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27-HYDROXYCHOLESTEROL PROMOTES BREAST CANCER PROGRESSION
THROUGH THE MODULATION OF MYELOID CELLS

BY

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DISSERTATION

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ABSTRACT

Breast cancer remains one of the leading causes of cancer mortality in the US. Elevated cholesterol is a major risk factor for breast cancer onset and recurrence, while cholesterol-lowering drugs, such as statins, are associated with a good prognosis. Previous work in murine models showed that cholesterol increases breast cancer metastasis, and the pro-metastatic effects of cholesterol were due to its primary metabolite, 27-hydroxycholesterol (27HC). Myeloid cells were found to be required for the pro-metastatic effects of 27HC, but their precise contribution remains unclear. We show that 27HC impairs T cell expansion and cytotoxic function through its actions on myeloid cells, including macrophages, in a liver X receptor (LXR)-dependent manner. Many oxysterols and LXR ligands had similar effects on T cell expansion. Moreover, their ability to induce the LXR target gene ABCA1 was associated with their effectiveness in impairing T cell expansion. Induction of T cell apoptosis was likely one mediator of this impairment. Interestingly, the enzyme responsible for the synthesis of 27HC, cytochrome P450 family 27 subfamily A member 1 (CYP27A1), is highly expressed in myeloid cells, suggesting that 27HC may have important autocrine or paracrine functions in these cells, a hypothesis supported by our finding that breast cancer metastasis was reduced in mice with a myeloid specific knockout of CYP27A1. Importantly, pharmacologic inhibition of CYP27A1 reduced metastatic growth and improved the efficacy of checkpoint inhibitor, α PD-L1. Previous reports also showed protective effect of synthetic activation of LXR in cancer models. To address this paradox, RNA sequencing of macrophages treated with 27HC and synthetic ligands of the LXR and estrogen receptor (ER) revealed the selective LXR modulatory potential of 27HC, similar to desmosterol, compared to the synthetic agonists/antagonists. Inside breast tumor microenvironment, single-cell RNAseq showed that 27HC also exerted distinct effects on even closely related myeloid-derived immune populations, compared to GW3965. Taken together, our work suggests that targeting the CYP27A1 axis in myeloid cells

may present therapeutic benefits and improve the response rate to immune therapies in breast cancer. It is also possible to devise synthetic selective modulators of LXR to augment the beneficial effects of its activation, while avoiding 27HC-driven immunosuppression. Our work ultimately provided a foundation for the future development of cancer therapeutics targeting cholesterol-LXR axis.

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LIST OF ABBREVIATIONS

A

ABC	ATP-Binding Cassette
Ag	Antigen
α PD-1	Anti-Programmed Death 1 Antibody
α PD-L1	Anti-Programmed Death-Ligand 1 Antibody
APC	Antigen-Presenting Cell
ARG1	Arginase 1
ATCC	American Type Culture Collection

B

BMDM	Bone Marrow-Derived Macrophages
BSA	Bovine Serum Albumin

C

CFA	Complete Freund's Adjuvant
CYP27A1	Cytochrome P450 Family 27 Subfamily A Member 1

E

ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen Receptor

F

FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum

G

GSEA	Gene-set Enrichment Analysis
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H

HER2	Human Epidermal Growth Factor Receptor 2
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I

IACUC	Institutional Animal Care and Use Committee
IDO1	Indoleamine 2,3-Dioxygenase 1
IFN	Interferon
IL	Interleukin

L

LPS	Lipopolysaccharide
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LXR	Liver X Receptor
SLXRM	Selective Liver X Receptor Modulator

M

M-CSF	Macrophage Colony Stimulating Factor
MHC	Major Histocompatibility Complex
M-MDSC	Monocytic Myeloid-Derived Suppressor Cells
MMP	Matrix Metalloproteinase

O

OVA	Ovalbumin
-----	-----------

P

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PR	Progesterone receptor

R

RNAseq	RNA sequencing
ROS	Reactive oxygen species

S

SERM	Selective ER modulator
shRNA	short hairpin RNA

T

TAM	Tumor-Associated Macrophages
TCGA	The Cancer Genome Atlas
TCR	T Cell Receptor
TGF	Transforming Growth Factor
TNBC	Triple Negative Breast Cancer
TNF	Tumor Necrosis Factor
Treg	T Regulatory Cell

V

VEGF	Vascular Endothelial Growth Factor
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W

WGCNA	Weighted Correlation Network Analysis
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Other

4 β HC	4 β -Hydroxycholesterol
7 β HC	7 β -Hydroxycholesterol
7KC	7-Ketocholesterol

20(S)HC	20(S)-Hydroxycholesterol
22(R)HC	22(R)-Hydroxycholesterol
24(S)HC	24(S)-Hydroxycholesterol
24,25EC	24,25-Epoxucholesterol
25HC	25-Hydroxycholesterol
27HC	27-Hydroxycholesterol

CHAPTER 1

INTRODUCTION

1.1 Breast Cancer

Among women in the United States, breast cancer is one of the the most common cancers and the second leading cause of cancer-related death (1). Similarly in UK, 1 in 12 women develops breast cancer (2). Worldwide, over 500,000 female lives were taken by breast cancer and 1.7 million cases were recorded in 2016 (3). Breast cancer is also heterogeneous in nature, comprising a variety of subtypes marked by distinct biological or pathological features, which then greatly impact treatment responses and prognosis (4). Traditionally and still used today, clinical and pathological information, including patient age, histological grade (tubule formation, nuclear pleomorphism and mitosis rate), tumor stage, and hormone receptors status have been used to stratify patients for targeted treatments (5, 6). Even though traditional stratification has provided prognostic value, a classification system incorporating the biological characteristics, rather than solely tumor morphology, could potentially further improve the prediction of clinical prognosis and drug response. First proposed by Perou *et al.*, the advent of molecular classification of breast cancer was made possible by DNA microarray data based on the tumor biology (5, 7). Based on gene expression profiles, breast cancer can be classified into 3 major subtypes: Luminal, HER2-positive and basal. Later, Kapp *et al.* found that these 3 major subtypes could be stably classified by solely using the genes associated with phenotypes of ER and HER2 as: ER-/HER2- (basal-like), HER2+(HER2-enriched), and ER+/HER2- (luminal A and B combined) (5, 8).

1.1.1 Breast Cancer Subtypes

The luminal A subtype is the most common subtype of breast cancer, representing 30-40% of all invasive breast cancers, and is often associated with a good prognosis (9). The status of luminal A subtype is defined by ER+ and/or PR+, HER2- and Ki67^{low} by immunohistochemistry. Like luminal A, luminal B subtype is most commonly ER+ (4). However, the HER2 status may vary. Contrary to luminal A, luminal B tumors are Ki67^{high} (4). This tumor type therefore is associated with poorer prognosis, in comparison to luminal A, and contributes to 20-30% of invasive breast cancer cases (9). HER2-enriched subtype accounts for 12-20% of breast cancer cases, and is generally more aggressive than luminal subtypes. As its name suggests, this subtype is characterized by high level of HER2 expression. The basal-like subtype are triple negative for the expression of ER, HER2 and PR, and therefore is also known as TNBC. Accounting for approximately 15-20% breast cancers, basal-like subtype has been associated with poor prognosis and higher risk of recurrence. Although all subtypes could develop distant metastasis, basal-like tumors have the lowest median duration of survival with distant metastasis (0.5 years) (10). Except for basal-like tumors, bone is the most prevalent metastatic site. More commonly, basal-like tumors develop brain, lung, and distant nodal metastases (10).

1.1.2 Breast Cancer Clinical Management

Endocrine therapy is the first line of defense for luminal subtypes of breast cancer, which works by blocking the effects of hormone or lowering the hormone level (11). Five types of endocrine therapies are commonly adopted: 1. ER modulator/degrader (e.g., tamoxifen and fulvestrant), 2. aromatase inhibitor (e.g., letrozol), 3. luteinizing hormone-releasing hormone (LHRH) analogs (e.g., goserelin). 4. In the event of developing resistance to hormonal therapy, CDK4/6 inhibitor and mTOR inhibitor are developed to be used in combination with hormonal therapies. 5. PI3K inhibitors (alpelisib) (11, 12).

Antibodies or small molecule inhibitors against HER2 receptors have been widely employed alone or as adjuvant therapy in HER2-enriched breast tumors (11). These include anti-HER2 monoclonal antibodies (e.g., trastuzumab) and tyrosine kinase inhibitors (e.g., lapatinib). Development of resistance to trastuzumab are observed in patients with progressive disease. In that case,

many novel therapies, such as PI3K/mTOR inhibitors, HER-family inhibitors, farnesyl transferase inhibitors, are under clinical trials to be used in conjunction with trastuzumab (11, 13).

In basal-like tumors, chemotherapy with surgery remains the standard of care. However, patients may also benefit from recent development of novel therapies. PARP inhibitors are beneficial for treating advanced stage TNBC with BRCA1 and BRCA2 mutation (14). Unlike other types of breast cancer, basal-like tumors that are PD-L1+ are the only one approved for immunotherapy against PD-1 and PD-L1. A study showed that atezolizumab (α PD-L1 antibody) with nab-paclitaxel significantly improved the overall survival, compared to chemotherapy alone (15). Nevertheless, despite durable therapeutic efficacy, only a subgroup of patients are responsive to checkpoint blockade, rendering the overall response rate of advanced TNBC to checkpoint inhibition alone or in combination with chemotherapy ranging from 5% to 56% (16).

