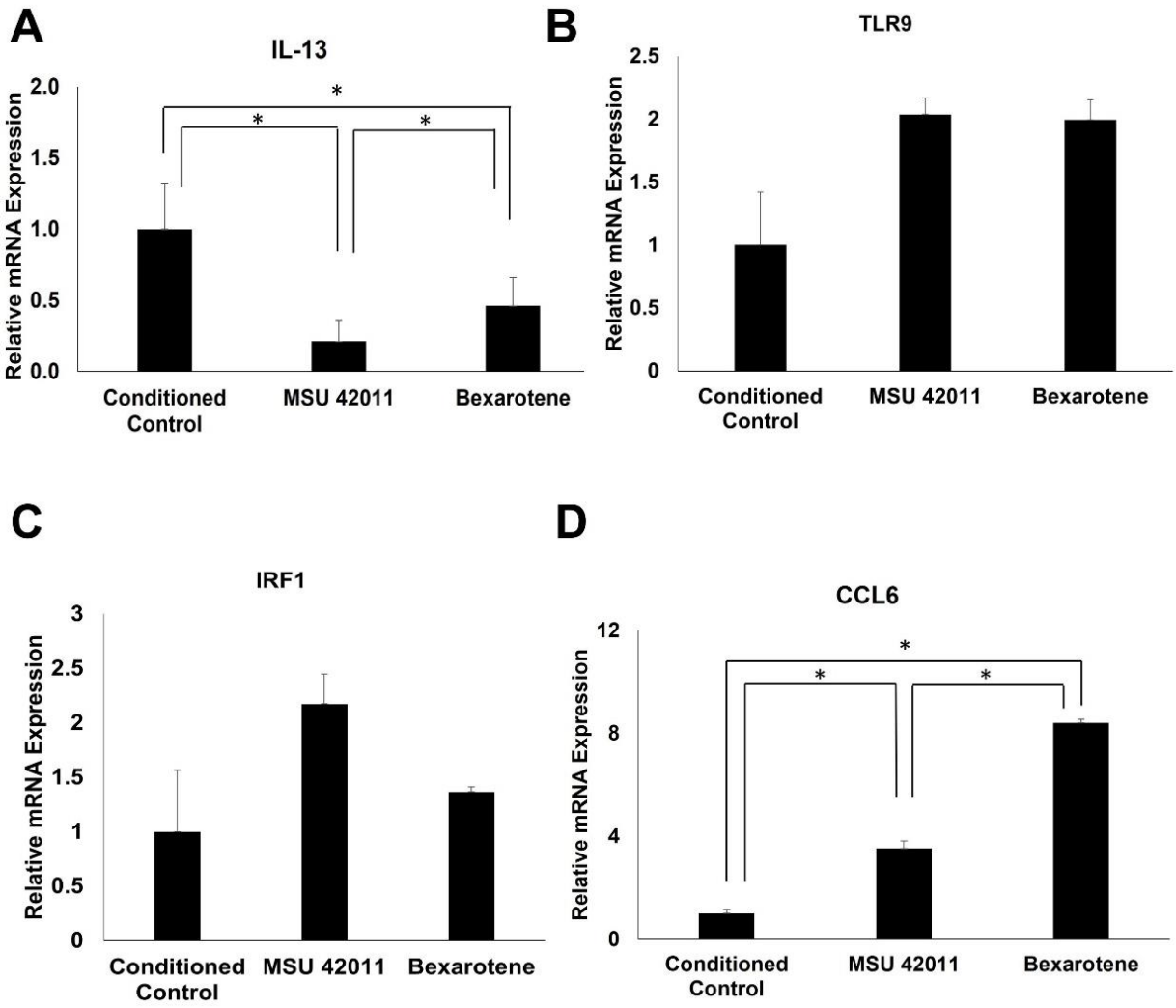

Figure 3.7. MSU 42011 differentially regulates genes in bone marrow-derived macrophages (BMDMs) relevant to polarization towards an anti-tumor phenotype. A- D. BMDMs were isolated from WT C57BL/6 mice and differentiated with MCSF. On day 5, BMDMs were treated with conditioned media from E18-14C-27 cells, either alone or with 300 nM RXR agonist. RNA was extracted using TRIzol reagent and gene expression was detected by qPCR on QuantStudio 7 Flex. Error bars represent standard error of 3 biological replicates. * $p < 0.05$

3.5: Discussion

RXR agonists are a class of drugs with anti-tumor activity in preclinical models of breast and lung cancer. While the known target of these drugs is the nuclear receptor RXR, different RXR agonists have markedly different effects on downstream gene

expression. The nature of nuclear receptors – their ability to homodimerize or to heterodimerize with other nuclear receptors, the diversity of the structures of their ligands, and the vast number of target genes – makes RXR an interesting drug target in its modularity.

For the first time, this paper characterized the downstream effects on gene expression of several novel RXR agonists in the MMTV-Neu model of breast cancer. Using RNA-seq, we compared pathway activation and biological activity of these molecules. Comparison of pathways activated by the novel RXR agonist MSU 42011 and the FDA-approved bexarotene reveals the regulation of many similar pathways, including focal adhesion and extracellular matrix components, are shared by these two molecules. Immune-related pathways such as cytokine signaling pathways, complement activation, and genes related to phagosome activity are also shared by both MSU 42011 and bexarotene. Interestingly, validation of individual genes within these pathways shows that while one RXR agonist upregulates an immune- or ECM-related gene, other RXR agonists do not – for example, MSU 42011 increases expression of IL-18 and Col6a3, but neither of these two gene products are increased in tumors treated with bexarotene.

Several pathways were identified through enrichment analysis that were unique to a single RXR agonist. For example, the ribosome pathway and the fatty acid biosynthesis pathway, through which macromolecules critical to cellular function are synthesized, were prominent in enrichment analysis for MSU 42011 but not bexarotene enrichment analysis. Conversely, the proteoglycans in cancer pathway, containing genes which code for matrix metalloproteinases (MMP), WNT signaling molecules, and

growth factors such as IGF1 and FGF2, is prominent in bexarotene differential expression analysis but not MSU 42011.

Immune response genes differentially expressed in tumors treated with RXR agonists, confirmed by qPCR and/or IHC, may be beneficial in the context of cancer treatment. The increase in *IL-18* in tumors treated with MSU 42011 suggests that this RXR agonist promotes a pro-inflammatory tumor microenvironment, which can be harnessed for breast cancer treatment. IL-18 has been investigated as a possible prognostic indicator in breast cancer patients[288] and augments the cytotoxicity of NK cells[289]. Further investigation into the mechanism of MSU 42011 is necessary to determine if IL-18 is a critical mediator of anti-tumor immune response, and if it can be used as an indicator of response to therapy. Furthermore, the increase in *H2-Aa* observed in MSU 42011-treated tumors provides further evidence of the immune modulatory effects of MSU 42011. H2-AA is an MHC class II component which is correlated with survival in ovarian cancer[290]. MHC II is responsible for antigen presentation to CD4+ T cells, which have more recently gained recognition for their roles in supporting the activation of cytotoxic T cells and mediating checkpoint inhibition response in cancer[291]. The MHC II pathway is involved in antitumor immunity in several cancer types and is upregulated by HDAC inhibitor treatment[292,293]. In triple negative breast cancer, high expression of genes associated with the MHC II pathway correlates with progression-free survival[294]. Pharmacologic means of augmenting MHC II signaling and component expression may be a valuable therapeutic strategy in enhancing anti-tumor immunity. The increase in expression in IL-18 and H2-AA seen in MSU 42011-

treated tumors but not bexarotene-treated tumors may provide insight into the unique immunomodulatory properties of these two RXR agonists.

While *COL6A3* expression has been explored as a prognostic biomarker in colorectal cancer[295], less is known about the role of COL6A3 in breast cancer. There is a trend of decreased *COL6A3* expression with increasing tumor stage in breast cancer patients[286], which suggests a propensity for invasion and metastasis in these tumors[296]. Further, increased expression of *COL6A3* in breast cancer after chemotherapy may predict for responsiveness to chemotherapy[297]. Finally, a cleavage fragment of COL6A3 known as endotrophin recruits macrophages through induction of monocyte chemoattractant protein-1 (MCP1) and increases IL-6 and TNFα in the tumor microenvironment[298]. Similarly, in obesity, collagen VI expression in omental white adipose tissue is correlated with expression of MCP-1, CD68, and CD86, providing further evidence that this collagen influences macrophage infiltration and phenotype[299]. As the role of COL6A3 is complex and can vary between cancer types and across tumor staging, the increase in expression of *Col6a3* in MSU 42011-treated tumors and resultant effect on invasion and immunity merits further investigation.

The expression of the microtubule-associated protein MAP9 is altered in both colorectal cancer and breast cancer, leading to cell cycle dysregulation[287]. *MAP9* hypermethylation in breast cancer leads to decreased expression and may have utility as an epigenetic biomarker[300]. Further, *MAP9* transcription is induced upon DNA damage, and MAP9 protein interacts with and stabilizes p53 in Sa-OS-2 cells, leading to increased tumor suppressor activity[301]. As expression of *Map9* is increased in tumors

treated with MSU 42011, an exploration of the effects of MSU 42011 on cell cycle control and the ways this may be exploited for therapeutic purposes is warranted. Based on MSU 42011's RNA Sequencing data, particularly differentially expressed genes and pathways relating to immunity, we investigated the effects of MSU 42011 treatment on gene expression in BMDMs. MSU 42011 induced a decrease in the expression of IL-13, an immunosuppressive cytokine, and an increase in expression of CCL6, a pro-inflammatory cytokine. Furthermore, treatment with MSU 42011 increased expression of TLR9 and IRF1, an interferon-regulatory factor known to be induced by ligation of TLR9. The TLR9-IRF1-IFN signaling axis has been implicated in macrophage polarization[302]. Taken together, this data provides supporting evidence that MSU 42011 skews macrophages away from a tumor-promoting, immunosuppressive phenotype, and toward an anti-tumor, proinflammatory phenotype. This effect on macrophages may be important for the anti-tumor activity of MSU 42011 and warrants further study.

In conclusion, RXR agonists are promising candidates for several types of cancer. Treatment with RXR agonists results in modulation of gene expression in ways that could be beneficial to cancer treatment. As a drug class, RXR agonists display a broad range of activities, regulating different genes and biological pathways. The diversity of these compounds may allow them to be utilized as targeted cancer therapy.

Acknowledgements

The authors thank the Investigative HistoPathology Laboratory at Michigan State University for paraffin embedding and sectioning of tissues for immunohistochemistry. RNA quality assessment on Agilent 2100 Bioanalyzer was done by the RTSF Genomics Core at Michigan State University. We thank Jonathan Diedrich and Omar Kana in the

Department of Pharmacology & Toxicology at Michigan State University for their assistance with coding in R. We thank Beth Lockwood, Joseph Jeong, Jessica Moerland, and Christopher Occhiuto for assistance with animal work. These studies were supported by the Breast Cancer Research Foundation (BCRF-20-096 to KL), a Targeted Support Grant for Technology Development (TSGTD to KL),.

Author Contributions

Conceptualization: L.A.R., A.S.L., E.E. and K.T.L.; Methodology: L.A.R., A.S.L., and K.T.L.; Conducting experiments: L.A.R., A.S.L.; Writing the original manuscript: L.A.R.; Review & editing the manuscript, L.A.R., A.S.L., E.E. and K.T.L; Funding acquisition, K.T.L.; Supervision K.T.L.

Competing Interest Statement

Patent applications covering the novel compounds described in this work have been applied for on behalf of Michigan State University; Ana S. Leal, Edmund Ellsworth, and Karen T. Liby are named inventors on the patent applications. Lyndsey A. Reich declares no competing interests.

Data Availability

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

CHAPTER 4

Conclusions and future directions

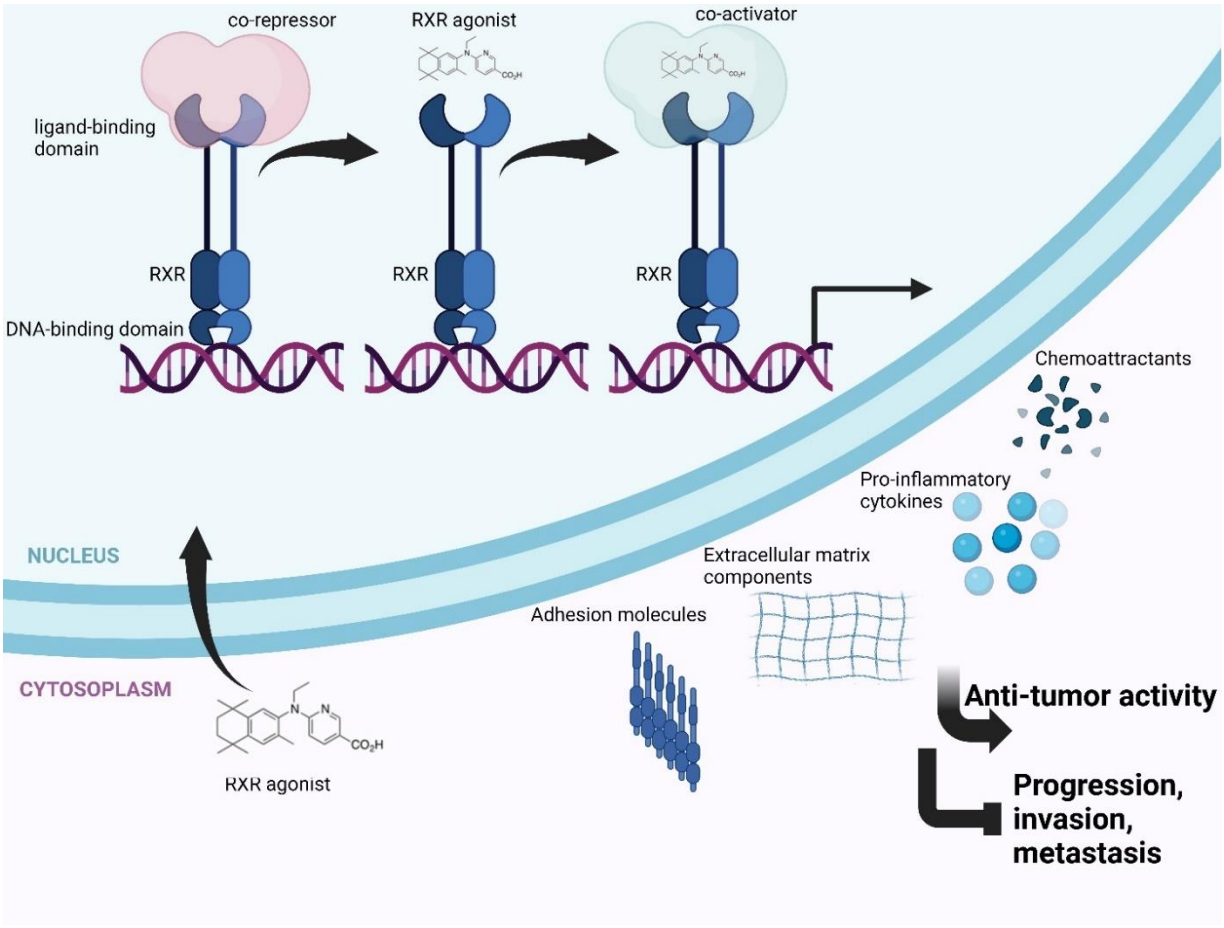

Figure 4.1. RXR agonists regulate cancer-relevant processes through control of gene expression. In the absence of ligand, the nuclear receptor RXR is bound to DNA, corepressors, and a partner nuclear receptor (homodimer or heterodimer). Upon binding of an RXR agonist, RXR recruits co-activators and initiates transcription of genes involved in cancer-relevant biological processes. These gene products include proinflammatory cytokines and chemoattractants for immune cells, as well as components like adhesion molecules and extracellular matrix proteins. Different RXR agonists result in different profiles of gene transcription in bulk tumors, necessitating further mechanistic studies.

4.1: Conclusions

In summary, this dissertation contributed to the field by investigating the efficacy of the new RXR agonist, V-125, in two mouse models, providing preliminary information about the mechanism of new RXR agonists, and using omics approaches to characterize the similarities and differences in gene expression in tumors treated with RXR agonists.

Chapter 2 describes the activity of the novel rexinoid V-125, which delayed tumor formation in the MMTV-Neu model of breast cancer and the A/J model of lung cancer and reduced tumor growth in a treatment study in the MMTV-Neu model[264]. The efficacy of V-125 in both models was significantly better than the only FDA-approved RXR agonist, bexarotene. Importantly, V-125 did not elevate triglycerides/cholesterol, a known adverse effect of bexarotene treatment.

Chapter 3 describes the effects of another novel RXR agonist, MSU 42011, on gene expression in MMTV-Neu tumors. Comparison of differentially regulated pathways with bexarotene-treated tumors revealed many common themes in gene expression altered by treatment with RXR agonists, such as focal adhesion and ECM-receptor interactions. Genes associated with these KEGG pathways, such as those that code for collagens, growth factors, integrins, and matrix proteins, participate in regulation of metastasis and invasion. Modulation of these pathways may have significant effects on cancer progression and metastasis. RNA Seq analysis also identified pathways unique to MSU 42011, such as rheumatoid arthritis and ribosomes.

Genes in the rheumatoid arthritis pathway differentially expressed in tumors treated with MSU 42011 include CXCL12, MMP3, IL-18, and genes coding for various MHC components. These genes code for cytokines and chemokines involved in immune regulation, providing further evidence that immunomodulation is a major component of the biological effects of RXR activation. More specifically, these signaling molecules play interesting roles in macrophage phenotype and function. CXCL12 is secreted by cancer cells, recruits monocytes, participates in macrophage polarization, and thereby affects tumor growth[303,304]. MMPs are secreted by macrophages in the tumor microenvironment, where they degrade ECM components and contribute to cancer progression and metastasis[305]. In addition to its relevance for cancer prognosis and treatment discussed in chapter 3 of this dissertation, IL-18 is secreted by activated macrophages, resulting in IFN secretion and regulation of angiogenesis[306]. Together, the genes in this pathway differentially regulated by MSU 42011 add to the collective evidence presented in previous Liby lab publications[105,265] that this RXR agonist promotes an anti-tumor phenotype in macrophages.

Another pathway differentially regulated by MSU 42011 is the ribosome pathway, which contains genes which regulate the synthesis of proteins. This pathway has been implicated in tumor progression and metastasis. In a study of metastasis in HER2+ breast cancer patients, RNA Sequencing of tumors from patients either with or without brain metastases yielded a list of differentially expressed pathways via KEGG differential expression analysis[285]. This list includes not only cell adhesion and extracellular matrix, but also plasma membrane components and ribosome[285]. The ribosome pathway in particular plays an important role in HER2+ breast cancer

pathogenesis, as tumor progression requires enhanced ribosomal component synthesis and protein translation[285,307]. Differential regulation of ribosomal synthesis is a feature of breast cancer cell lines with increased invasiveness[307]. As a pathway that is regulated by MSU 42011 treatment and correlates with tumor progression and invasion, this pathway may have significant implications for cancer treatment and may play a key role in mediating the anti-tumor effects of RXR agonists. To investigate these effects of MSU 42011, several methods exist to evaluate and quantify mRNA translation in treated versus untreated cells, including fluorescence in situ hybridization methods which visualize interactions between mRNA and ribosomes[308], fluorescent reporter assays[309], and novel single-cell microscopic imaging techniques[310]

We can utilize the data in chapters 2 and 3 to guide the development of novel RXR agonists - a critical area of research. In addition to cancer treatment, RXR agonists could be used to treat autoimmune and neurodegenerative diseases. Elucidating the mechanisms of RXR agonists, particularly the immunomodulatory effects, may lead to effective treatments for many diseases in which immune dysfunction plays an important role. RXR agonists are effective in preclinical models of obesity and type II diabetes, non-alcoholic fatty liver disease, Alzheimer's disease, intracerebral hemorrhage, rheumatoid arthritis, and inflammatory bowel disease[263]. To employ RXR agonists in other disease settings, a more comprehensive picture of the mechanism of action of RXR agonists would be useful.