1.2 Oxysterols and 27-Hydroxycholesterol

Oxysterols are derivatives of cholesterol produced by the oxidation of cholesterol molecules by enzymatic addition of hydroxyl, carbonyl or epoxide functional groups (17). Endogenous oxysterols are commonly produced by non-enzymatic mechanisms (18). Specifically, ROS, including alkoxy and peroxy radicals, can oxidize cholesterol molecules (17, 19). Cholesterol can also be oxidized by the leukocyte- H_2O_2 -HOCl system during inflammation (19). Non-enzymatic oxidation is primarily responsible for the generation of certain oxysterols such as 7KC and 7 β HC (19). However, producing oxysterols by the oxidation of cholesterol on the side-chain is typically catalyzed through enzymatic routes, which are mediated by cytochrome p450 family of enzymes (20, 21). 27HC is synthesized through this route by CYP27A1. Other examples include 24HC by CYP46A1, 25HC by cholesterol-25-hydroxylase and 22HC by CYP11A1 (19, 20) (**Figure 1.1**).

27HC has been described to bind to and modulate the activity of the LXRs, thereby completing an important homeostatic loop in cholesterol metabolism (described below). Besides, several recent studies have implicated 27HC in the modulation of other nuclear receptors such as the ERs, as well as non-nuclear receptor mechanisms. Furthermore, it is becoming increasingly apparent that 27HC play important roles in several pathologies, including cancers, cardiovascular diseases,

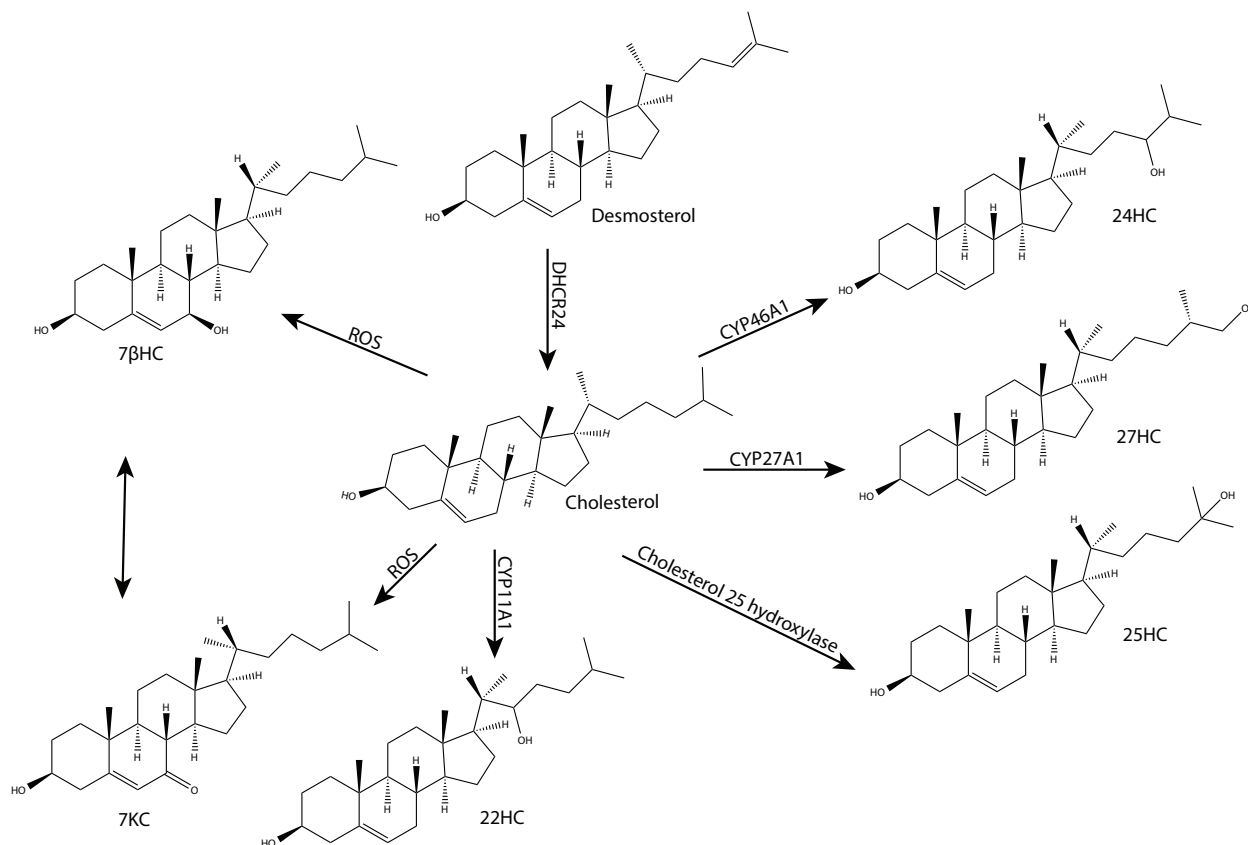


Figure 1.1: Chemical structures and metabolic pathways for several common oxysterols.

and neurodegenerative diseases (22).

1.2.1 Oxysterols, 27-Hydroxycholesterol and LXR Signaling

Of the nuclear receptors, the liver X receptors (LXRs) are thought to be the major target of oxysterols, especially in terms of the regulation of cholesterol metabolism. LXR α (NR1H3) and LXR β (NR1H2) are isoforms of LXR, which serve as cholesterol sensors for the regulation of cholesterol excess¹². LXR β is expressed fairly ubiquitously, while LXR α expression is highest in the liver, with lower expression to various degrees in other tissues^{13,14}. The two isoforms share significant sequence identity, and although speculated, clear evidence of specific biological differences have not been well described. The LXRs are known to heterodimerize with the retinoid X receptors (RXRs) and sit on various DNA response elements, with a direct repeat 4 (DR4) being recognized as the consensus sequence (5-AGGTCA-NNNN-AGGTCA-3) (23–25). In an unliganded state, it is thought that the heterodimer interacts with transcriptionally repres-

sive complex, such as with nuclear receptor corepressor (NCoR), thereby transrepressing the transcriptional activity of LXR (26–28). The transrepression elicited by NCoR complex plays an important role in LXR-mediated anti-inflammatory pathways, such as suppression of Toll-like receptor (TLR)-dependent pro-inflammatory signal expression in macrophages (29, 30). Upon ligand binding, corepressor proteins are exchanged for coactivators, including NAD⁺-dependent deacetylases Sirtuin 1 (SIRT1), which allows the RNA polymerase II complex to associate and initiate transcription (27, 28).

The first endogenous ligands to be described for the LXRs were 22(R)HC, 24(S)HC, and 24,25EC (31). Since then, it has been demonstrated that several oxysterols and certain precursors in the cholesterol-biosynthetic pathway can modulate the activity of the LXRs. These include 22(R)HC, 25HC, 27HC, 24(S)HC, 24,25EC and desmosterol (32–36). These metabolites have a wide range of affinities or potencies, with 24(S)HC having an EC₅₀ of 4 μ M and 3 μ M compared to 27HC at 85 nM and 71 nM for LXR α and LXR β , respectively (31, 36). For reference, 27HC is the most abundant oxysterol in circulation with concentrations ranging from 67 ng/mL (0.17 μ M) to 199 ng/mL (0.5 μ M) while 24(S)HC circulates from 39 ng/mL (0.1 μ M) to 91 ng/mL (0.23 μ M) (37).

Given the hepatic expression of CYP7A1, and more ubiquitous expression of CYP27A1 (with significant expression observed in cells of the myeloid immune lineage), it would be expected when cholesterol levels are high, so too is its metabolism into oxysterols. Indeed, there is a positive association between circulating cholesterol levels and 27HC in both mice and humans, while statin therapy is associated with decreased plasma 27HC (38, 39). Furthermore addition of cholesterol to macrophages resulted in increased secretion of 27HC, while inhibiting cholesterol synthesis with simvastatin abolished the production of 27HC by macrophages (40, 41). Although it is difficult to account for esterification, these studies suggest that increased level, of cholesterol could lead to increased production of 27HC, which can then engage LXRs. Upon binding of an endogenous ligand to the LXR-RXR heterodimer, the corepressor complex dissociates with commensurate recruitment of coactivation complexes, ultimately leading to a transcriptional program aimed at restoring cholesterol homeostasis. Specifically, LXR activation leads to the induction of target genes involved in cholesterol efflux (ABCA1/G1/G5/G8), apolipoproteins (APOA-1/E), cholesterol metabolism into bile acids (CYP7A1) and decreased intestinal cholesterol ab-

sorption (42). The removal of cholesterol by reverse cholesterol transport is facilitated by ABC transporters. ABCA1, expressed in multiple cell types, promotes the efflux of free cholesterol to apolipoprotein, in particular APOA1, leading to the formation of discoid lipid-poor HDL (24, 43). ABCG5 and ABCG8 are half transporters expressed on the apical membrane of enterocyte and on the canalicular membrane of hepatocytes (44). The formation of heterodimer by ABCG5 and ABCG8 on hepatocytes can promote the secretion of cholesterol into bile, while on enterocytes can facilitate the excretion of cholesterol back into intestinal lumen (23, 44). LXR activation is also involved in regulating lipid homeostasis by promoting triglyceride synthesis. Genes upregulated in this process are sterol regulatory element-binding protein 1, acetyl-CoA carboxylase, fatty acid synthase and stearoyl-CoA desaturase-1 (43, 45, 46). The relatively longer-term actions of LXR-mediated homeostasis work in concert with the quicker actions of cholesterol and oxysterols in maintaining sterol regulatory element-binding proteins (especially SREBP 2) in an inactive state bound to the endoplasmic reticulum, thereby reducing the transcription of genes associated with cholesterol metabolism and import (47).