4.2: Future Directions

4.2.1: Further synthesis of RXR agonists

This dissertation has documented the efficacy and safety of two novel RXR agonists, V-125 and MSU 42011. Building on existing effective RXR agonists, we can alter the structure of these compounds and use both *in vitro* assays and *in vivo* studies to determine which structural changes are most beneficial for our designated outcomes. For example, modifications to the ring of bexarotene which bears the carboxylic acid has yielded several compounds with varying potency and toxicity. Adding an electron withdrawing group to this ring, such as the nitrogen atom in the case of LG100268, increases compound potency, as does the cyclopropyl bridge of LG100268[311]. Adding groups which influence aromaticity of these compounds can alter pharmacological characteristics such as maximum serum concentration (C_{max}) in predictable ways based on structure-activity relationship (SAR) studies[311]. We can use *in vitro* assays to quickly identify characteristics of these newly synthesized compounds that produce relevant effects in preclinical models and in patients. For example, in the search for an RXR agonist with potent immunomodulatory activity, we can use the NO assay to identify compounds which reduce the secretion of nitric oxide from macrophage-like cells in response to an inflammatory stimulus. We can use a fluorescent binding assay to assess the compound's ability to bind to and activate RXR which is relevant to compound potency, and we can use qPCR to look at gene expression downstream of RXR. We also use the expression of SREBP in HepG2 cells as a surrogate for hypertriglyceridemia induced by these compounds, which is a known side effect of treatment with RXR agonists. This system of evaluating compounds *in vitro* is an

efficient means of identifying promising RXR agonist candidates, effectively expediting the development process. Using this platform, further synthesis and development of novel rexinoids could lead to even greater efficacy.

4.2.2: Mechanisms of action of RXR agonists

Immunomodulatory mechanisms

There is still much to be learned regarding the mechanism of RXR agonists in cancer models. Our previous publications on LG100268, MSU 42011 and V-125 have explored the immunomodulatory activity of these compounds, including but not limited to their effects on T cells. For example, the tool compound LG100268 increases the ratio of CD8/CD4+ activated T cells in MMTV-Neu mice, and reduces expression of Foxp3, altering the phenotype of T cells in the tumor microenvironment from immunosuppressive to pro-inflammatory[120]. Treatment with MSU 42011 in the A/J model in combination with carboplatin/paclitaxel increases infiltration of CD8+ T cells, and increases cell surface expression of the activation marker CD69 and the degranulation marker CD107a[105]. RNA Sequencing data on tumors treated with V-125 indicate that genes associated with the T cell receptor signaling pathway, such as JUN, PI3K signaling pathway members, TNF, RASGRP1, IL-4, and CD28, are differentially regulated by V-125. It has yet to be explored to what extent these effects on T cell function contribute to the anti-tumor activity of V-125.

We have also previously published on the effects of RXR agonists on macrophages in the tumor microenvironment. Treatment with MSU 42011 in combination with carboplatin/paclitaxel in the A/J model decreases CD206 expression on alveolar macrophages[105], suggesting a polarizing effect on macrophages away from

a tumor-promoting phenotype and towards a tumor-suppressive phenotype. As described in chapter 2 of this dissertation, 10 day-treatment with 100 mg/kg diet V-125 also decreased CD206 expression in MMTV-Neu tumors. Even if the number of macrophages in the tumor microenvironment or the expression of common macrophage cell surface markers are not altered, the phenotype and function of these cells may be affected – for example through the increase of phagocytic markers, and increased expression of survival genes[312]. The implementation of functional assays will help us determine the effects of RXR agonist treatment on macrophage activity, such as phagocytosis. Cancer cell phagocytosis assays can be performed using bright field microscopy or flow cytometry to evaluate this functional activity[313,314].

We have yet to determine the extent to which the effects on these immune cells contribute to the anti-tumor activity of RXR agonists. Immune cell depletion techniques can be used to answer this question. *In vivo* depletion antibodies directed against macrophage-specific markers such as CSF1R or depletion using liposomal clodronate would allow us to compare treatment efficacy of RXR agonists in wild-type animals versus macrophage-depleted animals.

Effects of nuclear receptor heterodimerization

While RXR agonists bind to and activate RXR, the biological nature of RXR requires either homodimerization or heterodimerization. As noted in chapter 3, the nuclear receptor binding partner of RXR will affect the gene transcription that occurs upon ligand binding. For example, RXR binding to PPAR results in transcription of genes such as PDK1 and malic enzyme gene (ME)[315]. RXR binding to LXR leads to transcription of genes including SREBP1c, FAS, and SCD1[316]. Nuclear receptor

screens, such as the Nuclear Hormone Receptor Panel offered by Eurofins[317] or Nuclear Receptor Profiling through Indigo Biosciences[318] would allow us to fully examine the complexity of receptor activation profiles and compare these profiles from one compound to the next. Furthermore, genetic knockout and/or pharmacologic inhibition of nuclear receptors may allow us to better attribute certain biological activity of an RXR agonist to activation of a particular nuclear receptor. In the case of LXR, while some of the adverse effects of RXR agonist treatment (hypertriglyceridemia and hypercholesterolemia) can be attributed to LXR activation, gene transcription downstream of this nuclear receptor may also contribute to the anti-tumor activity of RXR agonists. It is known that activation of LXR in macrophages contributes not only to metabolism of cholesterol, but also to cellular survival and function in innate immunity[319]. Evaluating the role of LXR activation in the mechanism of RXR agonists presents challenges, as whole-body KO of LXR in mice leads to significant morbidity, such as reduced fertility[320] and decreased myelination[25] and may interfere with study design. Furthermore, genetic KO of LXR may result in compensatory nuclear receptor activation and gene transcription. Therefore, the role of LXR in mediating the anti-tumor effects of RXR agonists should be evaluated using multiple experimental techniques. Tissue-specific knockout of LXR in the mammary gland may allow us to evaluate the role of LXR activation in the tumor microenvironment and how this contributes to RXR agonist efficacy. Co-treatment of a specific pharmacological antagonist of LXR such as GSK2033 and RXR agonist could also help us determine how much of the anti-tumor activity of rexinoids is mediated through LXR activation, and to what extent the immunomodulatory activity of rexinoids is dependent on LXR activation.

Signaling pathways involved in mediating RXR agonist activity

Many mechanistic questions have been raised by the RNA Seq data in chapter 3 of this dissertation and by the additional RNA Seq data in the appendix of this dissertation. Several signaling pathways which emerged from RNA Seq analysis merit further investigation, including the AGE-RAGE signaling pathway, cGMP-PKG signaling pathway, and the PI3K-Akt-mTOR pathway. The AGE-RAGE signaling pathway was identified in both V-125 vs control and bexarotene vs control analysis. This pathway, in which advanced glycation end products (AGE) bind to the receptor for advanced glycation end products (RAGE) and initiate a positive feedback loop which generates additional oxidative stress, is commonly associated with diabetes mellitus and other metabolic disorders. AGE-RAGE signaling has recently been implicated in cancer development and progression, which may provide a foundation for understanding the link between metabolic disorders and increased rates of cancer[321]. As we see differential expression of genes involves in flux through this pathway in tumors treated with RXR agonists, this may indicate a potential not only for cancer treatment, but for cancer prevention in high-risk populations, particularly patients with metabolic disorder.

4.2.3: Combination therapy

While we have seen striking single-agent efficacy in preclinical studies using RXR agonists, their use in combination therapies may further enhance anti-tumor activity. We have documented several instances in which treatment with RXR agonists in combination with chemotherapy provides increased efficacy over either treatment alone. Combination treatment using rexinoids and selective estrogen receptor modulators[182] or triterpenoids[68] enhance treatment efficacy in preclinical cancer models.

Combination treatment with MSU 42011 and anti-PD1 or MSU 42011 and anti-PD-L1 decreases tumor burden in the AJ model of lung cancer[265]. Combination treatment with LG100268 and anti-PD-L1 antibodies increases intratumoral infiltration of CD8+ T cells[120], contributing to increased cytotoxic anti-tumor activity. The increase in PD-L1 staining in MMTV-Neu tumors treated with V-125 by immunohistochemistry in chapter 2 of this dissertation may indicate a potential for synergistic anti-tumor activity in combination with anti-PD-L1 antibodies. Comprehensive evaluation of drug combinations featuring MSU 42011 and V-125 may uncover novel treatment combinations which lead to tumor prevention and/or regression.

REFERENCES

1 Zhang, D. *et al.* Testing Novel Pyrimidinyl Rexinoids: A New Paradigm for Evaluating Rexinoids for Cancer Prevention. *Cancer Prev Res* **12**, 211-224, doi:10.1158/1940-6207.Capr-18-0317 (2019).

2 Lánczky, A. & Győrffy, B. Web-Based Survival Analysis Tool Tailored for Medical Research (KMplot): Development and Implementation. *J. Med. Internet Res.* **23**, e27633, doi:10.2196/27633 (2021).

3 Ahuja, H., Szanto, A., Nagy, L. & Davies, P. J. The retinoid X receptor and its ligands: versatile regulators of metabolic function, cell differentiation and cell death. *J. Biol. Regul. Homeost. Agents* **17**, 29-45 (2003).

4 Bain, D. L., Heneghan, A. F., Connaghan-Jones, K. D. & Miura, M. T. Nuclear receptor structure: implications for function. *Annu. Rev. Physiol.* **69**, 201-220 (2007).

5 Vivat-Hannah, V., Bourguet, W., Gottardis, M. & Gronemeyer, H. Separation of retinoid X receptor homo-and heterodimerization functions. *Molecular and Cellular Biology* **23**, 7678-7688 (2003).

6 Sever, R. & Glass, C. K. Signaling by nuclear receptors. *Cold Spring Harb. Perspect. Biol.* **5**, a016709 (2013).

7 Cesario, R. M., Stone, J., Yen, W.-C., Bissonnette, R. P. & Lamph, W. W. Differentiation and growth inhibition mediated via the RXR: PPARγ heterodimer in colon cancer. *Cancer letters* **240**, 225-233 (2006).

8 Thompson, P. *et al.* Distinct retinoid X receptor activation function-2 residues mediate transactivation in homodimeric and vitamin D receptor heterodimeric contexts. *J. Mol. Endocrinol.* **27**, 211-227 (2001).

9 Altucci, L., Leibowitz, M. D., Ogilvie, K. M., de Lera, A. R. & Gronemeyer, H. RAR and RXR modulation in cancer and metabolic disease. *Nature reviews Drug discovery* **6**, 793-810 (2007).

10 Wagner, C. E. *et al.* Modeling, Synthesis and Biological Evaluation of Potential Retinoid X Receptor (RXR) Selective Agonists: Novel Analogues of 4-[1-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl]benzoic Acid (Bexarotene). *J Med Chem* **52**, 5950-5966, doi:10.1021/jm900496b (2009).

11 Evans, R. M. & Mangelsdorf, D. J. Nuclear Receptors, RXR, and the Big Bang. *Cell* **157**, 255-266, doi:10.1016/j.cell.2014.03.012 (2014).

12 Egea, P. F. *et al.* Crystal structure of the human RXRα ligand-binding domain

bound to its natural ligand: 9-cis retinoic acid. *The EMBO journal* **19**, 2592-2601 (2000).

13 Gampe, R. T. *et al.* Asymmetry in the PPARγ/RXRα Crystal Structure Reveals the Molecular Basis of Heterodimerization among Nuclear Receptors. *Mol. Cell* **5**, 545-555 (2000).

14 Bourguet, W. *et al.* Crystal structure of a heterodimeric complex of RAR and RXR ligand-binding domains. *Mol. Cell* **5**, 289-298 (2000).

15 Nahoum, V. *et al.* Modulators of the structural dynamics of the retinoid X receptor to reveal receptor function. *Proceedings of the National Academy of Sciences* **104**, 17323-17328 (2007).

16 Osz, J. *et al.* Structural basis of natural promoter recognition by the retinoid X nuclear receptor. *Scientific reports* **5**, 1-10 (2015).

17 Cao, X. *et al.* Retinoid X receptor regulates Nur77/TR3-dependent apoptosis [corrected] by modulating its nuclear export and mitochondrial targeting. *Molecular and cellular biology* **24**, 9705-9725 (2004).

18 Lin, X.-F. *et al.* RXRα acts as a carrier for TR3 nuclear export in a 9-cis retinoic acid-dependent manner in gastric cancer cells. *J. Cell Sci.* **117**, 5609-5621 (2004).

19 Gudas, L. J. & Wagner, J. A. Retinoids regulate stem cell differentiation. *J. Cell. Physiol.* **226**, 322-330 (2011).

20 Duester, G. in *Semin. Cell Dev. Biol.* 694-700 (Elsevier).

21 Delacroix, L. *et al.* Cell-specific interaction of retinoic acid receptors with target genes in mouse embryonic fibroblasts and embryonic stem cells. *Molecular and cellular biology* **30**, 231-244 (2010).

22 Kastner, P. *et al.* Genetic analysis of RXRα developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* **78**, 987-1003 (1994).

23 Sapin, V., Ward, S. J., Bronner, S., Chambon, P. & Dollé, P. Differential expression of transcripts encoding retinoid binding proteins and retinoic acid receptors during placentation of the mouse. *Developmental dynamics: an official publication of the American Association of Anatomists* **208**, 199-210 (1997).

24 Cermenati, G. *et al.* Liver X receptors, nervous system, and lipid metabolism. *J. Endocrinol. Invest.* **36**, 435-443 (2013).

25 Courtney, R. & Landreth, G. E. LXR regulation of brain cholesterol: from

development to disease. *Trends in Endocrinology & Metabolism* **27**, 404-414 (2016).

26 Wendling, O., Chambon, P. & Mark, M. Retinoid X receptors are essential for early mouse development and placentogenesis. *Proceedings of the National Academy of Sciences* **96**, 547-551 (1999).

27 Sucov, H. M. *et al.* RXR alpha mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes Dev.* **8**, 1007-1018 (1994).

28 Gruber, P. J. *et al.* RXR alpha deficiency confers genetic susceptibility for aortic sac, conotruncal, atrioventricular cushion, and ventricular muscle defects in mice. *The Journal of clinical investigation* **98**, 1332-1343 (1996).

29 Dyson, E. *et al.* Atrial-like phenotype is associated with embryonic ventricular failure in retinoid X receptor alpha-/-mice. *Proceedings of the National Academy of Sciences* **92**, 7386-7390 (1995).

30 Kastner, P. *et al.* Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development. *Development* **124**, 313-326 (1997).

31 Ruiz-Lozano, P. *et al.* Energy deprivation and a deficiency in downstream metabolic target genes during the onset of embryonic heart failure in RXRalpha−/− embryos. *Development* **125**, 533-544 (1998).

32 Ross, S. A., McCaffery, P. J., Drager, U. C. & De Luca, L. M. Retinoids in embryonal development. *Physiol. Rev.* **80**, 1021-1054 (2000).

33 Barak, Y. *et al.* PPARγ is required for placental, cardiac, and adipose tissue development. *Mol. Cell* **4**, 585-595 (1999).

34 Kastner, P. *et al.* Abnormal spermatogenesis in RXR beta mutant mice. *Genes Dev.* **10**, 80-92 (1996).

35 Krezel, W. *et al.* others. 1998. Impaired locomotion and dopamine signaling in retinoid receptor mutant mice. *Science* **279**, 864-867.

36 Szanto, A. *et al.* Transcriptional regulation of human CYP27 integrates retinoid, peroxisome proliferator-activated receptor, and liver X receptor signaling in macrophages. *Mol Cell Biol* **24**, 8154-8166, doi:10.1128/mcb.24.18.8154-8166.2004 (2004).

37 Imai, T., Jiang, M., Chambon, P. & Metzger, D. Impaired adipogenesis and lipolysis in the mouse upon selective ablation of the retinoid X receptor α mediated by a tamoxifen-inducible chimeric Cre recombinase (Cre-ERT2) in adipocytes.

Proceedings of the National Academy of Sciences **98**, 224-228 (2001).

38 Metzger, D. *et al.* Functional role of RXRs and PPARγ in mature adipocytes. *Prostaglandins, leukotrienes and essential fatty acids* **73**, 51-58 (2005).

39 Pathare, P. P., Lin, A., Bornfeldt, K. E., Taubert, S. & Van Gilst, M. R. Coordinate regulation of lipid metabolism by novel nuclear receptor partnerships. *PLoS Genet.* **8**, e1002645 (2012).

40 Wan, Y.-J. Y. *et al.* Peroxisome proliferator-activated receptor α-mediated pathways are altered in hepatocyte-specific retinoid X receptor α-deficient mice. *J. Biol. Chem.* **275**, 28285-28290 (2000).

41 Wan, Y.-J. Y. *et al.* Hepatocyte-specific mutation establishes retinoid X receptor α as a heterodimeric integrator of multiple physiological processes in the liver. *Molecular and cellular biology* **20**, 4436-4444 (2000).

42 Huang, J. *et al.* Prostatic intraepithelial neoplasia in mice with conditional disruption of the retinoid X receptor α allele in the prostate epithelium. *Cancer Research* **62**, 4812-4819 (2002).

43 Li, M. *et al.* Skin abnormalities generated by temporally controlled RXRα mutations in mouse epidermis. *Nature* **407**, 633-636, doi:10.1038/35036595 (2000).

44 Li, M. *et al.* RXR-alpha ablation in skin keratinocytes results in alopecia and epidermal alterations. *Development* **128**, 675-688, doi:10.1242/dev.128.5.675 (2001).

45 Gonzalez, H., Hagerling, C. & Werb, Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev.* **32**, 1267-1284, doi:10.1101/gad.314617.118 (2018).

46 Chaplin, D. D. Overview of the immune response. *Journal of Allergy and Clinical Immunology* **125**, S3-S23 (2010).

47 Gaudino, S. J. & Kumar, P. Cross-talk between antigen presenting cells and T cells impacts intestinal homeostasis, bacterial infections, and tumorigenesis. *Front. Immunol.* **10**, 360 (2019).

48 Speiser, D. E., Ho, P.-C. & Verdeil, G. Regulatory circuits of T cell function in cancer. *Nature Reviews Immunology* **16**, 599-611 (2016).

49 Du, X. *et al.* An essential role for RXRα in the development of Th2 responses. *Eur. J. Immunol.* **35**, 3414-3423 (2005).

50 Spilianakis, C. G., Lee, G. R. & Flavell, R. A. Twisting the Th1/Th2 immune

response via the retinoid X receptor: lessons from a genetic approach. *Eur. J. Immunol.* **35**, 3400-3404 (2005).

51 Stephensen, C. B., Borowsky, A. D. & Lloyd, K. C. Disruption of Rxra gene in thymocytes and T lymphocytes modestly alters lymphocyte frequencies, proliferation, survival and T helper type 1/type 2 balance. *Immunology* **121**, 484-498, doi:10.1111/j.1365-2567.2007.02595.x (2007).

52 Núñez, V. *et al.* Retinoid X receptor α controls innate inflammatory responses through the up-regulation of chemokine expression. *Proceedings of the National Academy of Sciences* **107**, 10626-10631 (2010).

53 Casanova-Acebes, M. *et al.* RXRs control serous macrophage neonatal expansion and identity and contribute to ovarian cancer progression. *Nature communications* **11**, 1-13 (2020).

54 Pérez-Martínez, D. Innate immunity in vertebrates: an overview. *Immunology* **148**, 125-139 (2016).

55 Rőszer, T., Menéndez-Gutiérrez, M. P., Cedenilla, M. & Ricote, M. Retinoid X receptors in macrophage biology. *Trends in Endocrinology & Metabolism* **24**, 460-468 (2013).

56 Thomas, M., Sukhai, M. A. & Kamel-Reid, S. An emerging role for retinoid X receptor α in malignant hematopoiesis. *Leukemia research* **36**, 1075-1081 (2012).

57 Kazawa, T. *et al.* Expression of liver X receptor α and lipid metabolism in granulocyte–macrophage colony-stimulating factor-induced human monocyte-derived macrophage. *Pathol. Int.* **59**, 152-160 (2009).

58 Safi, R. *et al.* Pharmacological manipulation of the RAR/RXR signaling pathway maintains the repopulating capacity of hematopoietic stem cells in culture. *Mol Endocrinol* **23**, 188-201, doi:10.1210/me.2008-0121 (2009).

59 Yunna, C., Mengru, H., Lei, W. & Weidong, C. Macrophage M1/M2 polarization. *Eur. J. Pharmacol.* **877**, 173090 (2020).

60 Daniel, B. *et al.* The nuclear receptor PPARγ controls progressive macrophage polarization as a ligand-insensitive epigenomic ratchet of transcriptional memory. *Immunity* **49**, 615-626. e616 (2018).

61 Huang, J. T. *et al.* Interleukin-4-dependent production of PPAR-γ ligands in macrophages by 12/15-lipoxygenase. *Nature* **400**, 378-382 (1999).

62 Söderström, M., Wigren, J., Surapureddi, S., Glass, C. K. & Hammarström, S. Novel prostaglandin D2-derived activators of peroxisome proliferator-activated

receptor-γ are formed in macrophage cell cultures. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1631**, 35-41 (2003).

63 Kiss, M., Czimmerer, Z. & Nagy, L. The role of lipid-activated nuclear receptors in shaping macrophage and dendritic cell function: From physiology to pathology. *Journal of Allergy and Clinical Immunology* **132**, 264-286 (2013).

64 Nagy, L., Szanto, A., Szatmari, I. & Széles, L. Nuclear hormone receptors enable macrophages and dendritic cells to sense their lipid environment and shape their immune response. *Physiol Rev* **92**, 739-789, doi:10.1152/physrev.00004.2011 (2012).

65 Roszer, T. *et al.* 681 Fischer, T, Ricote, M. 2011. Autoimmune kidney disease and impaired engulfment of apoptotic 682 cells in mice with macrophage peroxisome proliferator-activated receptor gamma or retinoid X 683 receptor alpha deficiency. *J. Immunol* **186**, 621-631.

66 Cramer, P. E. *et al.* ApoE-directed therapeutics rapidly clear β-amyloid and reverse deficits in AD mouse models. *Science* **335**, 1503-1506 (2012).

67 Liby, K. *et al.* Prevention and treatment of experimental estrogen receptor-negative mammary carcinogenesis by the synthetic triterpenoid CDDO-methyl Ester and the rexinoid LG100268. *Clin Cancer Res* **14**, 4556-4563, doi:10.1158/1078-0432.Ccr-08-0040 (2008).

68 Liby, K. *et al.* Triterpenoids CDDO-methyl ester or CDDO-ethyl amide and rexinoids LG100268 or NRX194204 for prevention and treatment of lung cancer in mice. *Cancer Prev Res* **2**, 1050-1058, doi:10.1158/1940-6207.Capr-09-0085 (2009).

69 Cao, M. *et al.* The Rexinoids LG100268 and LG101506 Inhibit Inflammation and Suppress Lung Carcinogenesis in A/J Mice. *Cancer Prev Res* **9**, 105-114, doi:10.1158/1940-6207.Capr-15-0325 (2016).

70 Liby, K. *et al.* A new rexinoid, NRX194204, prevents carcinogenesis in both the lung and mammary gland. *Clinical Cancer Research* **13**, 6237-6243, doi:10.1158/1078-0432.ccr-07-1342 (2007).

71 Kitagishi, Y., Kobayashi, M., Yamashina, Y. & Matsuda, S. Elucidating the regulation of T cell subsets. *Int. J. Mol. Med.* **30**, 1255-1260 (2012).

72 Colamatteo, A. *et al.* Molecular mechanisms controlling Foxp3 expression in health and autoimmunity: from epigenetic to post-translational regulation. *Front. Immunol.* **10**, 3136 (2020).

73 Josefowicz, S. Z., Lu, L.-F. & Rudensky, A. Y. Regulatory T cells: mechanisms of

differentiation and function. *Annu. Rev. Immunol.* **30**, 531 (2012).

74 Zhang, Q. *et al.* Aerosolized Bexarotene Inhibits Lung Tumorigenesis without Increasing Plasma Triglyceride and Cholesterol Levels in Mice. *Cancer Prevention Research* **4**, 270, doi:10.1158/1940-6207.CAPR-10-0246 (2011).

75 Smith, S. M. & Hayes, C. E. Contrasting impairments in IgM and IgG responses of vitamin A-deficient mice. *Proceedings of the National Academy of Sciences* **84**, 5878-5882 (1987).

76 Pino-Lagos, K., Benson, M. J. & Noelle, R. J. Retinoic acid in the immune system. *Ann. N. Y. Acad. Sci.* **1143**, 170-187 (2008).

77 Mark, M., Ghyselinck, N. B. & Chambon, P. Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. *Annu. Rev. Pharmacol. Toxicol.* **46**, 451-480 (2006).

78 Raverdeau, M. & Mills, K. H. Modulation of T cell and innate immune responses by retinoic acid. *The Journal of Immunology* **192**, 2953-2958 (2014).

79 Brown, C. C. & Noelle, R. J. Seeing through the dark: new insights into the immune regulatory functions of vitamin A. *Eur. J. Immunol.* **45**, 1287-1295 (2015).

80 Hall, J. A., Grainger, J. R., Spencer, S. P. & Belkaid, Y. The role of retinoic acid in tolerance and immunity. *Immunity* **35**, 13-22 (2011).

81 Al-Yassin, G. A. & Bretscher, P. A. Does T cell activation require a quorum of lymphocytes? *The Journal of Immunology* **201**, 2855-2861 (2018).

82 Rasooly, R. *et al.* Retinoid x receptor agonists increase bcl2a1 expression and decrease apoptosis of naive T lymphocytes. *The Journal of Immunology* **175**, 7916-7929 (2005).

83 Ishaq, M., Fan, M., Wigmore, K., Gaddam, A. & Natarajan, V. Regulation of Retinoid X Receptor Responsive Element-Dependent Transcription in T Lymphocytes by Ser/Thr Phosphatases: Functional Divergence of Protein Kinase C (PKC) θ and PKCα in Mediating Calcineurin-Induced Transactivation. *The Journal of Immunology* **169**, 732-738 (2002).

84 Ishaq, M., Fan, M. & Natarajan, V. Accumulation of RXRα during activation of cycling human T lymphocytes: modulation of RXRE transactivation function by mitogen-activated protein kinase pathways. *The Journal of Immunology* **165**, 4217-4225 (2000).

85 Ivanov, I. I. *et al.* Induction of intestinal Th17 cells by segmented filamentous

bacteria. *Cell* **139**, 485-498 (2009).

86 Takeuchi, H. *et al.* Efficient induction of CCR9 on T cells requires coactivation of retinoic acid receptors and retinoid X receptors (RXRs): exaggerated T Cell homing to the intestine by RXR activation with organotins. *The Journal of Immunology* **185**, 5289-5299 (2010).

87 Takeuchi, H. *et al.* Retinoid X receptor agonists modulate Foxp3+ regulatory T cell and Th17 cell differentiation with differential dependence on retinoic acid receptor activation. *The Journal of Immunology* **191**, 3725-3733 (2013).

88 Ohoka, Y., Yokota, A., Takeuchi, H., Maeda, N. & Iwata, M. Retinoic acid-induced CCR9 expression requires transient TCR stimulation and cooperativity between NFATc2 and the retinoic acid receptor/retinoid X receptor complex. *The Journal of Immunology* **186**, 733-744 (2011).

89 Macian, F. NFAT proteins: key regulators of T-cell development and function. *Nature Reviews Immunology* **5**, 472-484 (2005).

90 Banchereau, J. *et al.* Immunobiology of dendritic cells. *Annu. Rev. Immunol.* **18**, 767-811 (2000).

91 Geissmann, F. *et al.* Retinoids regulate survival and antigen presentation by immature dendritic cells. *The Journal of experimental medicine* **198**, 623-634 (2003).

92 Mohty, M. *et al.* All-trans retinoic acid skews monocyte differentiationinto interleukin-12-secreting dendritic-like cells. *Br. J. Haematol.* **122**, 829-836 (2003).

93 Zapata-Gonzalez, F. *et al.* 9-cis-Retinoic acid (9cRA), a retinoid X receptor (RXR) ligand, exerts immunosuppressive effects on dendritic cells by RXR-dependent activation: inhibition of peroxisome proliferator-activated receptor γ blocks some of the 9cRA activities, and precludes them to mature phenotype development. *The Journal of Immunology* **178**, 6130-6139 (2007).

94 E Wagner, C., W Jurutka, P., A Marshall, P. & C Heck, M. Retinoid X receptor selective agonists and their synthetic methods. *Curr. Top. Med. Chem.* **17**, 742-767 (2017).

95 de Almeida, N. R. & Conda-Sheridan, M. A review of the molecular design and biological activities of RXR agonists. *Med Res Rev.* **39**, 1372-1397 (2019).

96 Michellys, P. Y. *et al.* Design and synthesis of novel RXR-selective modulators with improved pharmacological profile. *Bioorg Med Chem Lett* **13**, 4071-4075, doi:10.1016/j.bmcl.2003.08.048 (2003).

97 Boehm, M. F. *et al.* Synthesis and structure-activity relationships of novel retinoid X receptor-selective retinoids. *Journal of medicinal chemistry* **37**, 2930-2941 (1994).

98 Vedell, P. T. *et al.* Effects on gene expression in rat liver after administration of RXR agonists: UAB30, 4-methyl-UAB30, and Targretin (Bexarotene). *Mol. Pharmacol.* **83**, 698-708 (2013).

99 Nakayama, M. *et al.* Discovery of a potent retinoid X receptor antagonist structurally closely related to RXR agonist NEt-3IB. *ACS Med. Chem. Lett.* **2**, 896-900 (2011).

100 T Liby, K. & B Sporn, M. Rexinoids for prevention and treatment of cancer: opportunities and challenges. *Curr. Top. Med. Chem.* **17**, 721-730 (2017).

101 Boehm, M. F. *et al.* Design and synthesis of potent retinoid X receptor selective ligands that induce apoptosis in leukemia cells. *Journal of medicinal chemistry* **38**, 3146-3155 (1995).

102 Pérez, E., Bourguet, W., Gronemeyer, H. & de Lera, A. R. Modulation of RXR function through ligand design. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1821**, 57-69 (2012).

103 Germain, P., Iyer, J., Zechel, C. & Gronemeyer, H. Co-regulator recruitment and the mechanism of retinoic acid receptor synergy. *Nature* **415**, 187-192 (2002).

104 Dominguez, M., Alvarez, S. & R de Lera, A. Natural and structure-based RXR ligand scaffolds and their functions. *Curr. Top. Med. Chem.* **17**, 631-662 (2017).

105 Moerland, J. A. *et al.* The novel rexinoid MSU-42011 is effective for the treatment of preclinical Kras-driven lung cancer. *Sci Rep* **10**, 22244 (2020).

106 Kawata, K. *et al.* RXR partial agonist produced by side chain repositioning of alkoxy RXR full agonist retains antitype 2 diabetes activity without the adverse effects. *Journal of medicinal chemistry* **58**, 912-926 (2015).

107 Ohsawa, F. *et al.* Mechanism of retinoid X receptor partial agonistic action of 1-(3, 5, 5, 8, 8-pentamethyl-5, 6, 7, 8-tetrahydro-2-naphthyl)-1 H-benzotriazole-5-carboxylic acid and structural development to increase potency. *Journal of medicinal chemistry* **56**, 1865-1877 (2013).

108 Kobayashi, T. *et al.* Positron emission tomography to elucidate pharmacokinetic differences of regioisomeric retinoid x receptor agonists. *ACS Med. Chem. Lett.* **6**, 334-338 (2015).

109 Takamatsu, K. *et al.* The first potent subtype-selective retinoid X receptor (RXR)

agonist possessing a 3-isopropoxy-4-isopropylphenylamino moiety, NEt-3IP (RXRα/β-dual agonist). *ChemMedChem: Chemistry Enabling Drug Discovery* **3**, 780-787 (2008).

110 Querfeld, C., Nagelli, L. V., Rosen, S. T., Kuzel, T. M. & Guitart, J. Bexarotene in the treatment of cutaneous T-cell lymphoma. *Expert Opin. Pharmacother.* **7**, 907-915 (2006).

111 Crunkhorn, S. RXR agonist reverses Alzheimer's disease. *Nature Reviews Drug Discovery* **11**, 271-271 (2012).

112 McFarland, K. *et al.* Low dose bexarotene treatment rescues dopamine neurons and restores behavioral function in models of Parkinson's disease. *ACS chemical neuroscience* **4**, 1430-1438 (2013).

113 Onuki, M. *et al.* A partial agonist for retinoid X receptor mitigates experimental colitis. *Int. Immunol.* **31**, 251-262 (2019).

114 D Muccio, D. *et al.* Translation of a tissue-selective rexinoid, UAB30, to the clinic for breast cancer prevention. *Curr. Top. Med. Chem.* **17**, 676-695 (2017).

115 Park, M.-C., Kwon, Y.-J., Chung, S.-J., Park, Y.-B. & Lee, S.-K. Liver X receptor agonist prevents the evolution of collagen-induced arthritis in mice. *Rheumatology* **49**, 882-890 (2010).

116 Boggild, A. K. *et al.* Use of peroxisome proliferator-activated receptor γ agonists as adjunctive treatment for Plasmodium falciparum malaria: a randomized, double-blind, placebo-controlled trial. *Clin. Infect. Dis.* **49**, 841-849 (2009).

117 Lewis, J. D. *et al.* Rosiglitazone for active ulcerative colitis: a randomized placebo-controlled trial. *Gastroenterology* **134**, 688-695 (2008).

118 Richards, D. B., Bareille, P., Lindo, E. L., Quinn, D. & Farrow, S. N. Treatment with a peroxisomal proliferator activated receptor gamma agonist has a modest effect in the allergen challenge model in asthma: a randomised controlled trial. *Respir. Med.* **104**, 668-674 (2010).

119 Cheng, H. S. *et al.* Exploration and development of PPAR modulators in health and disease: an update of clinical evidence. *Int. J. Mol. Sci.* **20**, 5055 (2019).

120 Leal, A. S. *et al.* Retinoid X receptor agonist LG100268 modulates the immune microenvironment in preclinical breast cancer models. *npj Breast Cancer* **5**, 39, doi:10.1038/s41523-019-0135-5 (2019).

121 Vitale, S. G. *et al.* Peroxisome proliferator-activated receptor modulation during metabolic diseases and cancers: master and minions. *PPAR research* **2016**

(2016).

122 Evans, R. M., Barish, G. D. & Wang, Y.-X. PPARs and the complex journey to obesity. *Nat. Med.* **10**, 355-361 (2004).

123 Borgeraas, H. *et al.* Association of time of obesity onset with comorbidities in treatment-seeking men and women with severe obesity. *Obesity science & practice* **4**, 427-436 (2018).

124 Mukherjee, R. *et al.* Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. *Nature* **386**, 407-410 (1997).

125 Pinaire, J. A. & Reifel-Miller, A. Therapeutic potential of retinoid x receptor modulators for the treatment of the metabolic syndrome. *PPAR research* **2007** (2007).

126 Liu, Y. *et al.* Retinoid X receptor agonists have anti-obesity effects and improve insulin sensitivity in Zucker fa/fa rats. *International journal of obesity* **24**, 997-1004 (2000).

127 Chen, H. *et al.* Cevoglitazar, a novel peroxisome proliferator-activated receptor-α/γ dual agonist, potently reduces food intake and body weight in obese mice and cynomolgus monkeys. *Endocrinology* **151**, 3115-3124 (2010).

128 Cesario, R. M. *et al.* The rexinoid LG100754 is a novel RXR: PPARγ agonist and decreases glucose levels in vivo. *Molecular Endocrinology* **15**, 1360-1369 (2001).

129 Forman, B. M. The Antidiabetic Agent LG100754 Sensitizes Cells to Low Concentrations of Peroxisome Proliferator-activated Receptor γ Ligands∗. *J. Biol. Chem.* **277**, 12503-12506 (2002).

130 Lenhard, J. *et al.* The RXR agonist LG100268 causes hepatomegaly, improves glycaemic control and decreases cardiovascular risk and cachexia in diabetic mice suffering from pancreatic beta-cell dysfunction. *Diabetologia* **42**, 545-554 (1999).

131 Li, Y., Jadhav, K. & Zhang, Y. Bile acid receptors in non-alcoholic fatty liver disease. *Biochem. Pharmacol.* **86**, 1517-1524 (2013).

132 Kong, B., Luyendyk, J. P., Tawfik, O. & Guo, G. L. Farnesoid X receptor deficiency induces nonalcoholic steatohepatitis in low-density lipoprotein receptor-knockout mice fed a high-fat diet. *Journal of Pharmacology and Experimental Therapeutics* **328**, 116-122 (2009).

133 Zhang, S., Wang, J., Liu, Q. & Harnish, D. C. Farnesoid X receptor agonist WAY-362450 attenuates liver inflammation and fibrosis in murine model of non-alcoholic steatohepatitis. *J. Hepatol.* **51**, 380-388 (2009).

134 Staels, B. *et al.* Hepatoprotective effects of the dual peroxisome proliferator-activated receptor alpha/delta agonist, GFT505, in rodent models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *Hepatology* **58**, 1941-1952 (2013).

135 Pawlak, M., Lefebvre, P. & Staels, B. Molecular mechanism of PPARα action and its impact on lipid metabolism, inflammation and fibrosis in non-alcoholic fatty liver disease. *J. Hepatol.* **62**, 720-733 (2015).

136 Lontchi-Yimagou, E., Sobngwi, E., Matsha, T. E. & Kengne, A. P. Diabetes mellitus and inflammation. *Curr. Diab. Rep.* **13**, 435-444 (2013).

137 P Koster, K. *et al.* Rexinoids as therapeutics for Alzheimer's disease: role of APOE. *Curr. Top. Med. Chem.* **17**, 708-720 (2017).

138 Cummings, J. L. *et al.* Double-blind, placebo-controlled, proof-of-concept trial of bexarotene in moderate Alzheimer's disease. *Alzheimers Res. Ther.* **8**, 1-9 (2016).

139 Yuan, C. *et al.* OAB-14, a bexarotene derivative, improves Alzheimer's disease-related pathologies and cognitive impairments by increasing β-amyloid clearance in APP/PS1 mice. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* **1865**, 161-180 (2019).

140 Bomfim, T. R. *et al.* An anti-diabetes agent protects the mouse brain from defective insulin signaling caused by Alzheimer's disease–associated Aβ oligomers. *The Journal of clinical investigation* **122**, 1339-1353 (2012).

141 Talbot, K. *et al.* Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *The Journal of clinical investigation* **122**, 1316-1338 (2012).

142 Pieper, A. A. *et al.* Discovery of a proneurogenic, neuroprotective chemical. *Cell* **142**, 39-51 (2010).

143 MacMillan, K. S. *et al.* Development of proneurogenic, neuroprotective small molecules. *J. Am. Chem. Soc.* **133**, 1428-1437 (2011).

144 Muñoz-Cabrera, J. M. *et al.* Bexarotene therapy ameliorates behavioral deficits and induces functional and molecular changes in very-old Triple Transgenic Mice model of Alzheimer´ s disease. *PLoS One* **14**, e0223578 (2019).