1.2.2 Oxysterols, 27-Hydroxycholesterol and ER Signaling

Oxysterols were definitively found to bind to and modulate the activity of the estrogen receptors just over 10 years ago (48, 49). There are two main subtypes of nuclear estrogen receptors in mammalian cells, ER α (NR3A1) and ER β (NR3A2). A membrane-associated G protein-coupled estrogen receptor 1 (GPER1, or GPR30) has also been described, but whether oxysterols can interact with this receptor remains unknown (50, 51). Nuclear ER signaling comprises ligand binding to ER, followed by dissociation of the heat shock protein from ER, revealing nuclear localization signal, and homo- or hetero-dimerization of two ER monomers (50). ER dimers then bind to the respective estrogen response element of the target gene, recruiting co-activators and/or co-repressors, leading to target gene transcription, including trefoil factor 1 (TFF1) and progesterone receptor (PR) (50, 51). So-called non-genomic or rapid ER signaling also exists, where cytosolic ERs engage in kinase-mediated signaling cascades including mobilization of intracellular calcium, stimulation of adenylate cyclase activity and epidermal growth factor receptor activation, ultimately resulting in rapid cellular changes (22, 51).

As early as in 2004, oxysterols, including β -epoxycholesterol, 20(S)-hydroxycholesterol and 22(R)HC, were shown to have estrogenic activity in ER α -expressing HeLa cells (52). More recently, a Gal4-ER co-transfection screen of several cholesterol metabolites revealed that 22(R)HC, 24(S)HC, 25HC and 27HC significantly inhibited estradiol (E2) activation of both ER α and β , with IC50s ranging from 1-5 μ M (49). 27HC was subsequently shown to be a selective ER modulator (SERM), with the ability to induce distinct ER conformational changes and to manifest either activating or inhibitory activities, depending on the tissue and cellular context (48). 27HC was found to activate the classic ERE-Luciferase reporter (3XERE-TATA-Luc) in transfected HeLa cells, with an EC50 of approximately 1 μ M, and when co-treated with estradiol had a Ki of 1.32 μ M (48). Demonstrating its selective-modulatory capabilities, it could activate the ER to promote the cellular proliferation of ER+ breast cancer cell lines, tumors or uteri, but inhibited estrogen-dependent expression of vascular nitric oxide synthase, thereby repressing carotid artery reendothelialization (48, 49, 53, 54).

Other oxysterols or cholesterol metabolites besides 27HC have also been found to modulate ER activities. 7KC was shown to decrease the cytotoxicity of doxorubicin in MCF7 cells, and this effect was dependent on ER α , as fulvestrant inhibition or ER α knockdown restored doxorubicin accumulation in MCF7 cells (55). 7KC also induced the expression of TFF1, an ER target gene (55). Structurally similar to 27HC, 25HC upregulates ER target genes, including TFF1, PR, cathepsin D, cyclin A and cyclin D1 in MCF7 and BG1 cells (56). Furthermore, 25HC promotes ER+ breast cancer cell proliferation and inhibits hypoxia-inducible factor-1 α (HIF-1 α) expression induced by hypoxia in cardiomyocytes to prevent cell apoptosis (56).

Subsequent studies have further confirmed the selective modulator capacity of 27HC. In hematopoietic stem cells (HSCs) during pregnancy, 27HC could induce ER α -dependent HSC mobilization and extramedullary hematopoiesis (57). In a cancer cell model, 27HC enhanced ER-mediated transcriptional activity and the recruitment of ER to TFF1 and growth regulating estrogen receptor binding 1 (GREB1) response elements in an ER+ cancer cell line (MCF7) adapted to long-term estrogen deprivation (58). However, in cardiovascular cells, 27HC reversed the cardioprotective effect of estrogen by inhibiting estrogen-induced nitric oxide production by vascular cells, indicating an antagonistic activity against ER (59). 27HC exhibited a partial-agonist effect on osteoblasts, but ultimately exerted a negative impact on bone quality that could be attenuated

by exogenous estrogen (60, 61). The negative effects on bone are likely a combination of signaling through the ERs and LXRs, and 27HC has been recently been reported to be associated with decreased bone mineral density in postmenopausal women when circulating estrogens are taken into account (62). Moreover, 27HC could serve as a partial agonist to ER in ER+ breast cancer cell lines other than MCF7 cells, including T47D and BT483 (48, 53). It has also been suggested that 27HC antagonizes the neuroprotective effects of estrogen signaling through ER α , as an increase in 27HC concentration in the brain is associated with increased neurodegeneration of the hippocampus, decreased expression of ER and a decreased level of hippocampal mitochondria (63). 27HC was also shown to exert effects through ER β : not only was ER β expression upregulated by 27HC in prostate cancer cell lines, LNCaP and PC3, but the 27HC-induced proliferation of prostate cancer cells was attenuated by the ER β -specific inhibitor, PHTPP (64).

1.3 Oxysterols, 27-Hydroxycholesterol and Cancer

Since 27HC was first described to be a selective estrogen receptor modulator, the effect of this oxysterol on breast cancer has now been extensively studied in human and pre-clinical models, which is not surprising given that approximately 60-75% of all breast cancers are ER α +. Enzymes responsible for generating and catabolizing 27HC has played documented roles in various tumor development. Higher level of CYP27A1 protein expression was associated with higher breast tumor grade, and reduced CYP7B1 mRNA expression was associated with increased intratumoral 27HC in thyroid cancer and was associated with a worse prognosis in breast and thyroid cancer, indicating a clinically relevant role for 27HC in cancer progression (22, 53, 65). Interestingly, studies revealed that 27HCs effects on tumor progression were both cancer cell-intrinsic and extrinsic.

1.3.1 Oxysterols, 27-Hydroxycholesterol and Cancer-intrinsic Roles

The ERs are a critical regulator of innumerable physiological functions and play recognized or suggestive roles in cancer progression of various cancer types, such as breast, lung and brain cancer (66–69). Since the discovery of 27HC as a natural ligand of the ERs, it has raised a possibility that 27HC could also play a number of important physiological roles by signaling

through this receptor. In 2008, soon after recognizing 27HC as an endogenous SERM, DuSell *et al.* first reported that 27HC could undertake similar roles as E2 in breast cancer cell lines, which promotes ER activation and subsequent cellular proliferation of ER α + cell lines (48). This suggests that 27HC signaling through ER α could facilitate cancer progression by cancer cell-intrinsic mechanisms. In support of this notion, Wu *et al.* found that 27HC induced ER α + breast cancer cell proliferation *in vitro*, and a selective ER α antagonist, methyl-piperidino-pyrazole, ablates this effect, suggesting the requirement of ER α for 27HC-driven breast cancer cell proliferation (54). Additionally, 27HC also stimulates the growth of ER α + breast tumors in preclinical mouse models (53, 54). The growth of MCF7 xenografts was stimulated by exogenous 27HC, and this effect was attenuated by co-treatment with the ER antagonist Fulvestrant (53). Germline deletion of CYP27A1 increased latency and decreased tumor growth in MMTV-PyMT mice, while loss of CYP7B1 (and subsequent increased circulating 27HC) increased growth (53). 7KC also plays a role in breast cancer in that 7KC decreased the cytotoxicity of doxorubicin in ER+ breast cancer cell line MCF7 by upregulating P-glycoprotein expression, while this effect was not observed in ER- cell line, MDA-MB-231 (55). The requirement of the ER α in 7KC's effect was further illustrated by knocking down ER α with siRNA, which restored the doxorubicin accumulation in MCF7 (55).

While the effects of 27HC and 7KC on breast cancer cells required ER α , ER β may also play an important role in 27HC signaling in cancer types. Raza *et al.* demonstrated that 27HC promoted prostate cancer cell proliferation *in vitro* in LNCaP and PC3 cells through the ER. Specifically, 27HC induced proliferation was significantly reduced in the presence of the ER inhibitor, ICI 182,780 (fulvestrant) (64). Likewise, the ER β -selective antagonist PHTPP reversed the proliferative effect of 27HC on prostate cancer cells, indicating that 27HC signals through ER β to promote their proliferation⁶⁵. However, these results were contradicted by another study, indicating that 27HC actually decreased the proliferation of prostate cancer cells, via an SREBP2-mediated mechanism (70). Furthermore, the expression of CYP27A1 was downregulated in prostate cancers compared to normal tissue, and elevated CYP27A1 expression is associated with a better prognosis for prostate cancer patients. Further work will be required to better characterize the impact of oxysterols on prostate cancer.

In addition to breast and prostate cancer, 27HC was shown to affect lung cancer develop-

ment. Using H1395 cell line as a lung cancer model, Hiramitsu *et al.* found that 27HC-advanced ER β + lung cancer cell proliferation via phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling (71). Chen *et al.* described the pro-metastatic activity of 25HC in lung adenocarcinoma (72). In A549 cells and NCL-H1975 cells, 25HC treatment enhanced Snail expression, a marker of EMT, in an LXR-dependent manner, which promoted invasion of those cancer cells (72).

Although many oxysterols have been reported to contribute to cancer progression, cholestane-3 β , 5 α , 6 β -triol (triol) was found to have anti-cancer activity (73). Not only was the triol treatment able to inhibit the growth of LNCaP CDXR3, DU145 and PC3 prostate cancer cell lines, but also oral administration of this oxysterol was able to slow the progression of xenograft PC3 in NUDE mice (73). Triol treatment also inhibited the invasive capacity of DU145 and PC3 cells and down-regulated the epithelial to mesenchymal transition-related genes, including N-cadherin, vimentin, Slug, focal adhesion kinase (FAK), phospho-FAK Ser722 and phospho-FAK Tyr861 (73). Another LXR modulator, dendrogenin A, was reported by de Medina *et al.* to possess anti-tumor property *in vivo* (74). This cholesterol metabolite was produced by 5,6 α EC reacting with histamine and was significantly downregulated in breast tumor, compared to normal tissues (74, 75). Treating cancer cells with dendrogenin A promoted cancer cell re-differentiation, inhibited OCDO production and induced lethal cancer cell autophagy (74, 75).

Interestingly, recent work has implicated LXR as a mediator of chemoresistance in ER-breast cancer (76). Both the synthetic ligand of LXR, GW3965, and oxysterols, including 27HC, were able to diminish the effect of epirubicin in TNBC cell lines, which could be attributed to the upregulation of chemotherapy efflux pump Pgp in an LXR α -dependent manner (76). An LXR α target gene signature was correlated with Pgp in human breast tumor samples. On the other hand, *in vitro* work indicated that 27HC did not alter sensitivity to doxorubicin in MCF7 cells (55). In ovarian cancer however, 27HC was also shown to confer chemoprotective property in mice grafted with MOE cells (77).

1.3.2 Oxysterols, 27-Hydroxycholesterol and Cancer-extrinsic Roles

There is rising awareness of the tumor microenvironments roles in cancer development and progression, and growing number of studies have revealed cancer-extrinsic factors involved in

dictating disease progression and response to therapy. Of those, tumor-infiltrating immune cell populations have been shown to have high predictive value in disease prognosis, even compared to traditional indicators in breast cancer, colorectal cancer, melanoma and pancreatic cancer (78–81). α PD1 (Pembrolizumab) and α PDL1 (Atezolizumab) are now approved for the treatment of several solid tumors, including a subset of patients with advanced TNBC (82–84). However, these two immune checkpoint inhibitions have limited efficacy in the treatment of many solid tumors, including breast and ovarian. The efficacy of immune checkpoint inhibitors relies on the functionalities of the host immune system and studies have shown that immune cell infiltration is associated with the response to immunotherapy (85–88). Therefore, it was of interest as to whether 27HC could modulate immune cell recruitment or function within the tumor microenvironments.

Previous work showed that human and mouse tumor-derived LXR ligands could inhibit C-C chemokine receptor 7 (CCR7) expression on mature dendritic cells, thereby hindering their migration to the lymphoid organs (89). These findings back the possibility of 27HC, as an LXR ligand, acting on the cells of myeloid origin at the tumor site during tumor progression. In support of this, Baek *et al.* showed that 27HC treatment of metastasis-bearing mice led to increased myeloid cell infiltration, both inflammatory monocytes and polymorphonuclear cells (PMNs), and a decrease of intratumoral CD8⁺ cytotoxic T cells (90). Follow-up experiments found that the pro-metastatic effect of 27HC required myeloid immune cells, and specifically PMNs. $\gamma\delta$ -T cells were also found to be critical, likely playing a role in conditioning the PMNs to be pro-tumorigenic (90).

Interestingly, in models of ovarian cancer, it was found that inflammatory monocytes (monocytic myeloid-derived suppressor cells, M-MDSCs, defined as CD11b⁺Ly6C⁺Ly6G⁻) and immature macrophages (CD11b⁺Ly6C⁺IA/IE⁺) were enriched post administration of 27HC (77). Tumors grown in CYP27A1^{-/-} mice failed to thrive and regressed to non-detectable levels in many of the CYP27A1^{-/-} mice. Bone marrow-derived cells were implicated as a marrow transplant from wildtype mice allowed tumors to establish and grow in CYP27A1^{-/-} mice. These results strongly implicate the immune system in mediating the effects of 27HC on cancer progression. They also support the notion that 27HC can work through different myeloid immune cell types to suppress adaptive immune function.

In preclinical models, mice lacking CYP27A1 in the myeloid cell population developed smaller breast tumors and exhibited less metastasis, indicating that targeting the production of

27HC or the cholesterol-27HC-LXR axis may attenuate the suppressive effects of 27HC in the tumor microenvironment. Such attenuation may eventually improve checkpoint inhibition efficacy and reduce tumor progression. Indeed, one study found that administration of a CYP27A1 inhibitor along with checkpoint inhibitor, α PDL1, was effective in reducing ovarian tumor burden in mice (77). Recent findings by Yu *et al.* also support the possible influence of cholesterol and/or cholesterol metabolites on macrophage-driven T cell death, as the study showed that F4/80+ cells isolated from liver metastasis, a high cholesterol microenvironment, induced apoptosis of OT-1 T cells (91). Another group of researchers showed that an inverse agonist of LXR promotes CD8+ T cell activity and enhances tumor destruction, although whether these effects were myeloid cell-driven was not investigated (92). Furthermore, inhibiting cholesterol synthesis by zaragozic acid A, which decreased LXR target gene expression in the tumor microenvironment, potentiates the tumoricidal ability of antitumor T cells *in vivo* (93). Similarly, blocking PCSK9 to reduce cholesterol metabolism synergized with immune checkpoint blockade, α PD1, to reduce the tumor burden of B16F10, MC38, 4T1 or CT26 models, some of which are known to be resistant to immune checkpoint monotherapy (94). Subsequent analysis showed that depleting PCSK9 could promote intratumoral infiltration of T cells (94). These data provide robust support for developing therapeutic targets along the cholesterol metabolic axis (cholesterol-CYP27A1-27HC-LXR) for bolstering the immune response to various cancers.

However, LXR signaling in myeloid cell populations and the subsequent effects on immune function may be ligand- and context-specific. Several studies also suggested the immune-protective roles of LXR acting through the myeloid cells. In 2018, Tavazoie *et al.* demonstrated that agonism of LXR led to decreased MDSC abundance in murine melanoma models as well as in human samples. This same ligand improved checkpoint blockade efficacy in the B16F10 mouse model (95). Recent work also showed that pharmacological activation of LXR using T0901317 downregulates the expression of Ccl7 in macrophages, a Treg-attracting chemokine (96). Another study suggested that activation of LXR through synthetic ligand, GW3965, could promote natural killer (NK) cell-mediated cytotoxicity against cancer cells (97). Outside cancer, an LXR agonist was found to augment the pro-inflammatory signal in human monocytes trained by Bacillus Calmette-Guerin (BCG) vaccine (98). However, an earlier study also suggested that activation of LXR through tumor-derived factors (oxysterols) dampened the maturation and therefore the

anti-tumor response of dendritic cells (89).

The conflicting roles of LXR in immunoregulation and anti-tumor functions could be due to several reasons: 1. the consequence of LXR activation is likely context-dependent, being tissue-type and cell-type specific. 2. Cholesterol metabolite signaling (e.g., 27HC) may result in the crosstalk between multiple receptors, so the observed effects may not be solely accredited to the activation of LXR. 3. LXR activation by synthetic agonists could yield a protective effect, yet natural ligands (oxysterols and cholesterol metabolites) may lead to pro-tumor consequences due to their selective modulatory activities (99). 4. There are possible off-target effects of synthetic LXR agonist that render enhanced immune surveillance and anti-tumor outcome (100). Nevertheless, further investigations are needed to better understand the immunoregulatory roles of LXR and its relationship to anti-tumor functions of immune cells. It is also important to delineate the differences in LXR activation and downstream signaling cascade driven by 27HC and other cholesterol metabolites versus the synthetic ligands in various tissue types and cancer types to better understand the pro-tumor and anti-tumor paradox of LXR.

1.3.3 Hypothesis and Objectives

In summary, 27HC's requirement of myeloid immune cells to exert pro-metastatic effects and the sometimes paradoxical effects of 27HC and GW3965 on cancer progression led me to hypothesize that 27HC modulates the myeloid immune cells to promote breast cancer progression, through its target nuclear receptors, ER and/or LXR, and this effect could be at least in part an outcome of the selective LXR modulatory capacity of 27HC, therefore distinctive to the effect of synthetic agonists, such as GW3965. In this thesis, I will illustrate how 27HC modulates macrophages to affect tumor progression in an LXR-dependent manner. I also will demonstrate that 27HC likely modulates the macrophages and other myeloid populations differentially from GW3965, potentially explaining the distinct yet paradoxical tumor progression outcomes driven by 27HC and GW3965.

I expect that the outcome of this study will validate the implementation of the preventative intervention for breast cancer progression by targeting the enzyme (CYP27A1) that is required for the production of 27HC (101). The current treatments targeting receptors responsible for breast

cancer mainly focus on the inhibition of tumor growth, while modulating tumor microenvironment to suppress tumor spread and recurrence may further reduce breast cancer-related mortality.

Note: this chapter cites heavily from two published review articles, "Oxysterols and nuclear receptors" (102) and "Our evolving understanding of how 27-hydroxycholesterol influences cancer" (103), including large sections of text that are directly quoted.

CHAPTER 2

27-HYDROXYCHOLESTEROL ACTS ON MYELOID IMMUNE CELLS TO INDUCE T CELL DYSFUNCTION, PROMOTING BREAST CANCER PROGRESSION

2.1 Introduction

Among women in the United States, breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related death (*1*). Worldwide, over 500,000 female lives were lost to breast cancer and 1.7 million incidences were recorded in 2016 (*104*). While the mortality rate of breast cancer has significantly declined since 1989, mainly due to screen-detection and improved therapeutics, the overall 5-year survival rate of stage IV metastatic breast cancer remains low (*3, 105*). Therefore, there is a continued need to identify targetable drivers of breast cancer, especially for late stage and therapy-refractory disease. Although immune checkpoint blockade (anti-programmed death-ligand 1; α PDL1) in combination with nab-paclitaxel has recently been approved for the treatment of patients with advanced TNBC staining positive for PDL1, a majority of these patients do not respond in a clinically meaningful way (*82, 106*). Furthermore, immune interventions for the treatment of the other breast cancer subtypes have shown very limited success (*107*). Although undoubtedly multifactorial, one major obstacle to immune-therapy is the highly immunosuppressive microenvironment of breast tumors a phenomenon that is strongly maintained by myeloid immune cells, including macrophages (*108, 109*). However, myeloid cells are also critical for a robust anti-tumor response (*110*). Thus, strategies to re-educate myeloid cells away from being pro-tumorigenic are warranted and starting to be explored, at least preclinically (*111–114*).