145 Loppi, S. *et al.* HX600, a synthetic agonist for RXR-Nurr1 heterodimer complex, prevents ischemia-induced neuronal damage. *Brain, behavior, and immunity* **73**, 670-681 (2018).

146 Zuo, Y. *et al.* Activation of retinoid X receptor by bexarotene attenuates neuroinflammation via PPARγ/SIRT6/FoxO3a pathway after subarachnoid

hemorrhage in rats. *J. Neuroinflammation* **16**, 1-15 (2019).

147 Tu, L. *et al.* Bexarotene attenuates early brain injury via inhibiting micoglia activation through PPARγ after experimental subarachnoid hemorrhage. *Neurol. Res.* **40**, 702-708 (2018).

148 Chang, C.-F., Massey, J., Osherov, A., Angenendt da Costa, L. H. & Sansing, L. H. Bexarotene enhances macrophage erythrophagocytosis and hematoma clearance in experimental intracerebral hemorrhage. *Stroke* **51**, 612-618 (2020).

149 Savage, J. C. *et al.* Nuclear receptors license phagocytosis by trem2+ myeloid cells in mouse models of Alzheimer's disease. *J. Neurosci.* **35**, 6532-6543 (2015).

150 Ting, S.-M. *et al.* Brain cleanup as a potential target for poststroke recovery: the role of RXR (retinoic X receptor) in phagocytes. *Stroke* **51**, 958-966 (2020).

151 Xu, J., Storer, P. D., Chavis, J. A., Racke, M. K. & Drew, P. D. Agonists for the peroxisome proliferator-activated receptor-α and the retinoid X receptor inhibit inflammatory responses of microglia. *J. Neurosci. Res.* **81**, 403-411 (2005).

152 Yuan, C. *et al.* Bexarotene improve depression-like behaviour in mice by protecting against neuro-inflammation and synaptic damage. *Neurochem. Res.* **45**, 1500-1509 (2020).

153 He, J. *et al.* Bexarotene protects against neurotoxicity partially through a PPARγ-dependent mechanism in mice following traumatic brain injury. *Neurobiol. Dis.* **117**, 114-124 (2018).

154 He, J. *et al.* Bexarotene promotes microglia/macrophages-specific brain-derived neurotrophic factor expression and axon sprouting after traumatic brain injury. *Exp. Neurol.* **334**, 113462 (2020).

155 Diab, A. *et al.* Ligands for the peroxisome proliferator-activated receptor-γ and the retinoid X receptor exert additive anti-inflammatory effects on experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **148**, 116-126 (2004).

156 Zhong, J. *et al.* Bexarotene protects against traumatic brain injury in mice partially through apolipoprotein E. *Neuroscience* **343**, 434-448 (2017).

157 Beehler, B. C. *et al.* Inhibition of disease progression by a novel retinoid antagonist in animal models of arthritis. *The Journal of Rheumatology* **30**, 355-363 (2003).

158 Brinckerhoff, C. E., Coffey, J. W. & Sullivan, A. C. Inflammation and collagenase production in rats with adjuvant arthritis reduced with 13-cis-retinoic acid. *Science* **221**, 756-758, doi:10.1126/science.6308759 (1983).

159 Haraoui B Fau - Wilder, R. L. *et al.* Dose-dependent suppression by the synthetic retinoid, 4-hydroxyphenyl retinamide, of streptococcal cell wall-induced arthritis in rats.

160 Li, Y. *et al.* Activation of RXR by bexarotene inhibits inflammatory conditions in human rheumatoid arthritis fibroblast-like synoviocytes. *Int J Mol Med* **44**, 1963-1970, doi:10.3892/ijmm.2019.4336 (2019).

161 Gravallese, E. M. *et al.* N-[4-hydroxyphenyl] retinamide in rheumatoid arthritis: a pilot study.

162 Brinckerhoff, C. E. & Sporn, M. B. Retinoids and rexinoids for the 21st century: a brave new world for arthritis. *The Journal of Rheumatology* **30**, 211-213 (2003).

163 Desreumaux, P. *et al.* Attenuation of Colon Inflammation through Activators of the Retinoid X Receptor (Rxr)/Peroxisome Proliferator–Activated Receptor γ (Pparγ) Heterodimer: A Basis for New Therapeutic Strategies. *Journal of Experimental Medicine* **193**, 827-838, doi:10.1084/jem.193.7.827 (2001).

164 Fujii, U. *et al.* Effect of a retinoid X receptor partial agonist on airway inflammation and hyperresponsiveness in a murine model of asthma.

165 Malemud, C. J. Defective T-Cell Apoptosis and T-Regulatory Cell Dysfunction in Rheumatoid Arthritis. *Cells* **7**, 223, doi:10.3390/cells7120223 (2018).

166 Neuman, M. G. Immune dysfunction in inflammatory bowel disease. *Translational Research* **149**, 173-186 (2007).

167 Gersemann, M., Wehkamp, J. & Stange, E. F. Innate immune dysfunction in inflammatory bowel disease. *Journal of Internal Medicine* **271**, 421-428, doi:10.1111/j.1365-2796.2012.02515.x (2012).

168 Yang, X., Chang, Y. & Wei, W. Endothelial Dysfunction and Inflammation: Immunity in Rheumatoid Arthritis. *Mediators of Inflammation* **2016**, 6813016, doi:10.1155/2016/6813016 (2016).

169 Chandraratna, R. A., Noelle, R. J. & Nowak, E. C. Treatment with retinoid X receptor agonist IRX4204 ameliorates experimental autoimmune encephalomyelitis. *Am J Transl Res* **8**, 1016-1026 (2016).

170 Uray, I. P., Dmitrovsky, E. & Brown, P. H. Retinoids and rexinoids in cancer prevention: from laboratory to clinic. *Semin. Oncol.* **43**, 49-64 (2016).

171 Tanaka, T. & De Luca, L. M. Therapeutic potential of “rexinoids” in cancer prevention and treatment. *Cancer research* **69**, 4945-4947 (2009).

172 Brtko, J. & Dvorak, Z. Natural and synthetic retinoid X receptor ligands and their role in selected nuclear receptor action. *Biochimie* **179**, 157-168 (2020).

173 Bischoff, E. D., Gottardis, M. M., Moon, T. E., Heyman, R. A. & Lamph, W. W. Beyond tamoxifen: the retinoid X receptor-selective ligand LGD1069 (TARGRETIN) causes complete regression of mammary carcinoma. *Cancer research* **58**, 479-484 (1998).

174 Agarwal, V. R., Bischoff, E. D., Hermann, T. & Lamph, W. W. Induction of adipocyte-specific gene expression is correlated with mammary tumor regression by the retinoid X receptor-ligand LGD1069 (targretin). *Cancer research* **60**, 6033-6038 (2000).

175 Bischoff, E. D., Heyman, R. A. & Lamph, W. W. Effect of the retinoid X receptor-selective ligand LGD1069 on mammary carcinoma after tamoxifen failure. *J. Natl. Cancer Inst.* **91**, 2118-2118 (1999).

176 Lubet, R. A. *et al.* Efficacy of Targretin on methylnitrosourea-induced mammary cancers: prevention and therapy dose–response curves and effects on proliferation and apoptosis. *Carcinogenesis* **26**, 441-448 (2005).

177 Yen, W.-c., Prudente, R. Y. & Lamph, W. W. Synergistic effect of a retinoid X receptor-selective ligand bexarotene (LGD1069, Targretin) and paclitaxel (Taxol) in mammary carcinoma. *Breast cancer research and treatment* **88**, 141-148 (2004).

178 Suh, N. *et al.* Prevention and treatment of experimental breast cancer with the combination of a new selective estrogen receptor modulator, arzoxifene, and a new rexinoid, LG 100268. *Clinical Cancer Research* **8**, 3270-3275 (2002).

179 Rendi, M. H. *et al.* The selective estrogen receptor modulator arzoxifene and the rexinoid LG100268 cooperate to promote transforming growth factor beta-dependent apoptosis in breast cancer. *Cancer Res* **64**, 3566-3571, doi:10.1158/0008-5472.Can-04-0234 (2004).

180 Hsu, W.-D., Lin, S.-L., Wu, F.-L., Chiang, C.-K. & Wu, K.-D. Topical antifungal treatment cures exit-site fungal infection. *Am. J. Kidney Dis.* **40**, e15. 11-e15. 12 (2002).

181 Wu, K. *et al.* Suppression of mammary tumorigenesis in transgenic mice by the RXR-selective retinoid, LGD1069. *Cancer Epidemiology Biomarkers & Prevention* **11**, 467-474 (2002).

182 Liby, K. *et al.* The Combination of the Rexinoid, LG100268, and a Selective Estrogen Receptor Modulator, Either Arzoxifene or Acolbifene, Synergizes in the Prevention and Treatment of Mammary Tumors in an Estrogen Receptor–Negative

Model of Breast Cancer. *Clin Cancer Res.* **12**, 5902-5909, doi:10.1158/1078-0432.Ccr-06-1119 (2006).

183 Yen, W.-C. & Lamph, W. W. The selective retinoid X receptor agonist bexarotene (LGD1069, Targretin) prevents and overcomes multidrug resistance in advanced breast carcinoma. *Molecular cancer therapeutics* **4**, 824-834 (2005).

184 Li, Y. *et al.* The rexinoid, bexarotene, prevents the development of premalignant lesions in MMTV-erbB2 mice. *British journal of cancer* **98**, 1380-1388 (2008).

185 Liby, K. *et al.* The rexinoid LG100268 and the synthetic triterpenoid CDDO-methyl amide are more potent than erlotinib for prevention of mouse lung carcinogenesis. *Molecular cancer therapeutics* **7**, 1251-1257, doi:10.1158/1535-7163.MCT-08-0023 (2008).

186 Grigg, C. & Rizvi, N. A. PD-L1 biomarker testing for non-small cell lung cancer: truth or fiction? *Journal for immunotherapy of cancer* **4**, 1-10 (2016).

187 van der Zanden, S. Y., Luimstra, J. J., Neefjes, J., Borst, J. & Ovaa, H. Opportunities for small molecules in cancer immunotherapy. *Trends Immunol.* **41**, 493-511 (2020).

188 Stanton, S. E., Gad, E., Corulli-Rastetter, L. R. & Disis, M. L. Bexarotene activates type 1 antigen presenting cells, increases tumor infiltrating CD8 T-cells, and augments the anti-tumor activity of chemotherapy in breast cancer. *Cancer Research* **77**, 3687-3687 (2017).

189 Knol, A. C. *et al.* Absence of modulation of CD4+CD25high regulatory T cells in CTCL patients treated with bexarotene. *Experimental Dermatology* **19**, e95-e102, doi:10.1111/j.1600-0625.2009.00993.x (2010).

190 Quail, D. F. & Joyce, J. A. Microenvironmental regulation of tumor progression and metastasis. *Nat. Med.* **19**, 1423-1437 (2013).

191 Kiss, M. *et al.* Retinoid X receptor suppresses a metastasis-promoting transcriptional program in myeloid cells via a ligand-insensitive mechanism. *Proceedings of the National Academy of Sciences* **114**, 10725-10730 (2017).

192 Waldman, A. D., Fritz, J. M. & Lenardo, M. J. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nature Reviews Immunology* **20**, 651-668 (2020).

193 Mangelsdorf, D. J., Ong Es Fau - Dyck, J. A., Dyck Ja Fau - Evans, R. M. & Evans, R. M. Nuclear receptor that identifies a novel retinoic acid response pathway. (1990).

194 Castaigne, S. *et al.* All-trans retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results.

195 Huang, M. E. *et al.* Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia.

196 Koch, H. F. Biochemical treatment of precancerous oral lesions: The effectiveness of various analogues of retinoic acid. *Journal of Maxillofacial Surgery* **6**, 59-63 (1978).

197 Minucci, S. *et al.* Retinoid X receptor (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid-dependent gene expression. *Molecular and cellular biology* **17**, 644-655, doi:10.1128/mcb.17.2.644 (1997).

198 Miller, V. A. *et al.* Initial clinical trial of a selective retinoid X receptor ligand, LGD1069. *J Clin Oncol.* **15**, 790-795, doi:10.1200/JCO.1997.15.2.790 (1997).

199 Budgin, J. B. *et al.* Biological Effects of Bexarotene in Cutaneous T-Cell Lymphoma. *Archives of Dermatology* **141**, 315-320, doi:10.1001/archpedi.161.4.356 (2005).

200 Duvic, M. *et al.* Phase 2 and 3 clinical trial of oral bexarotene (Targretin capsules) for the treatment of refractory or persistent early-stage cutaneous T-cell lymphoma. *Archives of dermatology* **137**, 581-593, doi:10.1099/ijs.0.63491-0 (2001).

201 Duvic, M. *et al.* Bexarotene is effective and safe for treatment of refractory advanced-stage cutaneous t-cell lymphoma: Multinational phase II-III trial results. *J Clin Oncol.* **19**, 2456-2471, doi:10.1200/JCO.2001.19.9.2456 (2001).

202 Hurst, R. E. Bexarotene ligand pharmaceuticals. *Curr Opin Investig Drugs* **1**, 514-523 (2000).

203 Benson, M. J., Pino-Lagos K Fau - Rosemblatt, M., Rosemblatt M Fau - Noelle, R. J. & Noelle, R. J. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation.

204 Wang, L. *et al.* RARα/RXR synergism potentiates retinoid responsiveness in cutaneous T-cell lymphoma cell lines. *Experimental dermatology* **26**, 1004-1011, doi:10.1111/exd.13348 (2017).

205 Esteva, F. J. *et al.* Multicenter phase II study of oral bexarotene for patients with metastatic breast cancer. *J Clin Oncol.* **21**, 999-1006, doi:10.1200/JCO.2003.05.068 (2003).

206 Brown, P. *et al.* Abstract CN04-04: Phase II trial of bexarotene in women at high risk of breast cancer: Comparison of protein and RNA biomarkers. *Cancer*

Prevention Research **1**, CN04-04 LP - CN04-04, doi:10.1158/1940-6207.PREV-08-CN04-04 (2008).

207 Thomas, P. *et al.* Abstract OT2-09-02: A phase I dose escalation study of topical bexarotene in women at high risk for breast cancer. *Cancer Research* **79**, OT2-09-02-OT02-09-02 (2019).

208 Dragnev, K. H. *et al.* A Proof-of-Principle Clinical Trial of Bexarotene in Patients with Non–Small Cell Lung Cancer. *Clinical Cancer Research* **13**, 1794 LP-1800, doi:10.1158/1078-0432.CCR-06-1836 (2007).

209 Govindan, R. *et al.* Phase II trial of bexarotene capsules in patients with advanced non–small-cell lung cancer after failure of two or more previous therapies. *Journal of clinical oncology* **24**, 4848-4854 (2006).

210 Rizvi, N., Hawkins, M. J., Eisenberg, P. D., Yocum, R. C. & Reich, S. D. Placebo-Controlled Trial of Bexarotene, a Retinoid X Receptor Agonist, as Maintenance Therapy for Patients Treated with Chemotherapy for Advanced Non–Small-Cell Lung Cancer. *Clinical Lung Cancer* **2**, 210-215, doi:10.3816/CLC.2001.N.005 (2001).

211 Khuri, F. R. *et al.* Multi-Institutional Phase I/II Trial of Oral Bexarotene in Combination With Cisplatin and Vinorelbine in Previously Untreated Patients With Advanced Non–Small-Cell Lung Cancer. *J Clin Oncol.* **19**, 2626-2637, doi:10.1200/JCO.2001.19.10.2626 (2001).

212 Roszkowski, K. *et al.* A multicenter, randomized, phase III study of docetaxel plus best supportive care versus best supportive care in chemotherapy-naive patients with metastatic or non-resectable localized non-small cell lung cancer (NSCLC).

213 Jassem, J. *et al.* A randomized phase III trial comparing bexarotene/cisplatin/vinorelbine versus cisplatin/vinorelbine in chemotherapy-naive patients with advanced or metastatic non-small cell lung cancer (NSCLC). *Journal of Clinical Oncology* **23**, LBA7024-LBA7024 (2005).

214 Blumenschein, G. R. *et al.* Phase III Trial Comparing Carboplatin, Paclitaxel, and Bexarotene With Carboplatin and Paclitaxel in Chemotherapy-Naïve Patients With Advanced or Metastatic Non–Small-Cell Lung Cancer: SPIRIT II. *Journal of Clinical Oncology* **26**, 1879-1885, doi:10.1200/JCO.2007.12.2689 (2008).

215 Wood, K., Hensing, T., Malik, R. & Salgia, R. Prognostic and Predictive Value in KRAS in Non-Small-Cell Lung Cancer: A Review. *JAMA Oncol* **2**, 805-812, doi:10.1001/jamaoncol.2016.0405 (2016).

216 Dragnev, K. H. *et al.* Bexarotene plus erlotinib suppress lung carcinogenesis independent of KRAS mutations in two clinical trials and transgenic models.

Cancer Prevention Research **4**, 818-828, doi:10.1158/1940-6207.CAPR-10-0376 (2011).

217 Shien, K., Papadimitrakopoulou, V. A. & Wistuba, II. Predictive biomarkers of response to PD-1/PD-L1 immune checkpoint inhibitors in non-small cell lung cancer.

218 Solinas, C. *et al.* Targeting immune checkpoints in breast cancer: an update of early results.

219 Münz, C., Lünemann, J. D., Getts, M. T. & Miller, S. D. Antiviral immune responses: triggers of or triggered by autoimmunity? *Nature Reviews Immunology* **9**, 246-258 (2009).

220 Tisoncik, J. R. *et al.* Into the eye of the cytokine storm. *Microbiology and Molecular Biology Reviews* **76**, 16-32 (2012).

221 Hu, B., Huang, S. & Yin, L. The cytokine storm and COVID-19. *J. Med. Virol.* **93**, 250-256 (2021).

222 Orienti, I., Gentilomi, G. A. & Farruggia, G. Pulmonary delivery of fenretinide: a possible adjuvant treatment in COVID-19. *Int. J. Mol. Sci.* **21**, 3812 (2020).

223 Tan, X., Sande, J. L., Pufnock, J. S., Blattman, J. N. & Greenberg, P. D. Retinoic acid as a vaccine adjuvant enhances CD8+ T cell response and mucosal protection from viral challenge. *J. Virol.* **85**, 8316-8327 (2011).

224 Esfahani, K. *et al.* A review of cancer immunotherapy: from the past, to the present, to the future. *Curr Oncol.* **27**, S87-S97, doi:10.3747/co.27.5223 (2020).

225 Pohlmann, P. R., Mayer, I. A. & Mernaugh, R. Resistance to Trastuzumab in Breast Cancer. *Clin Cancer Res* **15**, 7479-7491, doi:10.1158/1078-0432.CCR-09-0636 (2009).

226 Kim, E. S. Chemotherapy Resistance in Lung Cancer. *Adv Exp Med Biol* **893**, 189-209, doi:10.1007/978-3-319-24223-1_10 (2016).

227 Lin, J. J. & Shaw, A. T. Resisting Resistance: Targeted Therapies in Lung Cancer. *Trends Cancer* **2**, 350-364, doi:10.1016/j.trecan.2016.05.010 (2016).

228 Leal, A. S., Reich, L. A., Moerland, J. A., Zhang, D. & Liby, K. T. in *Adv. Pharmacol.* Vol. 91 (eds Bryan L. Copple & Cheryl E. Rockwell) 141-183 (Academic Press, 2021).

229 Chan, M. M., Lu, X., Merchant, F. M., Iglehart, J. D. & Miron, P. L. Gene expression profiling of NMU-induced rat mammary tumors: cross species comparison with

human breast cancer. *Carcinogenesis* **26**, 1343-1353, doi:10.1093/carcin/bgi100 (2005).