Cholesterol and control of its metabolism have emerged as potential therapeutic targets. An elevated level of circulating cholesterol has been associated with breast cancer recurrence both preclinically and clinically (*47, 115, 116*). Conversely, several studies have found that patients taking cholesterol-lowering drugs, such as statins, had improved prognosis, as demonstrated by decreased

breast cancer recurrence and increased progression-free survival (117–120). Thus, clinical observations strongly indicate that cholesterol is important for the progression of breast cancer.

Previously, work has shown that a primary metabolite of cholesterol, 27HC, has the capacity to activate the ERs and can directly promote the growth of ER α -positive tumors (53). Notably, we also found that 27HC can promote the metastatic colonization and progression of breast cancer, regardless of the expression of ER or breast cancer subtype (90). Furthermore, ER-negative models of ovarian cancer, 27HC was essential for growth (77). In the lung metastatic site of breast cancer, it was found that neutrophils and $\gamma\delta$ T cells were required for the full pro-metastatic effects of 27HC, while in ovarian cancer M-MDSCs (with the same surface markers as inflammatory monocytes) were required (77, 90). These observations suggest that 27HC is a critical factor in shaping myeloid cell function, directly or indirectly, and that the type of myeloid cell required for its pro-tumorigenic actions is dependent on the metastatic site and type of cancer.

Within the tumor microenvironment, macrophages are one of the most abundant subsets of the myeloid cell lineage (121, 122). Accumulation of this cell type at the tumor site is often associated with poor prognosis (123–125). TAMs are known to have a diverse set of polarities, with an M2-like phenotype dominating (126, 127). TAMs are known to promote tumor progression via a multitude of mechanisms, including providing angiogenesis-inducing factors (VEGF, proMMP-9), impairing effector T cells via reactive oxygen species, and recruiting T regulatory cells by producing IL-10, IL-2 and TGF- β (128). For both metastatic breast cancer and ovarian cancer models, 27HC resulted in decreased T cell abundance, indicating that immune suppression is a putative role for 27HC-myeloid cell-mediated pro-tumor actions (77, 90). A recent report showed that the use of statins to lower cholesterol led to reduced number of TAMs and better prognosis in human lung adenocarcinoma (129). This is particularly intriguing as macrophages highly express the enzyme responsible for the synthesis of 27HC, CYP27A1, suggesting that their pro-tumor function may require the conversion of cholesterol to 27HC.

Given the evidence suggesting that 27HC is critical for promoting the pro-tumorigenic properties of myeloid cells, and the abundance and plastic nature of macrophages within the tumor microenvironment, the goals of this work were to (1) establish the effects of 27HC on macrophages and (2) elucidate the mechanisms by which 27HC impacts macrophage regulation of T cell activity.

2.2 Materials and Methods

2.2.1 Reagents

27HC (purity >95%) and dendrogenin A (purity >98%) were synthesized by Sai Life (Hyderabad, India). 25HC, 4 β HC and Desmosterol were synthesized by Steraloids (Newport, RI) (purity >98%). 22(R)HC was synthesized by Avanti (Alabaster, AL) (purity >99%). 24(S)HC and 24,25-epoxycholesterol (24,25EC) were synthesized by Enzo (Farmingdale, NY) (purity $\geq$ 98%). GW3965, T0901317, GSK2033 and 7-ketocholesterol (7KC) were synthesized by Cayman (Ann Arbor, MI) (purity $\geq$ 98%). 20(S)HC was synthesized by Tocris (Bristol, UK) (purity >95%). 17 β -Estradiol (E2) was synthesized by MP Biomedicals (Irvine, California) (purity $\geq$ 98%). 27HC, dendrogenin A, 24(S)HC, 20(S)HC, GW3965, T0901317 and GSK2033 were dissolved in dimethyl sulfoxide (DMSO) and stored in -20°C. 7KC, 4 β HC, 22(R)HC, 24,25EC and 25HC were dissolved in ethanol and stored in -20°C. Desmosterol was dissolved in M-pyrol and stored in -20°C. Antibodies for flow cytometry were purchased from BD Biosciences and used at a working dilution of 1:100 in FACS buffer (2% FBS in PBS). Catalogue numbers were as follows: CD3: 555275, CD4: 553729, CD8: 557682. Annexin V-FITC/propidium iodide apoptosis assay was purchased from Thermo Fisher (catalogue number: V13242), and FAM-DEVD-FMK/propidium iodide apoptosis assay was purchased from Immunochemistry (catalogue number: 94).

2.2.2 Cell lines

E0771 and 4T1-luc cells were a gift from Mark Dewhirst (Duke University) and cultured in RPMI supplemented with 10% FBS (Corning), 1% non-essential amino acids (Corning), 1% sodium pyruvate (Corning) and 1% penicillin/streptomycin (Corning). E0771-OVA cells were a gift from Jun Yan (University of Louisville) and grown in the above-described condition. EL4 and E.G7-OVA cells were from ATCC and cultured with RPMI medium supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin and 0.05 mM 2-mercaptoethanol. E.G7-OVA cells were additionally cultured with 0.4 mg/ml G418 (Fisher). We did not culture any of these cell lines longer than 3 months after thawing the stocks. Cell lines

were tested for mycoplasma.

2.2.3 Preparation of BMDMs

Bone marrow cells were collected from mouse tibia and femur. Cells were passed through 70-micron cell strainer and subsequently cultured in the presence of 25 ng/mL of M-CSF (Novo-protein). Complete RPMI media supplemented with M-CSF was replaced every 3-4 days. On day 10 or 14, BMDMs were harvested for further experiments. For subsequent experiments, BMDMs were treated with DMSO or 27HC (solvent at a 1:1000 dilution) for 24hrs. Treatments were washed off prior to co-culturing with T cells.

2.2.4 T cell proliferation and apoptosis assay

CD3+ T cells were enriched from the spleen of wildtype, OT-I or OT-II mice, using Dynabeads Untouched mouse T cells kit (Thermo Fisher) according to the manufacturers instructions. Naive CD4+ T cells were isolated from wildtype mice, using mouse Naive CD4+ T Cell Isolation Kit (Miltenyi Biotec) according to manufacturers instruction. T cells were labeled with the vital dye CFSE according to the manufacturers instructions (BioLegend). Wildtype T cells activated chemically were treated with 10 nM PMA and 1 μ M ionomycin and cultured with washed BMDMs [as described in (130)]. OT-I or OT-II T cells activated by antigen presentation were also labeled and cultured with LPS- and OVA-primed BMDMs (131–133). BMDMs activating T cells from OT-I mice were primed with 50 μ g/ml OVA₂₅₇₋₂₆₄ (Bachem) and BMDMs activating T cells from OT-II mice were primed with 50 μ g/ml OVA₃₂₃₋₃₃₉ (Sigma). The proliferation of T cells was assessed by flow cytometry 72hrs after co-culture. Some OT-I mouse-derived T cells were harvested 48 or 72hrs after co-culture for RNA extraction (Thermo Fisher) and mRNA expression measurement by qPCR (BioRad). Some OT-I mouse-derived T cells were harvested 48 or 72hrs after co-culture for apoptosis assay using Annexin V-FITC and propidium iodide (Thermo Fisher), and some OT-I mouse-derived T cells were harvested 24, 48 or 72hrs after co-culture for apoptosis assay using FAM-DEVD-FMK (FAM-FLICA) against active caspase-3/7 and propidium iodide (ImmunoChemistry Technologies). Nave CD4+ T cells were isolated from wildtype mice, chem-

ically activated and cultured in conditioned media from treated BMDMs, harvested after 24 or 48hrs and assayed for apoptosis (FAM-FLICA + PI) as described above.

2.2.5 T cell cytolytic assay

T cells were isolated from the spleen of OT-I mice and activated for 72hrs by LPS- and OVA-primed BMDMs. E0771 and E0771-OVA or EL4 and E.G7-OVA cells were labeled with CFSE or Far Red cell tracker (Far Red) (Thermo Fisher), respectively, and then plated in a 96-well plate. Subsequently, activated T cells were harvested and counted to be co-cultured with cancer cell lines of interest at various ratios. 24hrs after co-culture, the cytolytic activity of activated T cells was assessed by the ratio between the OVA-expressing lines and the parental cell lines by flow cytometry. The level of granzyme B secreted in the co-culture was measured by ELISA according to the manufactures instruction (Thermo Fisher).

2.2.6 Gene silencing

For gene knockdown using siRNA, negative control and LXR/ER-complementary siRNA (Sigma, siRNA ID: SASI_Mm01_00189872, SASI_Mm01_00115295) were delivered to BMDMs for 48hrs using DharmaFECT transfection reagent 4 (GE) as previously described (61). For gene knockdown using shRNA, negative control and a set of 3 LXR-complementary shRNA (Horizon) were delivered to BMDMs using lentiviral particles following the manufactures instruction at MOI of 20 for 96hrs. The shRNA sequences targeting LXR α were: GTGAGCCTTCGCCATGTGG, GCGCCTGTTACACTGTTGC and GCAGGCGAAGGGCAAACAC. The shRNA sequences targeting LXR β were: ATGATGGCTAGCTCGGTGA, CTGCACATTAGGCCGATCG and AAC-CATTCCCAGGCACGGG. Expression of the target genes after the knockdown was assessed using qPCR.

2.2.7 Animal and animal studies

All protocols involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois Urbana-Champaign. Wildtype C57BL/6 and

BALB/C mice were purchased from Charles River Laboratory. Founder OT-I, OT-II and LysM-Cre mice were purchased from Jackson Laboratory and bred in-house. Founder CYP27A1^{fl/fl} mice were a kind gift from Donald P. McDonnell (Duke University). **Figure 2.7A:** CYP27A1^{fl/fl};LysMCre+ and age-matched LysMCre+ mice were orthotopically grafted with 500,000 E0771-OVA cells in Matrigel (Corning) into the mammary fat pad. The resulting tumor growth was measured by caliper 5 times a week. After the tumor size reached 200 mm³ in either group, 7 million T cells from OT-I mice were grafted retro-orbitally into each tumor-bearing mouse in that particular group. One day before T cell adoptive transfer, each tumor-bearing mouse was immunized intraperitoneally with 25 µg of OVA₂₅₆₋₂₆₄ in Complete Freund's Adjuvant (CFA) (Sigma). For **Figure 2.7B-C:** 200,000 E0771 cells were grafted intravenously as described previously [23]. Mice were euthanized 3 weeks post-graft, and exterior, visible metastatic nodules were assessed and scored based on number and size. The metastatic lesions were quantified with the following scoring criteria: 1) The metastatic nodules were counted and then scored according to their diameters using photos taken. Specifically, a nodule with diameter greater than or equal to 0.5 in, 0.3 in, 0.2 in and 0.1 in received a score of 4, 3, 2 and 1, respectively. 2). If a metastatic lesion was discovered outside the lung (such as liver), the lesion would also receive a score of 4. For **Figure 2.7D:** BALB/C mice were grafted intravenously with 4T1-luc cells, which are 4T1 cells stably expressing a luciferase plasmid construct. Bioluminescent signal was used to quantify resulting metastatic burden (134). GW273297X treatment commenced 3d post-graft at 100mg/kg/day (90). αPDL1 treatment started at the same time and followed the same regimen as described previously (77).

2.2.8 Data Analysis

RNA sequencing data (RSEM, batched-normalized) for breast invasive carcinoma that were generated from the Cancer Genome Atlas (TCGA, <https://www.cell.com/pb-assets/consortium/pancanceratlas/pancani3/index.html>) were downloaded from the cBioPortal (<http://www.cbioportal.org>) using R package cgdscr (135, 136). Data included 1084 samples in **Figure 2.3I-N** and **Supplemental Figure 3.3**. Correlations between gene expression were assessed by log2 transformation and Pearson's correlation in **Figure 2.3I-N** and **Supplemental Figure 3.3**, and the best fit line was determined by linear regression between the explana-

tory (x axis) and the response variable (y axis). Two-tailed t-test followed by Benjamini-Hochberg false discovery rate correction was performed for comparing cytokine secretion by BMDMs in **Supplemental Table 3.1-3.2** (137). Two-way ANOVA followed by Bonferroni's multiple comparisons test was used in **Figure 2.6B**. Fisher's exact test was performed for proportion testing in **Figure 2.7A-C**. For **Figure 2.7D**, luminescence was normalized to the mean placebo + α -IgG, and one-way ANOVA was performed followed by the Student Newman-Keuls post-hoc test for significance. For the rest of the results, two-tailed t-test or non-parametric Mann-Whitney U-test was performed to compare the effect of vehicle and 27HC on BMDMs and, subsequently, T cells.

2.3 Results

2.3.1 27HC modulates gene expression in macrophages.

Oxysterols are a large group of compounds, with a diverse spectrum of biological activities (102). Many oxysterols including 27HC have been described as ligands of the liver x receptors (LXRs), the activation of which promotes cholesterol homeostatic machinery, such as cholesterol efflux and cholesterol metabolism (102). LXR activation, by various ligands including synthetic agonists, has also been implicated in regulating various aspects of the immune system. In general, many reports indicate that LXRs promote an anti-inflammatory phenotype, although these conclusions are largely based on synthetic ligands. This has led the field to believe that LXR agonists are generally anti-inflammatory. However, endogenous oxysterols and other LXR agonists are likely to have diverse, or even opposing actions (102, 138, 139). It is well established that LXR induction in T cells impairs proliferative expansion and activity (140). However, the actions of LXRs on the immune function of myeloid cells are less clear. Therefore, to further explore these putative immunomodulatory effects on macrophages, we investigated the effect of 27HC on the expression of genes associated with cholesterol homeostasis and inflammation. In order to simulate an inflammatory state, we evaluated the effects of 27HC in the absence as well as presence of LPS. LPS would be expected to polarize the macrophages towards an M1-like phenotype.

BMDMs were prepared from female C57BL/6 mice. As expected, a 24-hour treatment of BMDMs with 27HC resulted in an induction of the LXR target genes ABCA1 and ABCG1, re-

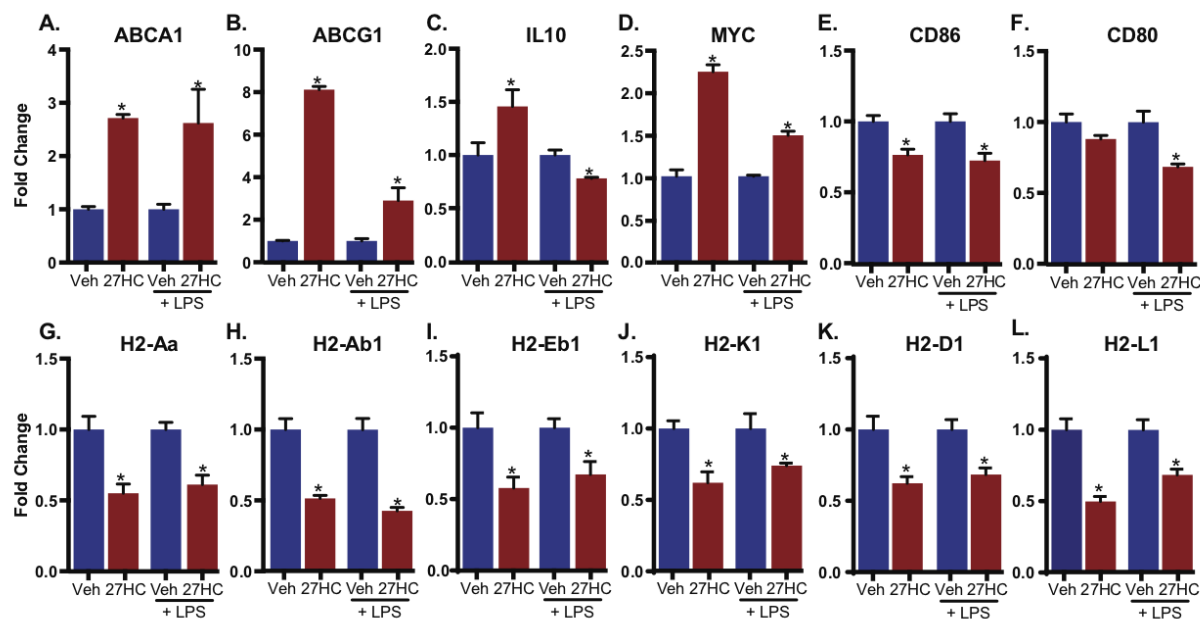


Figure 2.1: 27HC results in altered expression of genes associated with macrophage function and interaction with T cells. Murine BMDMs were treated with vehicle (DMSO) or 10 μ M 27HC in the absence or presence of LPS. Transcripts were detected and quantified by qPCR and displayed as mean $\pm$ SEM. Data are presented as normalized to respective vehicle control. Asterisks (*) indicate statistical significance from the respective vehicle control (N=3/group for all samples treated with LPS, N=6/group for all samples treated with vehicle only, N=5 for all samples treated with 27HC only). (two-tailed t-test, $p < 0.05$)

regardless of the presence of the inflammatory stimulus LPS (**Figure 2.1A-B**). IL-10, a cytokine previously implicated in immune suppression in the tumor microenvironment (*141*) was found to be induced by 27HC in the absence of LPS, but modestly suppressed in the presence of LPS, suggesting that 27HC may selectively regulate genes depending on the inflammatory state of BMDMs (**Figure 2.1C**). Similarly, 27HC induced the expression of MYC, a known factor involved in the alternative M2 polarization of macrophages (*142*), and this induction was most pronounced in the absence of LPS (**Figure 2.1D**). Interestingly, 27HC decreased the expression of antigen-presentation/co-stimulation makers, namely MHCII (H2-Aa, H2-Ab1 and H2-Eb1) and B7 (CD80 and CD86) (**Figure 2.1E-I**). MHC I proteins (H2-K1, H2-D1 and H2-L1) were also decreased by 27HC, regardless of whether the BMDMs were exposed to LPS (**Figure 2.1J-L**). Because macrophages serve as antigen-presenting cells bridging innate and adaptive immunity, such suppression of antigen-presentation and co-stimulation markers indicates that 27HC may decrease the capacity of macrophages to present and activate T cells.

2.3.2 27HC-treated macrophages inhibit T cell expansion.

The regulation by 27HC of genes in BMDMs that are associated with antigen presentation and cross-presentation to T cells (**Figure 2.1**) suggests that 27HC likely influences myeloid cell-T cell crosstalk. Therefore, I investigated whether treating BMDMs with 27HC impacts T cell activation and function. When T cells are activated, they undergo rapid clonal expansion, with proliferative activity therefore, being a biological readout of activation.

For these experiments, I utilized T cells isolated from OT-I or OT-II mice. These mice have been genetically modified so that their TCRs recognize specific ovalbumin peptides in the context of the MHCI and MHCII molecules (H2-Kb or I-Ab) respectively (*143*). T cells were enriched from the spleen of these mice, with a purity of 60-80% CD3+ T cells as assessed by flow cytometry (data not shown). When the cells were purified from OT-I mice, 50-60% of all cells were CD8+ (data not shown). Wildtype murine BMDMs were cultured in the presence of the activating agent LPS and ovalbumin peptides (OVA₂₅₇₋₂₆₄ or OVA₃₂₃₋₃₃₉), which serve as defined antigens. Simultaneously, BMDMs were treated with either vehicle or 27HC for 24hrs. At this time, BMDMs were washed to preclude the direct effects of 27HC on T cells, and subsequently co-cultured with CFSE-labeled CD3+ T cells isolated from OT-I or OT-II mice.