230 Gottardis, M. M. *et al.* Chemoprevention of Mammary Carcinoma by LGD1069 (Targretin): An RXR-selective Ligand. *Cancer Res.* **56**, 5566-5570 (1996).

231 Anzano, M. A. *et al.* Prevention of Breast Cancer in the Rat with 9-cis-Retinoic Acid as a Single Agent and in Combination with Tamoxifen. *Cancer Res.* **54**, 4614 (1994).

232 Teplitzky, S. R. *et al.* Chemoprevention of NMU-induced rat mammary carcinoma with the combination of melatonin and 9-cis-retinoic acid. *Cancer Lett.* **168**, 155-163 (2001).

233 Apfel, C. *et al.* A retinoic acid receptor alpha antagonist selectively counteracts retinoic acid effects. *Proc. Natl. Acad. Sci.* **89**, 7129, doi:10.1073/pnas.89.15.7129 (1992).

234 Jordan, V. C. Alternate antiestrogens and approaches to the prevention of breast cancer. *J Cell Biochem Suppl* **22**, 51-57, doi:10.1002/jcb.240590808 (1995).

235 Wu, K. *et al.* The Retinoid X Receptor-Selective Retinoid, LGD1069, Prevents the Development of Estrogen Receptor-Negative Mammary Tumors in Transgenic Mice. *Cancer Res.* **62**, 6376-6380 (2002).

236 Forkert, P.-G. Mechanisms of lung tumorigenesis by ethyl carbamate and vinyl carbamate. *Drug Metab. Rev.* **42**, 355-378, doi:10.3109/03602531003611915 (2010).

237 El Osta, B. *et al.* Characteristics and Outcomes of Patients With Metastatic KRAS-Mutant Lung Adenocarcinomas: The Lung Cancer Mutation Consortium Experience. *J Thorac Oncol* **14**, 876-889, doi:10.1016/j.jtho.2019.01.020 (2019).

238 Schmeltz, I., Chiong, K. G. & Hoffmann, D. Formation and Determination of Ethyl Carbamate in Tobacco and Tobacco Smoke. *J. Anal. Toxicol.* **2**, 265-268, doi:10.1093/jat/2.6.265 (1978).

239 Jurutka, P. W. *et al.* Modeling, synthesis, and biological evaluation of potential retinoid X receptor (RXR) selective agonists: novel analogues of 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl]benzoic acid (bexarotene) and (E)-3-(3-(1,2,3,4-tetrahydro-1,1,4,4,6-pentamethylnaphthalen-7-yl)-4-hydroxyphenyl)acrylic acid (CD3254). *J Med Chem* **56**, 8432-8454, doi:10.1021/jm4008517 (2013).

240 Heck, M. C. *et al.* Modeling, Synthesis, and Biological Evaluation of Potential Retinoid X Receptor (RXR)-Selective Agonists: Analogues of 4-[1-(3,5,5,8,8-

Pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl]benzoic Acid (Bexarotene) and 6-(Ethyl(5,5,8,8-tetrahydronaphthalen-2-yl)amino)nicotinic Acid (NEt-TMN). *J Med Chem* **59**, 8924-8940, doi:10.1021/acs.jmedchem.6b00812 (2016).

241 Guy, C. T. *et al.* Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci.* **89**, 10578-10582, doi:10.1073/pnas.89.22.10578 (1992).

242 Metsalu, T. & Vilo, J. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res.* **43**, W566-W570, doi:10.1093/nar/gkv468 (2015).

243 Hanish, B. J. *et al.* A novel gene expression analytics-based approach to structure aided design of rexinoids for development as next-generation cancer therapeutics. *Steroids* **135**, 36-49, doi:10.1016/j.steroids.2018.04.009 (2018).

244 Mallick, S. *et al.* Evaluating Novel RXR Agonists That Induce ApoE and Tyrosine Hydroxylase in Cultured Human Glioblastoma Cells. *ACS Chem. Neurosci.* **12**, 857-871, doi:10.1021/acschemneuro.0c00707 (2021).

245 Hernandez, L. G. & Forkert, P. G. Inhibition of vinyl carbamate-induced lung tumors and Kras2 mutations by the garlic derivative diallyl sulfone. *Mutat Res* **662**, 16-21, doi:10.1016/j.mrfmmm.2008.11.013 (2009).

246 Fry, E. A., Taneja, P. & Inoue, K. Clinical applications of mouse models for breast cancer engaging HER2/neu. *Integr Cancer Sci Ther* **3**, 593-603, doi:10.15761/ICST.1000210 (2016).

247 Wong, A. W., Dunlap, S. M., Holcomb, V. B. & Nunez, N. P. Alcohol Promotes Mammary Tumor Development via the Estrogen Pathway in Estrogen Receptor Alpha-Negative HER2/neu Mice. *Alcoholism: Clinical and Experimental Research* **36**, 577-587 (2012).

248 Khazal, K. F., Hill, D. L. & Grubbs, C. J. Effect of Withania somnifera root extract on spontaneous estrogen receptor-negative mammary cancer in MMTV/Neu mice. *Anticancer Res.* **34**, 6327-6332 (2014).

249 Sporn, M. B. & Liby, K. T. A Mini-Review of Chemoprevention of Cancer——Past, Present, and Future. *Progress in Chemistry* **25**, 1421 (2013).

250 Zhang, Q. *et al.* Optimized Bexarotene Aerosol Formulation Inhibits Major Subtypes of Lung Cancer in Mice. *Nano Lett.* **19**, 2231-2242, doi:10.1021/acs.nanolett.8b04309 (2019).

251 Mommers, E. C. M., van Diest, P. J., Leonhart, A. M., Meijer, C. J. L. M. & Baak, J. P. A. Balance of cell proliferation and apoptosis in breast carcinogenesis. *Breast*

Cancer Res. Treat. **58**, 163-169, doi:10.1023/A:1006396103777 (1999).

252 Mantovani, A., Marchesi, F., Malesci, A., Laghi, L. & Allavena, P. Tumour-associated macrophages as treatment targets in oncology. *Nat. Rev. Clin. Oncol.* **14**, 399-416, doi:10.1038/nrclinonc.2016.217 (2017).

253 Najafi, M. *et al.* Macrophage polarity in cancer: A review. *J. Cell. Biochem.* **120**, 2756-2765 (2019).

254 Baptista, M. Z., Sarian, L. O., Derchain, S. F. M., Pinto, G. A. & Vassallo, J. Prognostic significance of PD-L1 and PD-L2 in breast cancer. *Hum. Pathol.* **47**, 78-84 (2016).

255 Wimberly, H. *et al.* PD-L1 Expression Correlates with Tumor-Infiltrating Lymphocytes and Response to Neoadjuvant Chemotherapy in Breast Cancer. *Cancer Immunol. Res.* **3**, 326, doi:10.1158/2326-6066.CIR-14-0133 (2015).

256 Schmid, P. *et al.* Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer. *N. Engl. J. Med.* **379**, 2108-2121, doi:10.1056/NEJMoa1809615 (2018).

257 Costa, F. F. *et al.* Epigenetic silencing of the adhesion molecule ADAM23 is highly frequent in breast tumors. *Oncogene* **23**, 1481-1488, doi:10.1038/sj.onc.1207263 (2004).

258 Schmid, B. C. *et al.* The neuronal guidance cue Slit2 induces targeted migration and may play a role in brain metastasis of breast cancer cells. *Breast Cancer Research and Treatment* **106**, 333-342, doi:10.1007/s10549-007-9504-0 (2007).

259 Dallol, A. *et al.* SLIT2, a human homologue of the Drosophila Slit2 gene, has tumor suppressor activity and is frequently inactivated in lung and breast cancers. *Cancer Res* **62**, 5874-5880 (2002).

260 Kumari, N., Dwarakanath, B. S., Das, A. & Bhatt, A. N. Role of interleukin-6 in cancer progression and therapeutic resistance. *Tumour Biol.* **37**, 11553-11572, doi:10.1007/s13277-016-5098-7 (2016).

261 Rosenfeld, M. G., Lunyak, V. V. & Glass, C. K. Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev.* **20**, 1405-1428 (2006).

262 O'Malley, B. W., Malovannaya, A. & Qin, J. Minireview: nuclear receptor and coregulator proteomics—2012 and beyond. *Molecular endocrinology* **26**, 1646-1650 (2012).

263 Leal, A. S., Reich, L. A., Moerland, J. A., Zhang, D. & Liby, K. T. Potential

therapeutic uses of rexinoids. (2021).

264 Reich, L. A. *et al.* The rexinoid V-125 reduces tumor growth in preclinical models of breast and lung cancer. *Scientific Reports* **12**, 293, doi:10.1038/s41598-021-04415-0 (2022).

265 Leal, A. S. *et al.* The RXR Agonist MSU42011 Is Effective for the Treatment of Preclinical HER2+ Breast Cancer and Kras-Driven Lung Cancer. *Cancers (Basel)* **13**, doi:10.3390/cancers13195004 (2021).

266 Andrechek, E. R. *et al.* Gene Expression Profiling of Neu-induced Mammary Tumors from Transgenic Mice Reveals Genetic and Morphological Similarities to ErbB2-expressing Human Breast Cancers1. *Cancer Research* **63**, 4920-4926 (2003).

267 Rennhack, J. P. *et al.* Integrated analyses of murine breast cancer models reveal critical parallels with human disease. *Nature Communications* **10**, 3261, doi:10.1038/s41467-019-11236-3 (2019).

268 Shiota, G. & Kanki, K. Retinoids and their target genes in liver functions and diseases. *J. Gastroenterol. Hepatol.* **28**, 33-37 (2013).

269 Nielsen, R. *et al.* Genome-wide profiling of PPARgamma:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. *Genes Dev* **22**, 2953-2967, doi:10.1101/gad.501108 (2008).

270 Ding, N. *et al.* A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* **153**, 601-613, doi:10.1016/j.cell.2013.03.028 (2013).

271 Abba, M. C. *et al.* Transcriptomic signature of Bexarotene (Rexinoid LGD1069) on mammary gland from three transgenic mouse mammary cancer models. *BMC Med. Genomics* **1**, 40, doi:10.1186/1755-8794-1-40 (2008).

272 Zhang, D., Rennhack, J., Andrechek, E. R., Rockwell, C. E. & Liby, K. T. Identification of an Unfavorable Immune Signature in Advanced Lung Tumors from Nrf2-Deficient Mice. *Antioxidants & Redox Signaling* **29**, 1535-1552, doi:10.1089/ars.2017.7201 (2018).

273 Crowe, A. R. & Yue, W. Semi-quantitative Determination of Protein Expression using Immunohistochemistry Staining and Analysis: An Integrated Protocol. *Bio Protoc* **9**, doi:10.21769/BioProtoc.3465 (2019).

274 Gheban, B.-A. *et al.* Techniques for digital histological morphometry of the pineal gland. *Acta Histochem.* **124**, 151897 (2022).

275 Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550, doi:10.1186/s13059-014-0550-8 (2014).

276 Anders, S. & Huber, W. Differential expression of RNA-Seq data at the gene level–the DESeq package. *Heidelberg, Germany: European Molecular Biology Laboratory (EMBL)* **10**, f1000research (2012).

277 Krämer, A., Green, J., Pollard, J., Jr. & Tugendreich, S. Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics* **30**, 523-530, doi:10.1093/bioinformatics/btt703 (2014).

278 Győrffy, B. Survival analysis across the entire transcriptome identifies biomarkers with the highest prognostic power in breast cancer. *Computational and Structural Biotechnology Journal* **19**, 4101-4109 (2021).

279 Wei, C. *et al.* MicroRNA-330-3p promotes brain metastasis and epithelial-mesenchymal transition via GRIA3 in non-small cell lung cancer. *Aging (Albany N. Y.)* **11**, 6734-6761, doi:10.18632/aging.102201 (2019).

280 Heger, L. *et al.* CLEC10A Is a Specific Marker for Human CD1c+ Dendritic Cells and Enhances Their Toll-Like Receptor 7/8-Induced Cytokine Secretion. *Front. Immunol.* **9**, doi:10.3389/fimmu.2018.00744 (2018).

281 Jiang, T. *et al.* FNDC1 Promotes the Invasiveness of Gastric Cancer via Wnt/β-Catenin Signaling Pathway and Correlates With Peritoneal Metastasis and Prognosis. *Frontiers in Oncology* **10**, doi:10.3389/fonc.2020.590492 (2020).

282 Abudureyimu, S. *et al.* Essential Role of Linx/Islr2 in the Development of the Forebrain Anterior Commissure.

283 Navab, R. *et al.* Integrin α11β1 regulates cancer stromal stiffness and promotes tumorigenicity and metastasis in non-small cell lung cancer.

284 Jeong, S. *et al.* Liver X Receptor β Related to Tumor Progression and Ribosome Gene Expression in Papillary Thyroid Cancer. *Endocrinol Metab (Seoul)* **35**, 656-668, doi:10.3803/EnM.2020.667 (2020).

285 Lu, X. *et al.* Identification of the key pathways and genes involved in HER2-positive breast cancer with brain metastasis. *Pathology - Research and Practice* **215**, 152475 (2019).

286 Schenck, J. & Fitzgerald, J. Analysis of COL6A3, COL6A5 and COL6A6 gene expression in breast and prostate tumors. *Advances in Clinical and Translational Research* **2**, 1-10 (2018).

287 Rouquier, S., Pillaire, M.-J., Cazaux, C. & Giorgi, D. Expression of the Microtubule-Associated Protein MAP9/ASAP and Its Partners AURKA and PLK1 in Colorectal and Breast Cancers. *Dis. Markers* **2014**, 798170, doi:10.1155/2014/798170 (2014).

288 Aguiar, M. A. N. *et al.* Interleukin-18 (IL-18) is equally expressed in inflammatory breast cancer and noninflammatory locally advanced breast cancer: A possible association with chemotherapy response. *Asia Pac. J. Clin. Oncol.* **14**, e138-e144 (2018).

289 Senju, H. *et al.* Effect of IL-18 on the Expansion and Phenotype of Human Natural Killer Cells: Application to Cancer Immunotherapy. *Int. J. Biol. Sci.* **14**, 331-340, doi:10.7150/ijbs.22809 (2018).

290 Turner, T. B. *et al.* Epigenetic modifiers upregulate MHC II and impede ovarian cancer tumor growth. *Oncotarget* **8**, 44159-44170, doi:10.18632/oncotarget.17395 (2017).

291 Axelrod, M. L., Cook, R. S., Johnson, D. B. & Balko, J. M. Biological Consequences of MHC-II Expression by Tumor Cells in Cancer. *Clinical Cancer Research* **25**, 2392-2402, doi:10.1158/1078-0432.CCR-18-3200 (2019).

292 Magner, W. J. *et al.* Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. *J. Immunol.* **165**, 7017-7024, doi:10.4049/jimmunol.165.12.7017 (2000).

293 Cycon, K. A., Mulvaney, K., Rimsza, L. M., Persky, D. & Murphy, S. P. Histone deacetylase inhibitors activate CIITA and MHC class II antigen expression in diffuse large B-cell lymphoma. *Immunology* **140**, 259-272, doi:10.1111/imm.12136 (2013).

294 Forero, A. *et al.* Expression of the MHC Class II Pathway in Triple-Negative Breast Cancer Tumor Cells Is Associated with a Good Prognosis and Infiltrating Lymphocytes. *Cancer Immunology Research* **4**, 390-399, doi:10.1158/2326-6066.CIR-15-0243 (2016).

295 Liu, W., Li, L., Ye, H., Tao, H. & He, H. Role of COL6A3 in colorectal cancer. *Oncol. Rep.* **39**, 2527-2536, doi:10.3892/or.2018.6331 (2018).

296 Zhang, L., Wang, L., Yang, H., Li, C. & Fang, C. Identification of potential genes related to breast cancer brain metastasis in breast cancer patients. *Biosci. Rep.* **41**, BSR20211615, doi:10.1042/BSR20211615 (2021).

297 Al Amri, W. S. *et al.* Identification of candidate mediators of chemoresponse in breast cancer through therapy-driven selection of somatic variants. *Breast Cancer Research and Treatment* **183**, 607-616, doi:10.1007/s10549-020-05836-7 (2020).

298 Wang, J. & Pan, W. The Biological Role of the Collagen Alpha-3 (VI) Chain and Its Cleaved C5 Domain Fragment Endotrophin in Cancer. *Onco Targets Ther.* **13**, 5779-5793, doi:10.2147/OTT.S256654 (2020).

299 Guglielmi, V. *et al.* Omental adipose tissue fibrosis and insulin resistance in severe obesity. *Nutr. Diabetes* **5**, e175-e175, doi:10.1038/nutd.2015.22 (2015).

300 Dehbid, M., Aleyasin, S. & Vaziri, H. Evaluation of DNA Methylation of MAP9 Gene in Breast Cancer as Epigenetic Biomarker. *J Mol Biomarkers Diagn S* **8**, 2 (2016).

301 Basbous, J. *et al.* Induction of ASAP (MAP9) contributes to p53 stabilization in response to DNA damage. *Cell Cycle* **11**, 2380-2390, doi:10.4161/cc.20858 (2012).

302 Ma, B. *et al.* Modular bioinformatics analysis demonstrates that a Toll-like receptor signaling pathway is involved in the regulation of macrophage polarization. *Mol Med Rep* **18**, 4313-4320, doi:10.3892/mmr.2018.9486 (2018).

303 Beider, K. *et al.* Multiple myeloma cells recruit tumor-supportive macrophages through the CXCR4/CXCL12 axis and promote their polarization toward the M2 phenotype. *Oncotarget* **5**, 11283-11296, doi:10.18632/oncotarget.2207 (2014).

304 Babazadeh, S. *et al.* Macrophage polarization by MSC-derived CXCL12 determines tumor growth. *Cell. Mol. Biol. Lett.* **26**, 30, doi:10.1186/s11658-021-00273-w (2021).

305 Decock, J., Thirkettle, S., Wagstaff, L. & Edwards, D. R. Matrix metalloproteinases: protective roles in cancer. *J Cell Mol Med* **15**, 1254-1265, doi:10.1111/j.1582-4934.2011.01302.x (2011).

306 Wahl, L. M. & Kleinman, H. K. Tumor-Associated Macrophages as Targets for Cancer Therapy. *JNCI: Journal of the National Cancer Institute* **90**, 1583-1584, doi:10.1093/jnci/90.21.1583 (1998).

307 Shenoy, N. *et al.* Alterations in the ribosomal machinery in cancer and hematologic disorders. *J. Hematol. Oncol.* **5**, 1-9 (2012).

308 Burke, K. S., Antilla, K. A. & Tirrell, D. A. A Fluorescence in Situ Hybridization Method To Quantify mRNA Translation by Visualizing Ribosome–mRNA Interactions in Single Cells. *ACS Central Science* **3**, 425-433, doi:10.1021/acscentsci.7b00048 (2017).

309 Capece, M. C., Kornberg, G. L., Petrov, A. & Puglisi, J. D. A simple real-time assay for in vitro translation. *RNA* **21**, 296-305, doi:10.1261/rna.047159.114 (2015).

310 Khuperkar, D. *et al.* Quantification of mRNA translation in live cells using single-

molecule imaging. *Nat. Protoc.* **15**, 1371-1398, doi:10.1038/s41596-019-0284-x (2020).

311 Marshall, P. A. *et al.* Analysis of differential secondary effects of novel rexinoids: select rexinoid X receptor ligands demonstrate differentiated side effect profiles. *Pharmacology Research & Perspectives* **3**, e00122 (2015).

312 Robertson Remen, K. M., Gustafsson, J.-Å. & Andersson, G. The liver X receptor promotes macrophage differentiation and suppresses osteoclast formation in mouse RAW264.7 promyelocytic leukemia cells exposed to bacterial lipopolysaccharide. *Biochemical and Biophysical Research Communications* **430**, 375-380 (2013).