Intriguingly, flow cytometry showed that 27HC-treated BMDMs failed to promote antigen-specific T cell expansion, a hallmark of T cell activation. Impaired expansion was observed regardless of the T cell subtype considered: CD4+ helper T cells (OT-II) or CD8+ cytotoxic T cells (OT-I) (**Figure 2.2A and B**), respectively; representative histograms in **Supplemental Figure 3.1A**). Therefore, 27HC-treated BMDMs result in decreased T cell expansion.

Although the ability of 27HC to regulate MHCII and B7 expression (**Figure 2.1**) is consistent with the decreased expansion of T cells resulting from a deficit in antigen presentation or co-stimulation, it is well known that macrophages can also impair T cell activity via several distinct mechanisms, including those independent of antigen presentation (*144*). To assess this possibility, I evaluated the impact of 27HC-treated BMDMs on the expansion of chemically activated T cells. Specifically, BMDMs were treated with vehicle or 27HC, along with or without stimulation signals, LPS and BSA. After the treatments were washed off, those BMDMs were then co-cultured with CFSE-labeled T cells that were activated chemically by PMA and ionomycin [as described

in (130)]. These activated T cells should be highly proliferative due to the activation of the PKC pathway without the need for specific antigen-presentation. The induction of T cell proliferation by PMA and ionomycin was confirmed in assays with T cells only (verified in each experiment, **Figure 2.2C-H**).

Under these co-culture conditions, I found that 27HC treatment of BMDMs resulted in suppression of the expansion of chemically activated T cells (**Figure 2.2C**). This impaired expansion was observed whether or not the BMDMs were previously stimulated by LPS and BSA (**Figure 2.2C-D**). The suppression was found to be dose-dependent: when higher concentrations of 27HC were used to treat BMDMs, there was a concomitant decrease in subsequent T cell expansion (**Figure 2.2E**). The decreased expansion was also apparent when conditioned media was cultured with naive CD4⁺ T cells (**Supplemental Figure 3.1B**). Therefore, the effects of 27HC on the macrophage-mediated impairment of T cell expansion did not require MHC-cognate antigen-TCR ligation, indicating that any macrophage exposed to 27HC within proximity to a T cell could suppress its expansion. Furthermore, this would suggest that 27HC-induced macrophages could impair T cell expansion regardless of how they are activated (as in, even if they were activated by dendritic cells), since the chemical activation strategy targets downstream cell surface interactions.

Given that the enzyme responsible for the synthesis of 27HC, CYP27A1 is highly expressed in macrophages, it was of interest whether an autocrine source of 27HC was sufficient to impair T cell expansion. Therefore, I evaluated the capacity of BMDMs isolated from CYP27A1^{-/-} mice to influence T cell expansion. In support of our hypothesis, I found that compared to BMDMs from wildtype mice, co-culture of T cells with CYP27A1^{-/-} BMDMs resulted in increased expansion (**Figure 2.2F**). This finding is important, given that CYP27A1 is amenable to targeting with a small molecule inhibitor, and one such molecule was previously shown to have anti-metastatic effects (90).

Since we previously demonstrated the requirement of neutrophils and $\gamma\delta$ T cells for the pro-metastatic effects of 27HC (90), we were interested in whether these cell types could influence the 27HC-mediated BMDM impairment of T cell expansion. Thus, I co-cultured 27HC-treated BMDMs, bone marrow-derived neutrophils or both cell types with chemically activated T cells. I found that 27HC-treated neutrophils alone could impair T cell expansion (**Figure 2.2G**). There was no obvious additive or synergistic effect of co-culturing both neutrophils and BMDMs on T

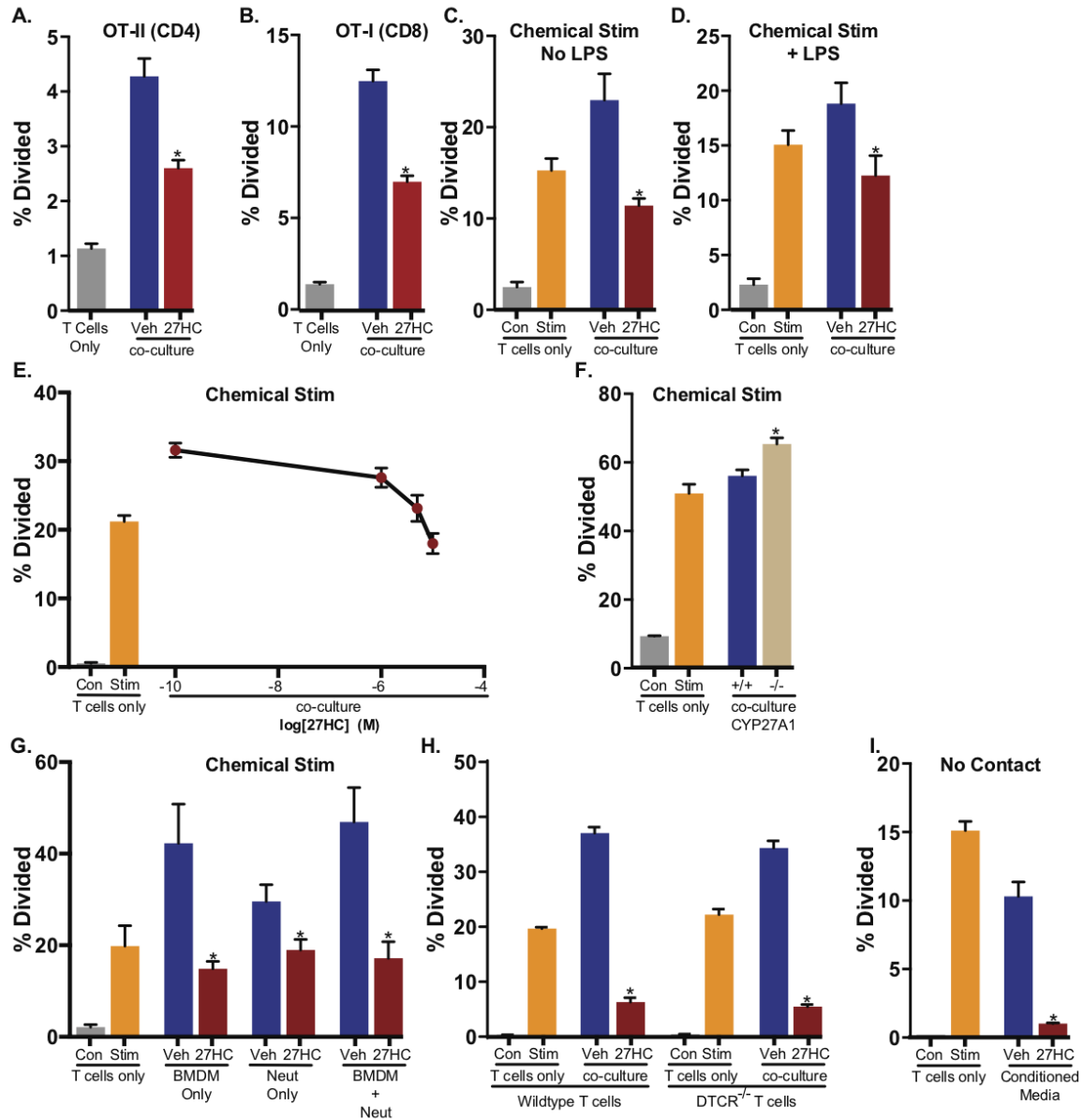


Figure 2.2: 27HC-treated macrophages decrease T cell activation and expansion. Murine BMDMs were cultured with OVA peptide in the presence or absence of indicated ligands in A and B. The media was then replaced and BMDMs were co-cultured with CFSE-labeled T cells from (A) OT-II mice (where TCRs of CD4+ T cells are engineered to recognize OVA), or (B) OT-I mice (where TCRs of CD8+ T cells are engineered to recognize OVA) for 72hrs. Subsequent T cell proliferation was quantified by flow cytometry for CFSE dilution. For subsequent panels (C-I), T cells from wildtype mice were activated by chemical stimulation with 10 nM PMA and 1 μ M ionomycin and co-cultured with BMDMs. (F) BMDMs from wildtype or CYP27A1^{-/-} were used for co-culture with T cells. (G) The same inhibitory effects of 27HC-treated BMDMs were observed with 27HC-treated neutrophils (PMNs). (H) $\gamma\delta$ T cells are dispensable for the effects of 27HC-treated BMDMs on T cell expansion; T cells isolated from DTCCR^{-/-} mice do not have $\gamma\delta$ T cells. (I) Direct contact between 27HC-treated BMDMs and T cells is not required for its inhibitory effects. Conditioned media from treated BMDMs was used to culture chemically activated T cells. Data are presented as mean $\pm$ SEM and asterisks (*) indicate statistical significance from the respective vehicle control (N=3 for all T cell only controls. For co-culture conditions, N=4/group in (A-D), N=5/group in (E), and N=6/group in (F-I)). (two-tailed t-test, $p < 0.05$)

cell expansion (**Figure 2.2G**). Culture of T cells from mice incapable of producing $\gamma\delta$ T cells (DTCCR^{-/-} mice) continued to be suppressed by 27HC-treated BMDMs, suggesting that, at least in

vitro, this cell type is dispensable for the immunosuppressive activities of 27HC (**Figure 2.2H**). Collectively, these results indicate that (A) $\gamma\delta$ T cells are not required, and (B) provide support for our hypothesis that 27HC results in impaired acquired immunity, regardless of what myeloid cell subtype it is working through, and the specific myeloid cell of importance is likely tumor type, tumor site and tumor-stage dependent (77, 90).