313 Liu, R. *et al.* CD47 promotes ovarian cancer progression by inhibiting macrophage phagocytosis. *Oncotarget* **8**, 39021 (2017).

314 Barkal, A. A. *et al.* CD24 signalling through macrophage Siglec-10 is a target for cancer immunotherapy. *Nature* **572**, 392-396 (2019).

315 Ijpenberg, A. *et al.* In vivo activation of PPAR target genes by RXR homodimers. *The EMBO Journal* **23**, 2083-2091 (2004).

316 Lalloyer, F. *et al.* Rexinoid Bexarotene Modulates Triglyceride but not Cholesterol Metabolism via Gene-Specific Permissivity of the RXR/LXR Heterodimer in the Liver. *Arteriosclerosis, Thrombosis, and Vascular Biology* **29**, 1488-1495, doi:doi:10.1161/ATVBAHA.109.189506 (2009).

317 Roychowdhury, S., Ward, C. M., Kennedy, K. J. & Zhao, Y. in *Drug Metab. Pharmacokinet.* S50-S50 (JAPANESE SOC STUDY XENOBIOTICS INT MED INF CENTER SHINANOMACHI RENGAKAN, 35 …).

318 Mitchell, C. A., Dasgupta, S., Zhang, S., Stapleton, H. M. & Volz, D. C. Disruption of Nuclear Receptor Signaling Alters Triphenyl Phosphate-Induced Cardiotoxicity in Zebrafish Embryos. *Toxicol. Sci.* **163**, 307-318, doi:10.1093/toxsci/kfy037 (2018).

319 Joseph, S. B. *et al.* LXR-Dependent Gene Expression Is Important for Macrophage Survival and the Innate Immune Response. *Cell* **119**, 299-309 (2004).

320 Bełtowski, J. & Semczuk, A. Liver X receptor (LXR) and the reproductive system — a potential novel target for therapeutic intervention. *Pharmacol. Rep.* **62**, 15-27, doi:10.1016/S1734-1140(10)70239-5 (2010).

321 Abe, R. & Yamagishi, S.-i. AGE-RAGE system and carcinogenesis. *Curr. Pharm. Des.* **14**, 940-945 (2008).

322 Samulin Erdem, J., Arnoldussen, Y. J., Skaug, V., Haugen, A. & Zienolddiny, S. Copy number variation, increased gene expression, and molecular mechanisms of neurofascin in lung cancer. *Mol. Carcinog.* **56**, 2076-2085, doi:10.1002/mc.22664 (2017).

323 Querol, L. *et al.* Neurofascin IgG4 antibodies in CIDP associate with disabling tremor and poor response to IVIg. *Neurology* **82**, 879-886 (2014).

324 Duan, J. J., Cai, J., Guo, Y. F., Bian, X. W. & Yu, S. C. ALDH1A3, a metabolic target for cancer diagnosis and therapy. *Int. J. Cancer* **139**, 965-975, doi:10.1002/ijc.30091 (2016).

325 Caron, E. Cellular functions of the Rap1 GTP-binding protein: a pattern emerges. *J. Cell Sci.* **116**, 435-440, doi:10.1242/jcs.00238 (2003).

326 Zhang, Y. L., Wang, R. C., Cheng, K., Ring, B. Z. & Su, L. Roles of Rap1 signaling in tumor cell migration and invasion. *Cancer Biol Med* **14**, 90-99, doi:10.20892/j.issn.2095-3941.2016.0086 (2017).

327 Maldonado, L. Y., Arsene, D., Mato, J. M. & Lu, S. C. Methionine adenosyltransferases in cancers: Mechanisms of dysregulation and implications for therapy. *Experimental Biology and Medicine* **243**, 107-117, doi:10.1177/1535370217740860 (2017).

328 Tomasi, M. L. *et al.* S-adenosyl methionine regulates ubiquitin-conjugating enzyme 9 protein expression and sumoylation in murine liver and human cancers. *Hepatology* **56**, 982-993 (2012).

329 Simile, M. M. *et al.* Chemopreventive N -(4-hydroxyphenyl)retinamide (fenretinide) targets deregulated NF-κB and Mat1A genes in the early stages of rat liver carcinogenesis. *Carcinogenesis* **26**, 417-427, doi:10.1093/carcin/bgh315 (2005).

330 Ahirwar, D. K. *et al.* Slit2 Inhibits Breast Cancer Metastasis by Activating M1-Like Phagocytic and Antifibrotic Macrophages. *Cancer Res* **81**, 5255-5267, doi:10.1158/0008-5472.Can-20-3909 (2021).

APPENDIX A – SUPPLEMENTAL FIGURES

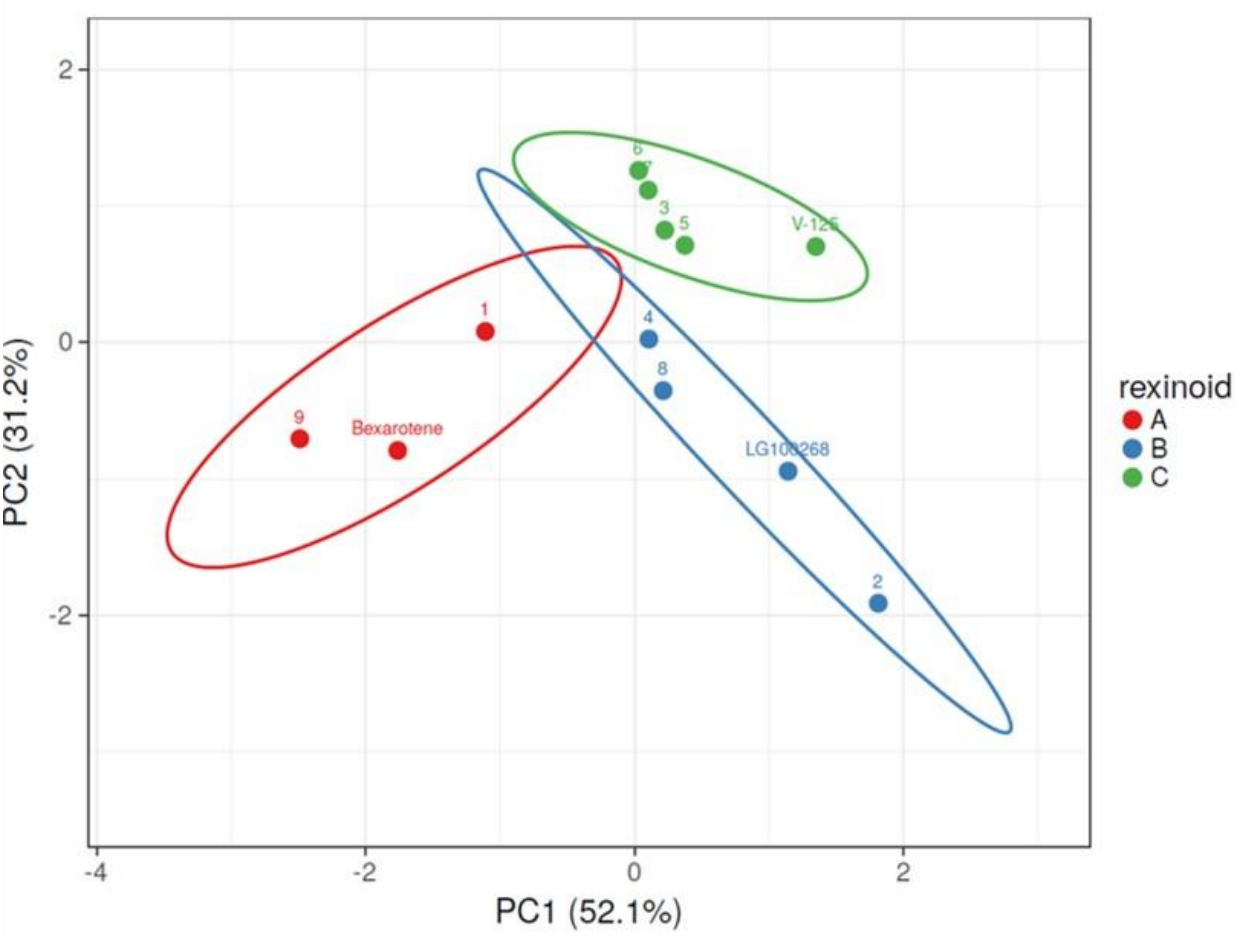

Figure S.1. V-125 displays unique activity in principal component analysis (PCA). Unit variance scaling is applied to rows; singular value decomposition (SVD) with imputation is used to calculate principal components. X and Y axis show principal component 1 and principal component 2 that explain 52.1% and 31.2% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. N = 12 data points.

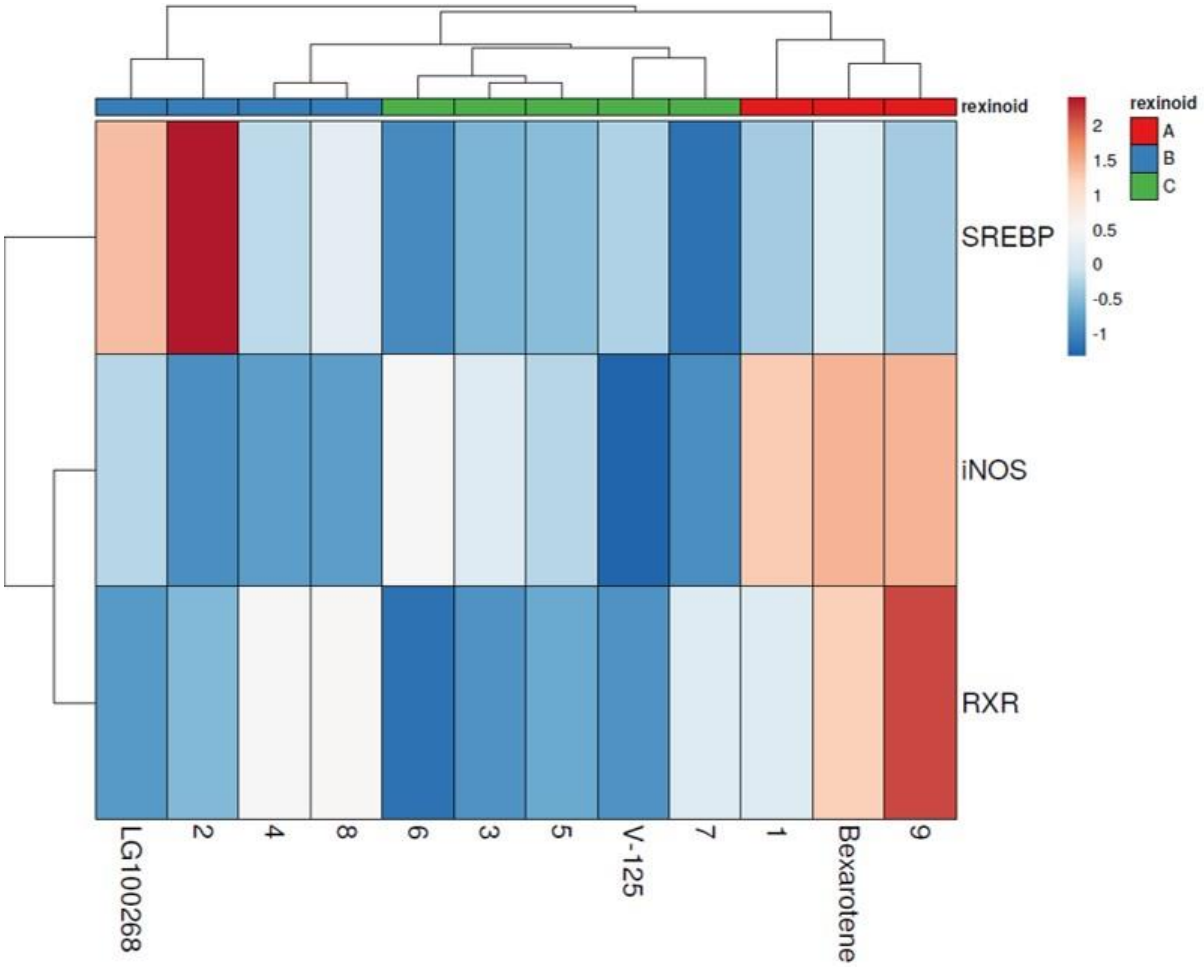

Figure S.2. Heat map of activity of novel rexinoids in *in vitro* screening assays. Rows are centered; unit variance scaling is applied to rows. Rows are clustered using correlation distance and average linkage. Columns are clustered using maximum distance and average linkage. 3 rows, 12 columns. SREBP = SREBP activation. RXR = RXR activation. iNOS = iNOS suppression. Scale is centered around average value for each column.

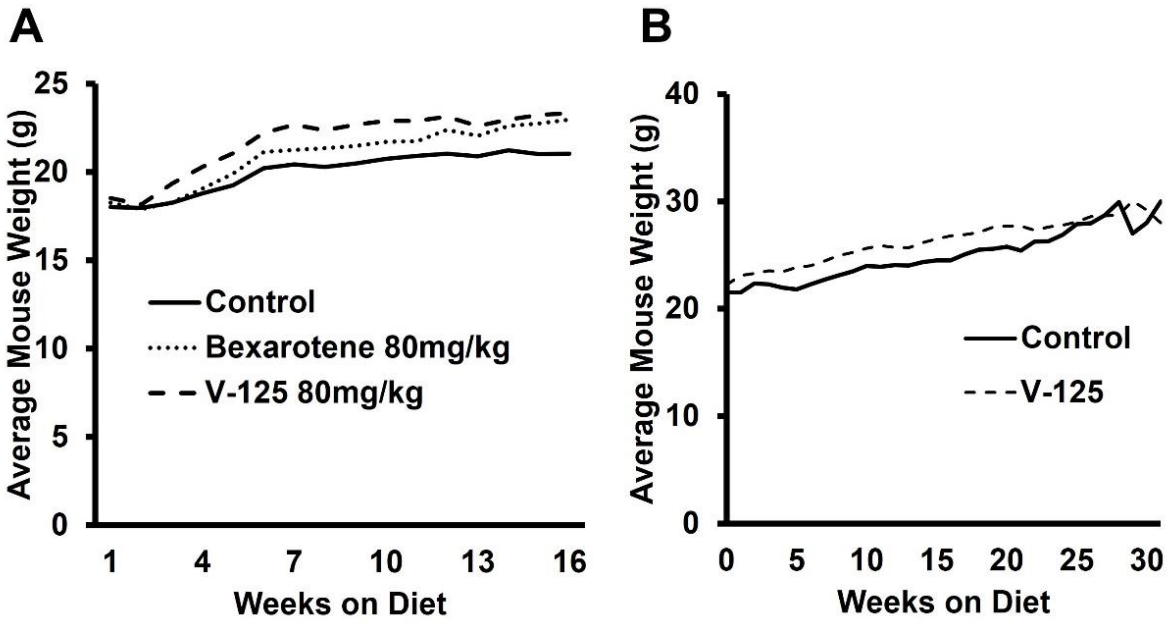

Figure S.3. V-125 does not decrease weight of A/J or MMTV-Neu mice in prevention studies. A/J mice (A) were fed bexarotene (80 mg/kg diet), V-125 (80 mg/kg diet) or control diet for 16 weeks, as described in Figure 2.2. Mice were weighed weekly throughout the course of the study. MMTV-Neu mice (B) were fed control or V-125 (30 mg/kg diet) in a prevention study, as described in Figure 2.3. A. N = 14-15 mice/group. B. N = 14 mice/group.

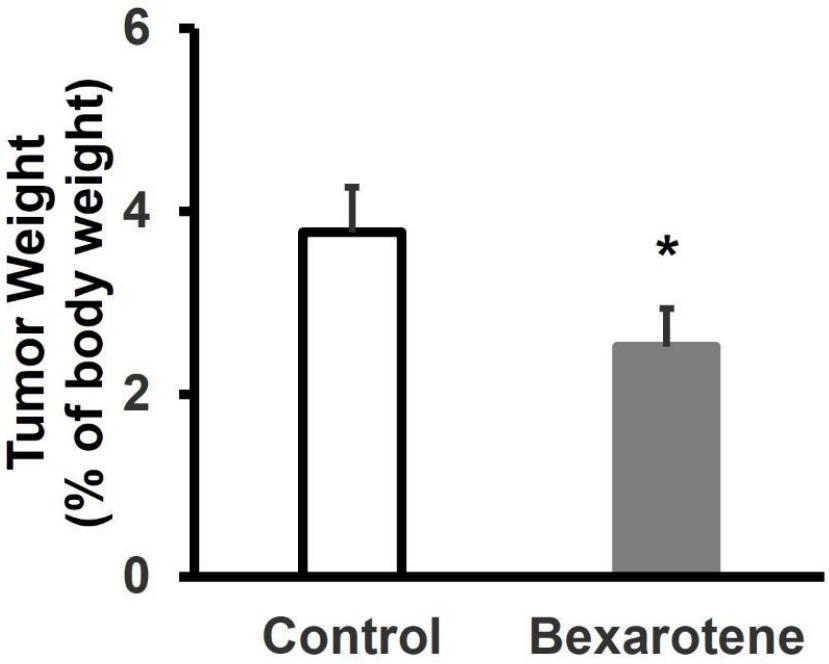

Figure S.4. Bexarotene reduces total tumor weight in MMTV-Neu mice. Female MMTV-Neu mice with established mammary tumors 5 mm in diameter were placed on control or bexarotene (100 mg/kg diet) for 10 days. Tumor weight at the end of 10 days is presented as a percent of mouse body weight. *, $P < 0.05$ vs. control; N=8-9 mice per group.

Position	Symbol	Fold Change
A01	Abcb1a	1.03
A02	Abcg2	**0.19**
A03	Adam23	**48.48**
A04	Akt1	1.09
A05	Apc	1.50
A06	Ar	1.20
A07	Atm	0.97
A08	Bad	0.95
A09	Bcl2	1.94
A10	Birc5	1.06
A11	Brca1	0.93
A12	Brca2	1.25
B01	Ccna1	1.00
B02	Ccnd1	1.38
B03	Ccnd2	1.05
B04	Ccne1	1.86
B05	Cdh1	0.81
B06	Cdh13	0.96
B07	Cdk2	1.00
B08	Cdkn1a	0.91
B09	Cdkn1c	**2.27**
B10	Cdkn2a	**0.35**
B11	Csf1	1.79
B12	Cst6	1.16
C01	Ctnnb1	0.60
C02	Ctsd	0.97
C03	Egf	1.99
C04	Egfr	1.63
C05	Erbb2	1.00
C06	Esr1	**2.13**
C07	Esr2	1.29
C08	Foxa1	1.06
C09	Gata3	1.24
C10	Gli1	**3.75**
C11	Grb7	**2.29**
C12	Gstp1	1.32
D01	Hic1	**2.25**
D02	Id1	1.23
D03	Igf1	1.00
D04	Igf1r	**0.36**
D05	Igfbp3	1.25
D06	Il6	**0.31**
D07	Jun	**0.50**
D08	Krt18	0.94
D09	Krt19	**14.75**
D10	Krt5	0.65
D11	Krt8	1.02
D12	Mapk1	1.28
E01	Mapk3	1.10
E02	Mapk8	1.06
E03	Mgmt	0.70
E04	Mki67	0.85
E05	Mlh1	0.86
E06	Mmp2	**2.89**
E07	Mmp9	1.21
E08	Muc1	1.24
E09	Myc	1.32
E10	Nme1	1.46
E11	Notch1	0.55
E12	Nr3c1	0.89
F01	Pgr	1.61
F02	Plau	1.06
F03	Prdm2	0.56
F04	Pten	**0.46**
F05	Ptgs2	**0.28**
F06	Pycard	1.50
F07	Rarb	0.88
F08	Rassf1	0.82
F09	Rb1	0.96
F10	Serpine1	**0.33**
F11	Sfn	0.97
F12	Sfrp1	**0.36**
G01	Slc39a6	0.91
G02	Slit2	**5.43**
G03	Snai2	1.59
G04	Src	1.33
G05	Tff3	0.93
G06	Tgfb1	1.36
G07	Thbs1	**0.42**
G08	Trp53	1.20
G09	Trp73	0.59
G10	Twist1	**4.16**
G11	Vegfa	0.72
G12	Xbp1	0.72
H01	Actb	0.75
H02	B2m	0.76
H03	Gapdh	0.81
H04	Gusb	1.59
H05	Hsp90ab1	1.43

Figure S.5. V-125 differentially regulates 20 genes on PCR array. Female MMTV-Neu mice with established mammary tumors 5 mm in diameter were placed on control or V-125 (100 mg/kg diet) for 10 days. Total RNA was extracted from tumors for PCR array. Analysis was performed using GeneGlobe software (Qiagen).

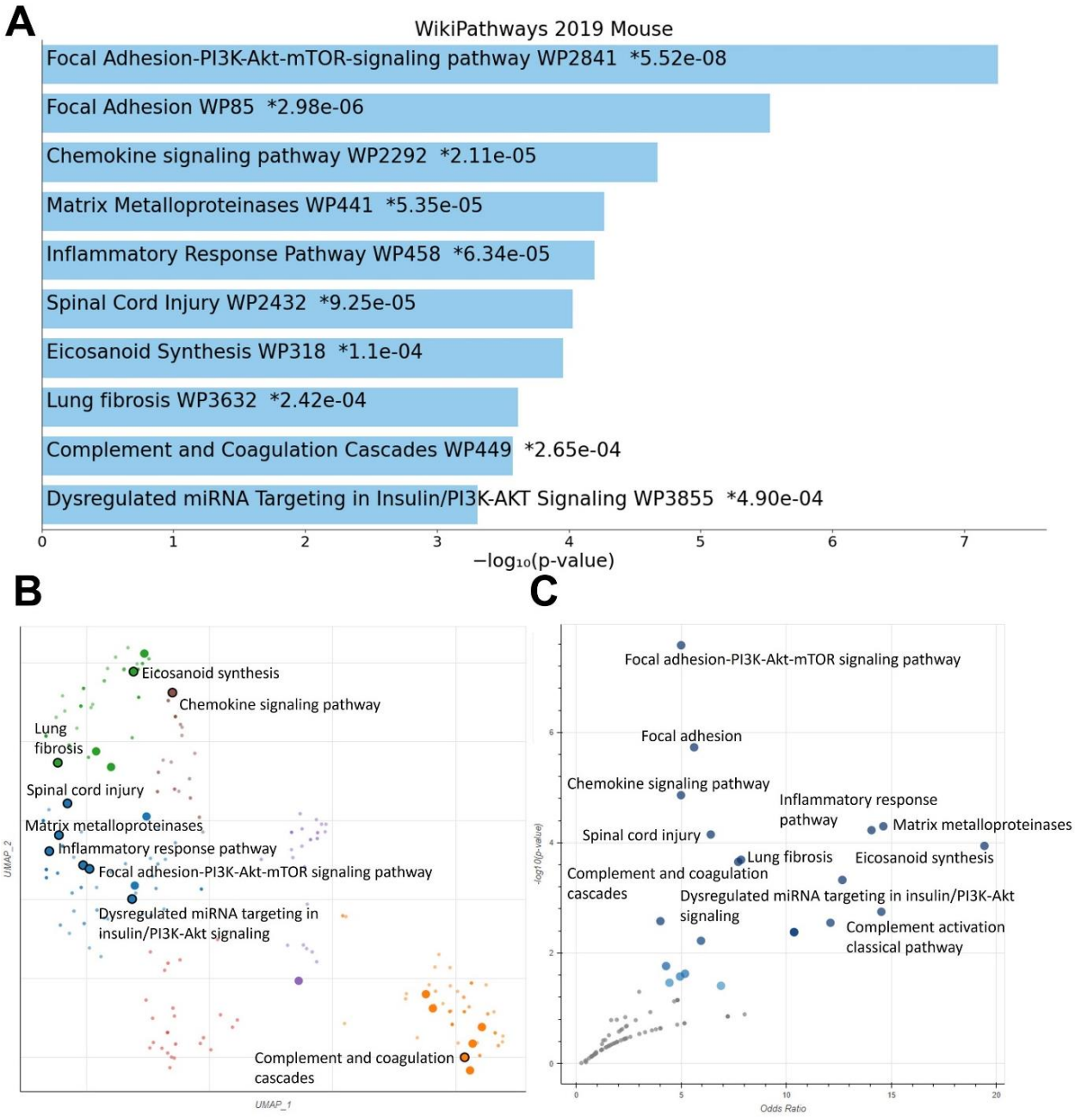

Figure S.6. MSU 42011 and bexarotene regulate cancer-relevant biological pathways in MMTV-Neu tumors. Female MMTV-Neu mice with established mammary tumors 4 mm in diameter were fed control diet, bexarotene (100 mg/kg in diet), or MSU 42011 (100 mg/kg in diet) for 10 days. The Wikipathways 2019 mouse database was used for enrichment analysis. **A**. Bar graph of top differentially expressed pathways. Scatterplot (**B**) and volcano plot (**C**) of top differentially expressed pathways.

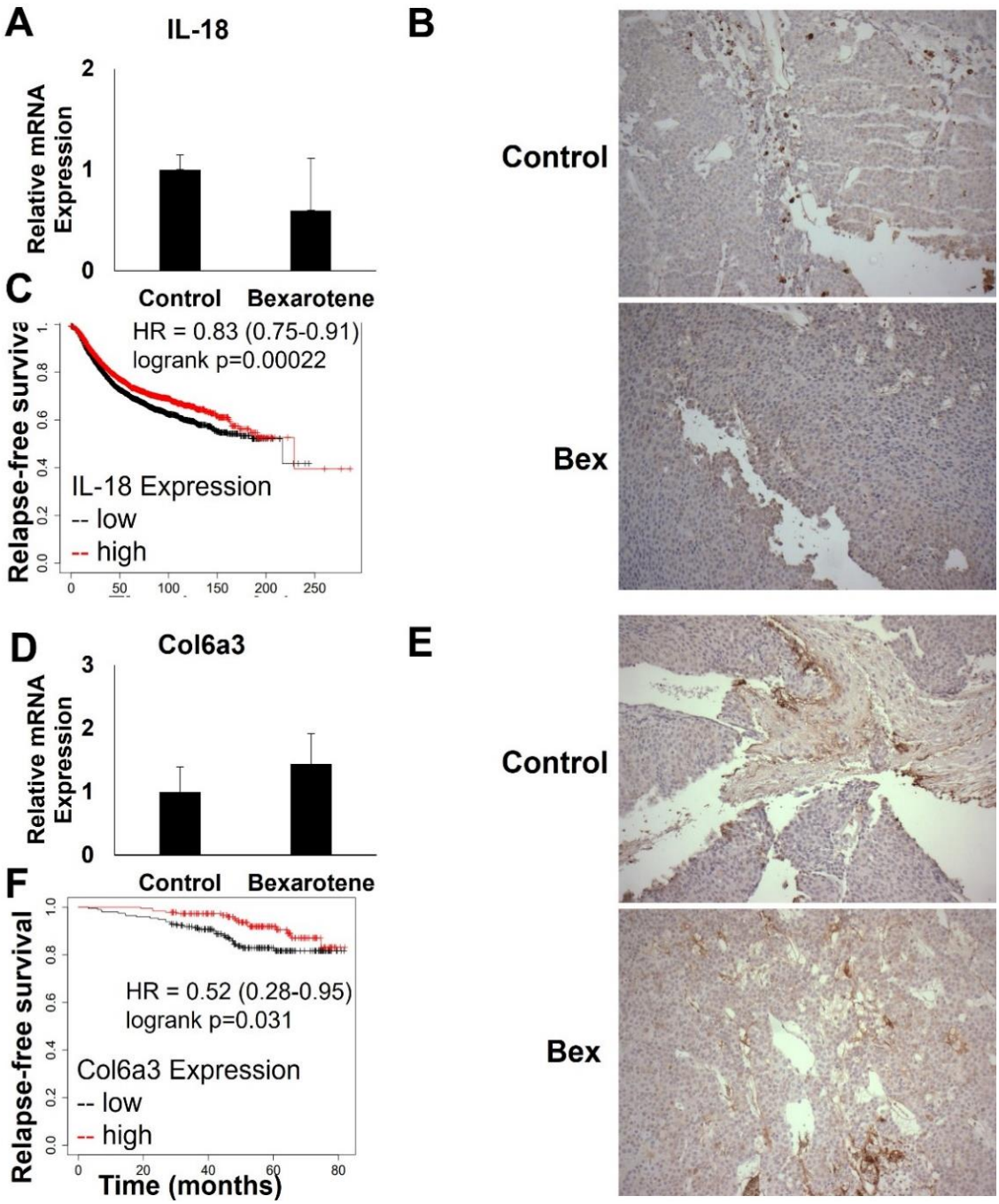

Figure S.7. Bexarotene does not increase expression of IL-18 and Col6a3 in mouse mammary tumors. Tumors treated with bexarotene (100 mg/kg diet) or control were harvested and flash-frozen. Tumors were homogenized and RNA extracted. **A, D**. Gene expression was detected by qPCR on QuantStudio 7 Flex. N=5 mice/group. Error bars represent standard error. * $p < 0.05$ **B, E**. Immunohistochemical staining for IL-18 and Col6a3, representative of 3 mice per group. **C, F**. Kmplot survival curves correlate expression of **C**. Il-18 with survival in breast cancer patients (n = 4929; 2456 high, 2473 low) and **F**. Col6a3 with survival in HER2+ breast cancer patients (n = 379; 183 high, 196 low).

APPENDIX B – SUPPLEMENTAL DATA: THE NOVEL RXR AGONIST V-125 REGULATES GENE EXPRESSION IN MMTV-NEU TUMORS

As shown in Chapter 2 of this dissertation, the novel RXR agonist has striking efficacy and is well tolerated in multiple preclinical cancer models. As with MSU 42011, we used RNA Sequencing as a tool to generate hypotheses regarding the mechanisms of anti-tumor activity of V-125.

Materials and Methods

Drugs: V-125 was synthesized as previously described[264], and bexarotene was procured from LC Laboratories. For *in vivo* studies, RXR agonists were dissolved in a vehicle of 1 part ethanol:3 parts Neobee oil (Thermo Fisher Scientific). 50 mL vehicle or drug dissolved in vehicle was mixed into 1 kg of powdered 5002 rodent chow (PMI Nutrition) in a KitchenAid mixer. Stability of rexinoids in powdered mouse chow for up to 6 weeks at 4°C has been confirmed using liquid chromatography-mass spectrometry.

***In vivo* experiments:** Animal studies were approved by the Institutional Animal Care and Use Committee at Michigan State University (IACUC protocol number 202100188). MMTV-Neu mice[241] from our breeding colony (founders were purchased from Jackson Laboratory) were fed pelleted chow and palpated for tumors. Once tumors were detected, mice were switched to powder 5002 chow. Tumors were measured twice weekly with a caliper until reaching 5 mm in diameter, at which time mice were randomized and fed control diet or 100 mg per kg diet of RXR agonist (V-125 or bexarotene) diet (~25 mg per kg body weight) for 10 days. Tumors were harvested after 10 day treatment and sections were either flash frozen for RNA-seq/qPCR or saved in neutral buffered formalin for immunohistochemistry

RNA Sequencing: Frozen tumor sections (4 samples per treatment group) were weighed and homogenized. RNA was extracted using RNeasy Mini Kit (Qiagen), and RNA integrity was analyzed using Agilent Bioanalyzer through the RTSF Genomics Core at Michigan State University. RNA sequencing was completed by Novogene (Sacramento, CA) as described previously[272]. Raw read counts were processed using the DESeq2 package in R to generate differential expression data, and EnrichR was used for enrichment analysis.

qPCR: RNA harvested from frozen tumor sections was normalized across samples using Nanodrop, and 500 ng of RNA was used to synthesize cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was run on QuantStudio 7 Flex using SYBR green fluorescence. PCR data was analyzed using the delta-delta CT method using the housekeeping control gene GAPDH. Error bars represent standard error of the mean of biological replicates. The following forward/reverse primers (Integrated DNA Technologies) were used: Slit2, 5' – CCATGTAAAAATGATGGCACCTG – 3' / 5' – ATCACAGTCCTGACCCTTGAA – 3', Mat1a, 5' – GTGCTGGATGCTCACCTCAAG – 3' / 5' – CCACCCGCTGGTAATCAACC – 3'.

Immunohistochemistry: Formalin-fixed tissues were embedded in paraffin and sectioned by the Histology Core at Michigan State University. Boiling citrate buffer was used for antigen retrieval, and hydrogen peroxide was used to quench endogenous peroxidase activity. Tissue sections were stained with antibodies against Mat1a (Thermo Fisher Scientific) and Slit2 (Thermo Fisher Scientific) as described[120]. Sections were then labeled with biotinylated secondary antibodies (anti-rabbit, Cell Signaling;

anti-rat, Vector Labs), as previously described.[120] A DAB substrate (Cell Signaling) was used for signal detection, as per manufacturer-provided protocols, and sections were counterstained with hematoxylin (Vector Labs). The Fiji ImageJ image processing package was used for quantification of intensity of DAB staining by the color deconvolution method[273] and mean gray value was used to calculate optical density by the formula OD = log (max intensity/mean intensity, with a maximum intensity of 255 for 8–bit images[274].

Statistical analysis: Results were expressed as the mean ± standard error. Data from tumor qPCR experiments were analyzed by one-tailed t test. $p < 0.05$ was considered statistically significant throughout all experiments. For RNAseq, differential expression analysis was performed using DESeq2. P values were adjusted to correct for multiple comparisons, and padj < 0.05 was considered statistically significant.

Results

Top genes differentially expressed in tumors treated with V-125 are correlated with survival in breast cancer patients

Genes differentially expressed in V-125 versus control were given a log fold change expression cutoff of 2 and sorted by adjusted p value. From the top 10 differentially expressed genes, genes in Figure S.8 were altered in V-125 tumors in a manner correlated with significantly increased overall survival in breast cancer patients (i.e. upregulation of NFASC in V-125-treated tumors) as per Kmplot data[2].

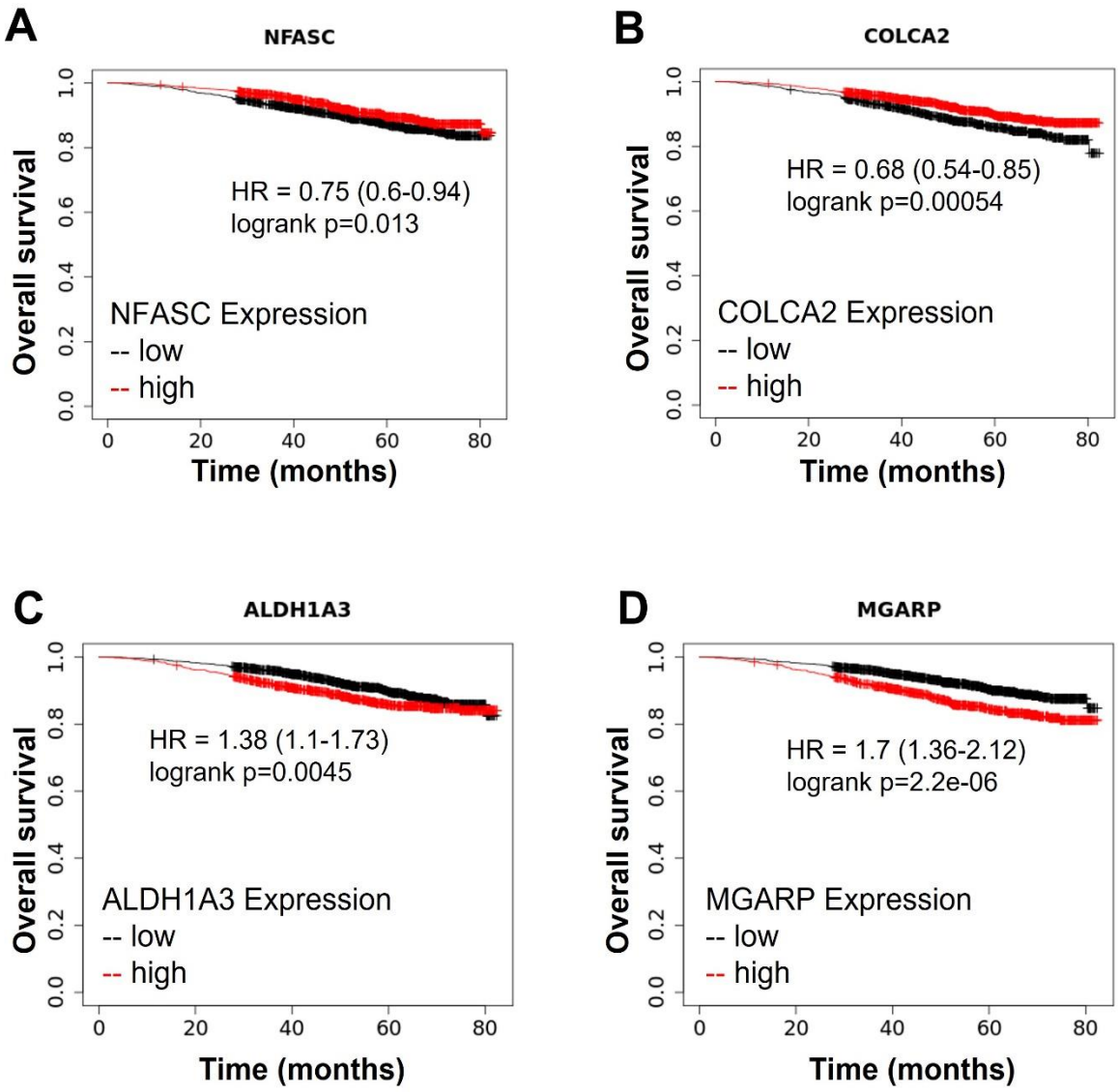

Figure S.8. Top genes differentially expressed in tumors treated with V-125 correlate with increased survival in breast cancer patients. Kaplan-Meier survival curves from the top 10 differentially expressed genes from V-125 vs control in breast cancer patients (n=2976; 1342 high 1634 low in **A**, 1858 high 1118 low in **B**, 1091 high 1885 low in **C**, 1039 high 1937 low in **D**). Data generated using KMPlot (http://www.kmplot.com). No stratification strategies were used.

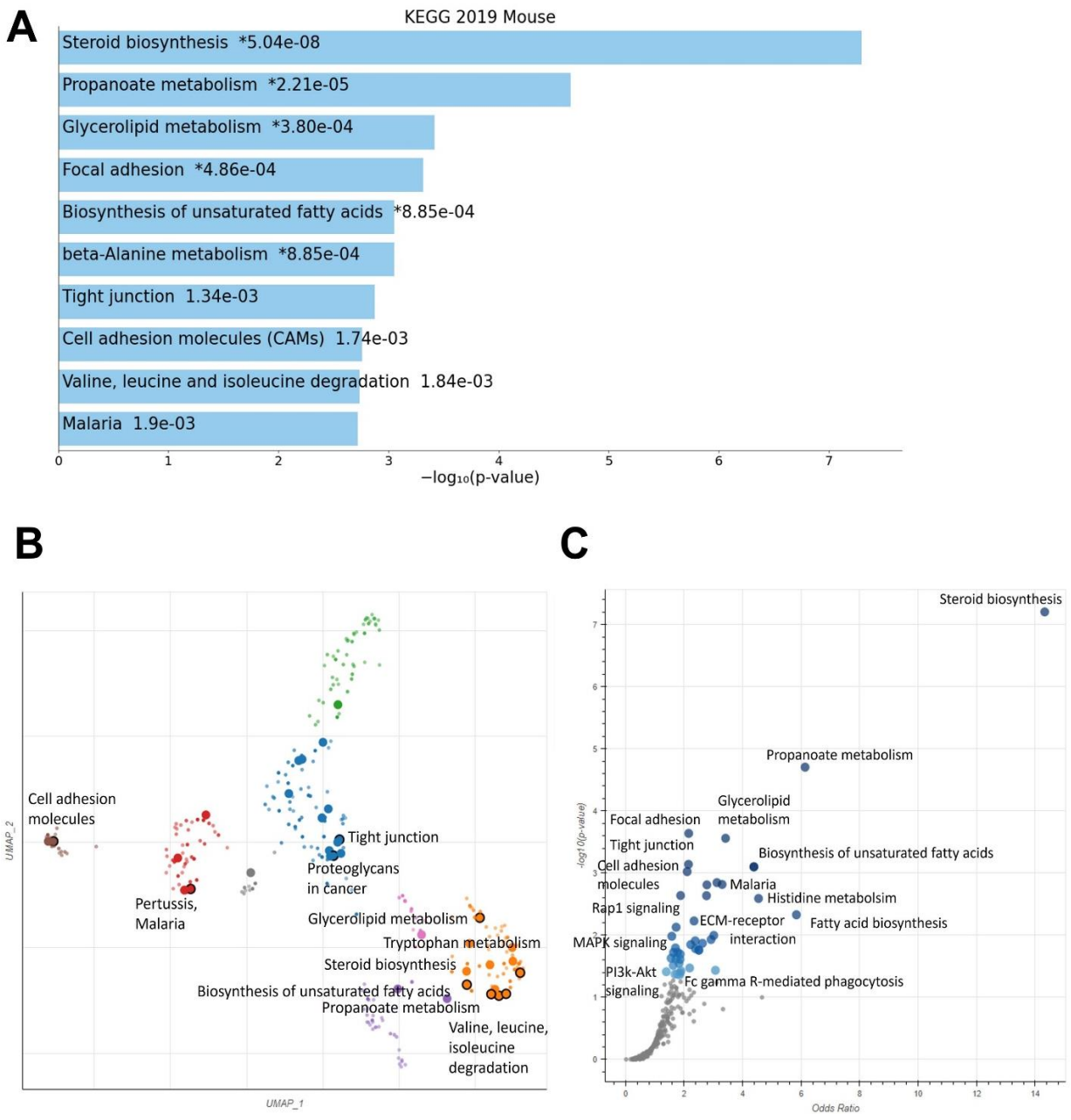

Figure S.9. V-125 and bexarotene regulate cancer-relevant pathways in MMTV-Neu tumors. Female MMTV-Neu mice with established mammary tumors 5 mm in diameter were fed control diet, bexarotene (100 mg/kg in diet), or V-125 (100 mg/kg in diet) for 10 days. KEGG 2019 mouse was used for enrichment analysis. **A.** Bar graph of top differentially expressed pathways. Scatterplot (**B**) and volcano plot (**C**) of top differentially expressed pathways.

V-125 and bexarotene regulate cancer-relevant biological pathways in MMTV-Neu tumors

MMTV-Neu mice with established tumors measuring 5 mm in diameter were placed on V-125 at a dose of 100 mg/kg diet (~25 mg/kg body weight) for a period of 10 days, followed by RNA isolation and RNA Sequencing. Enrichment analysis on control vs V-125 vs bexarotene differential expression data using EnrichR reveals a set of pathways enriched by treatment with RXR agonists (Figure S.9). These pathways include genes associated with cell adhesion molecules and macromolecule biosynthesis and metabolism. Genes within these pathways code for macromolecules involved in cellular structure and function, cellular behavior such as adhesion and migration, cellular proliferation and maintenance. KEGG 2019 mouse was used as a database for these analyses.

V-125 and bexarotene enrich both similar and distinct pathways in treated tumors

Enrichment analysis reveals pathways differentially regulated by V-125-treated tumors versus control (Figure S.10A, S.10C, S.10E) and by bexarotene-treated tumors versus control (Figure S.10B, S.10D, S.10F). This analysis highlights pathways enriched in both treatment groups, such as cell adhesion molecules, focal adhesion, and Rap1 signaling. This analysis also highlights pathway enrichment unique to V-125 treatment, such as biosynthesis of unsaturated fatty acids, glycerolipid metabolism, and the prominence of steroid biosynthesis as a differentially regulated pathway (Figure S.10E).

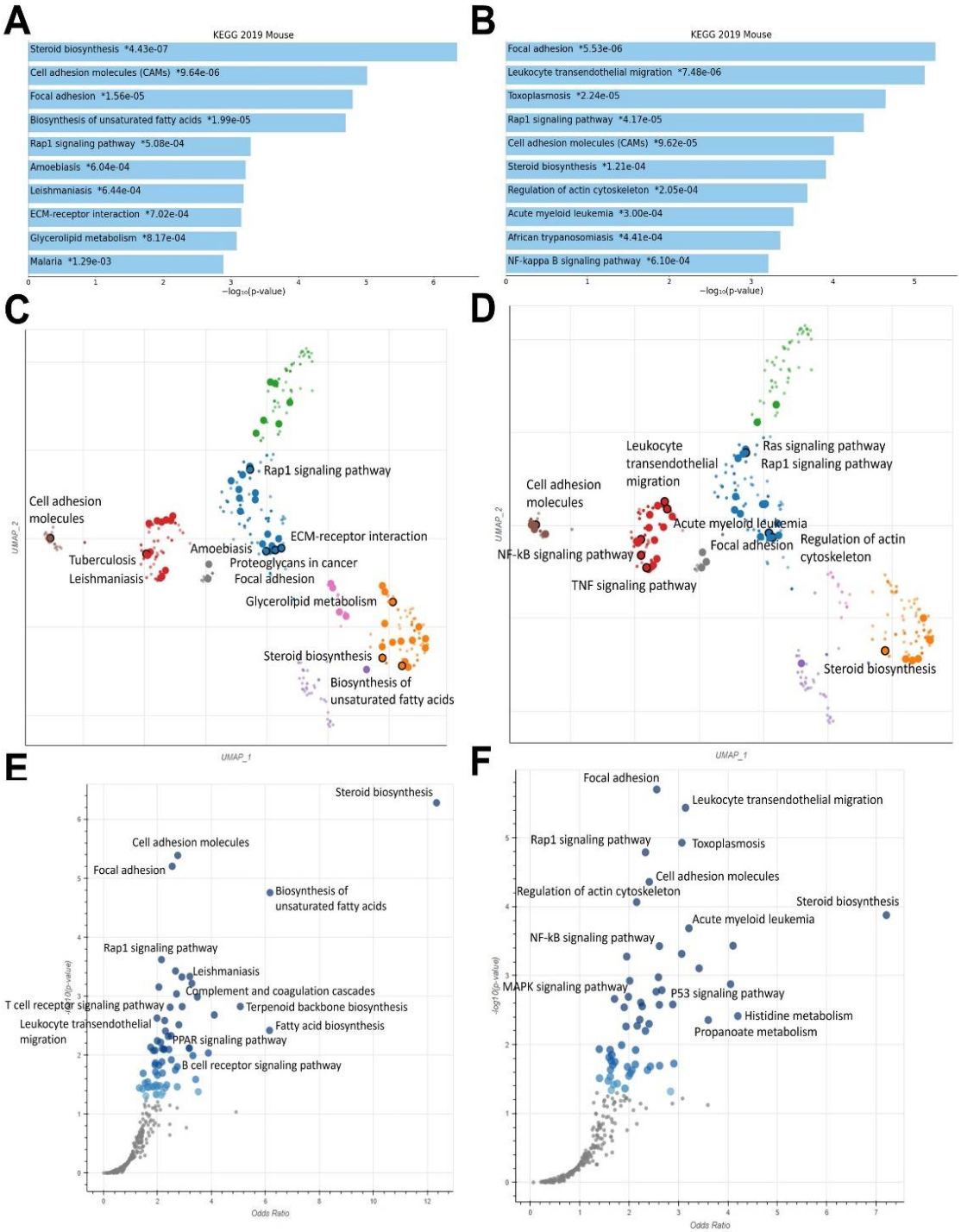

Figure S.10. V-125 and bexarotene enrich both similar and distinct pathways in treated tumors. A-B. Bar graph of differentially regulated pathways in V-125-treated vs control tumors (**A**) and bexarotene-treated vs control tumors (**B**) using the KEGG 2019 Mouse database in Enrichr. C-F. Scatterplot (**C,D**) and volcano plot (**E,F**) depictions of differentially regulated pathways in V-125-treated vs control tumors (**C,E**) and bexarotene-treated vs control tumors (**D,F**).

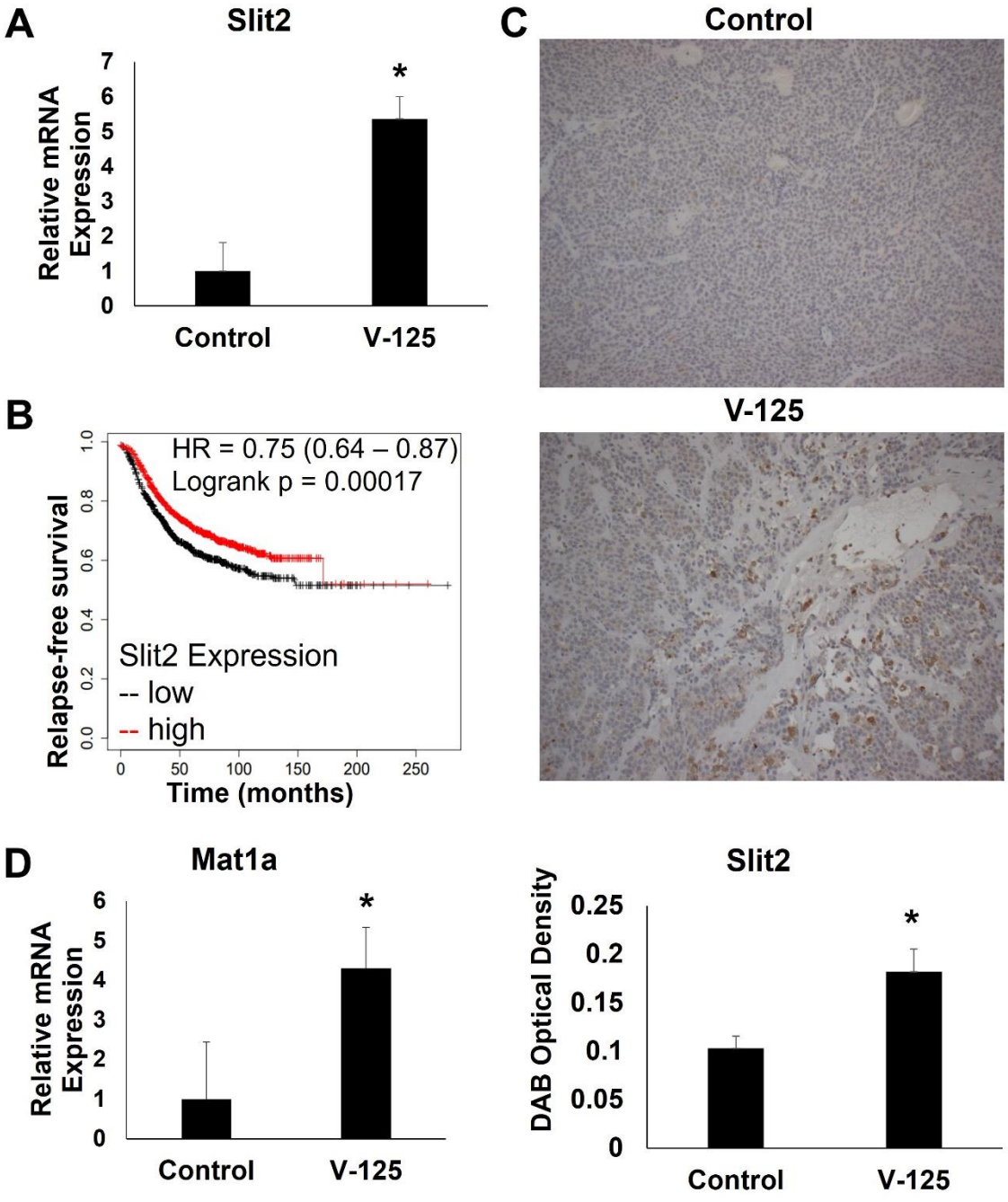

Figure S.11. Novel RXR agonist V-125 regulates immune- and cancer-related genes in mouse mammary tumors. Tumors treated with V-125 (100 mg/kg diet) or control were harvested and flash-frozen. Tumors were homogenized and RNA was extracted. **A, D**. qPCR was performed on QuantStudio 7 Flex. N=5 mice/group. Error bars represent standard error of biological replicates. * $p < 0.05$. **B**. Kmplot survival curve correlates expression of Slit2 with survival in breast cancer patients (n = 2032; 1013 high, 1019 low). **C**. Immunohistochemical staining for Slit2 in MMTV-Neu tumor tissue, representative of 3 mice/group.

Novel RXR agonist V-125 regulates immune- and cancer-related genes in mouse mammary tumors

Following 10 day treatment, tumors were harvested and either flash frozen for RNA Sequencing and qPCR validation, or preserved in formalin for immunohistochemistry. Slit2, a gene upregulated in V-125 tumors by RNA Sequencing, was also found to be significantly upregulated in V-125-treated tumors by qPCR (Figure S.11A) and by immunohistochemistry of sectioned tumor tissue (Figure S.11C). High Slit2 expression is positively correlated with increased relapse-free survival in breast cancer patients, as per Kmplot (Figure S.11B). Mat1a, another gene found to be upregulated in V-125-treated tumors by RNA Sequencing, was also significantly upregulated in tumors evaluated by qPCR (Figure S.11D).

Discussion

The studies in chapter 3 of this dissertation provided the first comparative RNA Sequencing data on tumors treated with novel RXR agonists generated by the lab. The studies contained within this appendix sought to further explore the similarities and differences in gene expression induced by an additional novel RXR agonist, V-125.

Genes identified by differential expression analysis using DESeq2 provides an interesting list of top genes differentially expressed by treatment with V-125, shown in Figure S.8. These genes, NFASC, COLCA2, ALDH1A3, and MGARP, were all altered by V-125 in the direction which is correlated with increased survival in breast cancer patients. Information in the literature suggests that these genes play important roles in tumor biology and the tumor microenvironment. NFASC, or neurofascin, is an adhesion molecule which regulates cell migration in NSCLC cell lines[322]. Outside the setting of

cancer, a subset of patients with chronic inflammatory demyelinating polyneuropathy (CIDP) are characterized as having anti-neurofascin antibodies, which are associated with worsened symptoms such as disabling tremor, poor response to therapy, and overall poor prognosis[323]. As antibodies against neurofascin are critical to prognosis and disease progression in CIDP, this suggests that a drug which regulates the expression of neurofascin might be clinically useful, particularly one which also has immunomodulatory properties. ALDH1A3, also identified as a gene differentially expressed in tumors treated with V-125, is an enzyme involved in retinoid metabolism which has also been implicated in cancer development and progression[324]. Further investigation of the role of ALDH1A3 in mediating not only metabolism of V-125 but also anti-tumor activity may yield interesting data.

Pathways identified as differentially regulated by V-125 include several metabolic pathways, such as steroid biosynthesis, biosynthesis of unsaturated fatty acids, and glycerolipid metabolism. The rapid rates of cellular proliferation involved in cancer progression require increased rates of macromolecule synthesis, and V-125's effect on these processes may indicate that some of the anti-tumor effects of RXR agonist treatment are metabolic in nature. Further investigation into the metabolic effects of RXR agonist treatment is warranted.

As previously noted in chapter 3 of this manuscript, RXR agonist effects on focal adhesion, extracellular matrix, and other pathways associated with cellular adhesion are common themes across the drugs we've studied in this class. Based on this information, some of the anti-tumor activity of RXR agonists may be attributed to their effects on invasion, migration, and metastasis. Further studies on the effects of RXR agonists on

these processes, such as scratch assays and other migration assays, would be a good way to begin evaluating these hypotheses generated through RNA Sequencing.

V-125 induces differential regulation of genes associated with the disease states Amoebiasis and Leishmaniasis. While this may seem unusual, some of the genes in these pathways include ITGAM, TGFβ, TNF, JUN, and IL-4. These genes have known roles in cancer and immunity and would be excellent genes to validate in future mechanistic studies of V-125.

The theme of Rap1 signaling emerging as a RXR agonist-regulated pathway is a potentially exciting observation. We have noted in previous publications[105] that RXR agonists are effective in preclinical cancer models with elevated Ras signaling activity. Rap1, or Ras-associated protein 1, is another GTPase with notable sequence homology to Ras[325]. The role of Rap1 in promotion of cancer invasion and metastasis has been an area of recent interest[326]. Rap1 interacts with other proteins which function in cellular adhesion, proliferation, and cytoskeletal maintenance. Genes in the Rap1 enrichment pathway include RRAS, growth factors, PI3K signaling pathway members, and MAPK pathway members. Studies focused on RXR agonist effects on Rap1 signaling may provide insight into our previous observations about Ras signaling activity and into critical mechanistic features of these drugs.

Tumors treated with V-125 demonstrate a significant ($p = 0.02145$) upregulation of *Slit2* in comparison to control tumors (Figure S.11A). Interestingly, upregulation of *Slit2* was not seen in MSU 42011 or bexarotene-treated tumors (data not shown). Furthermore, high expression of *Slit2* is correlated with increased survival in breast cancer patients (Figure S.11B) and is relevant to anti-tumor immunity. V-125 also

induced a significant upregulation of *Mat1a*, which codes for a metabolic enzyme which is involved in cellular response to oxidative stress by catalyzing the formation of the methyl donor S-adenosylmethionine (SAM)[327] (Figure S.11D). As methylation is required for epigenetic regulation of gene transcription, V-125-mediated upregulation of *Mat1a* may have significant implications for cancer treatment. In the liver, SAM deficiency creates oxidative stress, and *Mat1a* knockout mice have an increased susceptibility to developing hepatocellular carcinoma[328]. Low levels of MAT1A are found in precancerous lesions in a rat model of liver carcinogenesis, and this downregulation can be overcome by treatment with the retinoid N – (4 – hydroxyphenyl)retinamide (HPR), possibly due to crosstalk between MAT1A and NF-κB, and the effects of retinoids on NF-κB related pathways[329]. The upregulation of *Mat1a* induced by V-125 treatment may provide insight into the mechanism of anti-tumor activity of V-125.

Slit2, also upregulated in tumors treated with V-125, plays an established role in anti-tumor immunity. *Slit2* is inactivated in breast and lung cancer, preventing SLIT2 signaling through the Robo1 receptor[259]. SLIT2 activates M1-like tumor-associated macrophages in the tumor microenvironment and modulates IL-6 and matrix metalloprotease expression[330]. This confirmed upregulation of *Slit2* expression indicates that macrophages may play an important role in mediating the anti-tumor activity of V-125. Future studies are needed to determine if V-125's effects on macrophages are necessary for the anti-tumor activity of the compound, such as an *in vivo* macrophage depletion in MMTV-Neu mice treated with V-125. Furthermore, the role of SLIT2 in tumors treated with V-125 can be confirmed by *Slit2* conditional knockout, and analysis

of the treatment efficacy of V-125 in wild-type vs. *Slit2* knockout mice, as well as analysis of macrophage polarization markers in wild-type vs. *Slit2* knockout mice.

In conclusion, RNA Sequencing data in this appendix adds to the pool of genomic data collected in this dissertation regarding the effects of RXR agonists on gene expression in mammary tumors. This information will aid us in developing hypotheses to further explore the mechanisms of anti-tumor activity of RXR agonists.