Our observations that BMDMs treated with 27HC impaired the ability of chemically activated T cells to proliferate, suggest that antigen-presentation is not required for 27HC-treated BMDMs to inhibit T cell expansion. Therefore, it was of interest as to whether direct contact between these two cell types was required and whether a soluble factor was mediating these effects. To address this question, I treated BMDMs with vehicle or 27HC for 24hrs and then washed the treatments off. BMDMs were then cultured in fresh media for another 72hrs, before I harvested supernatant from them. The conditioned media was subsequently used in culture with chemically activated T cells. Intriguingly, even without the presence of BMDMs, conditioned media alone exerted T cell suppression (representative experiments in **Figure 2.2I** and **Supplemental Figure 3.1C**). This is consistent with our findings that 27HC-pretreated mice continue to have increased metastatic burden post intravenous graft of mammary cancer cells, even when MHCII has been neutralized by an antibody (**Supplemental Figure 3.2**). Thus, the 27HC-macrophage-mediated T cell impairment does not require cell-cell contact, and is mediated by a soluble factor(s).

2.3.3 T cells co-cultured with 27HC-treated macrophages have decreased cytotoxic activity.

Our data indicate that 27HC-treated BMDMs severely impair T cell expansion. However, it was also of interest whether those T cells that did expand had altered functional activity. In this regard, when I controlled for input post exposure to 27HC-treated BMDMs (**Figure 2.3A**), I observed downregulated mRNA expression of cytotoxic T cell effector proteins, granzyme B and perforin (**Figure 2.3B-C**). Subsequent ELISA analysis of T cell-conditioned media confirmed that cytotoxic T cells exposed to 27HC-treated BMDMs had decreased secretion of granzyme B (**Figure 2.3D**). These findings indicate that the effector function of T cells was impaired after exposure to 27HC-treated BMDMs.

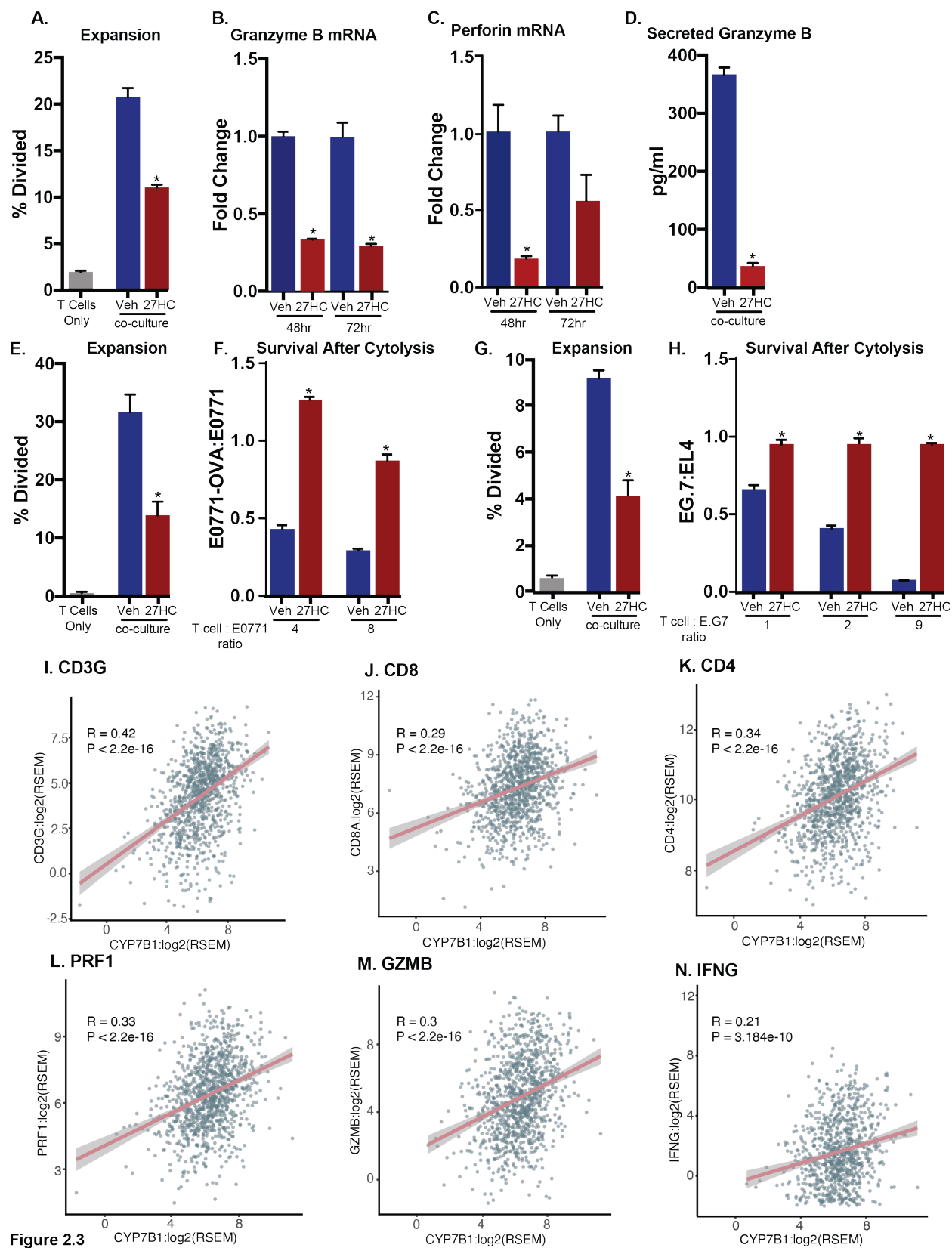


Figure 2.3

Figure 2.3: (Cont.) T cells co-cultured 27HC-treated macrophages have decreased cytotoxic activity. T cells co-cultured with BMDMs treated with 27HC for 72hrs have decreased expansion (A) and the resulting expanded T cells also have decreased granzyme B and perforin mRNA expression through time after co-culture as measured by qPCR (B-C) and secretion of granzyme B as measured by ELISA (D). Data in (A-C) are presented as normalized to respective vehicle control. (E and G) When OT-I T cells are co-cultured with BMDMs treated with indicated ligands as well as OVA, 27HC treatment results in decreased expansion, and these T cells have decreased cytolytic activity against (F) E0771 mammary cancer cells or (H) EG.7-OVA lymphoma cells. Both of these lines were engineered to express OVA. Specifically, the parental cell lines (E0771 or EL4) were labeled with CFSE and derivative cell lines (E0771-OVA and E.G7-OVA) were labeled with Far Red. Equivalent cell number of the respective parental and derivative cell lines were plated and co-cultured with OT-I T cells activated by vehicle- or 27HC-treated BMDMs at indicated ratios. The cytolytic activity of T cells was assessed via flow cytometry as represented by the ratio of derivative (Far Red)/parental (CFSE) cell lines. (I-N) Human RNA sequencing data from TCGA Pan Cancer for invasive breast carcinoma indicated a moderately positive and significant correlation between CYP7B1, an enzyme responsible for catabolizing 27HC, expression and the expression of 6 T cell genes (CD3G, CD8A, CD4, perforin 1, granzyme B, INF- γ) that are related to immune competence. mRNA expressions were log2 transformed and Pearson's correlation was assessed (N=1059, 1081, 1082, 1077 and 905, respectively). Data in (A-H) are presented as mean $\pm$ SEM and asterisks (*) indicate statistical significance from the respective vehicle control (two-tailed t-test in (A-H), $p < 0.05$). A: N=3/group for control and N=6/group for co-culture conditions, B: N=3-4/group, C: N=3-4/group, D: N=5-6/group, E: N=4/group, F: N=4/group, G: N=4/group, H: N=5/group.

To specifically test whether anti-cancer T cell function was impaired by 27HC-treated BMDMs, I performed tumor cytolysis assays. T cells derived from OT-I mice were co-cultured with BMDMs exposed to LPS and OVA peptide, in the presence or absence of 27HC. As expected, 27HC resulted in decreased T cell expansion (**Figure 2.3E**). The resulting T cells were harvested, counted, and then co-cultured with E0771 murine mammary cancer cells (controlling for T cell input) for 24hrs. Two different sublines of E0771 were used: the parental line that was stained with CFSE, and E0771-OVA cells that ectopically express OVA₂₅₇₋₂₆₄ and were stained with Far Red [development of E0771-OVA cells described in (145)]. In this way, antigen-specific cytolytic activity could be determined as a ratio between E077-OVA (Far Red) : parental E0771 (CFSE). A low ratio indicates strong cytolytic activity of T cells, while a high ratio suggests otherwise. Importantly, T cells harvested from co-culture with 27HC-treated BMDMs were less efficient at killing E0771-OVA cells (**Figure 2.3E-F**). In order to ensure that these effects were not specific to E0771 cells, I repeated this experiment using the murine lymphoma cell line EL4 and its OVA₂₅₇₋₂₆₄-expressing derivative, E.G7-OVA (146). As expected, T cells harvested from co-culture with 27HC-treated BMDMs had significantly reduced capacity to eliminate this cell type, as the ratio between E.G7-OVA and EL4 remained high and unchanged, even with increasing numbers of antigen-specific T cells added to the co-culture (**Figure 2.3G-H**). Collectively, our data indicate that 27HC-treated BMDMs not only impair T cell expansion, but also their functional activity, potentially explaining the robust increase in metastasis observed in 27HC-treated mice (90).

Elevated level of CYP7B1, the enzyme responsible for catabolizing 27HC, expression has been associated with improved progression-free survival among breast cancer patients, and is reflective of intra-tumoral 27HC concentrations (65, 147). In an effort to demonstrate the relevance

of our findings to human health, I surveyed TCGA as accessed through the Invasive Breast Carcinoma from Pan-Cancer Atlas. I assessed tumoral CYP7B1 mRNA, the enzyme responsible for the catabolism of 27HC, expecting that higher CYP7B1 levels would result in decreased 27HC levels, as recently demonstrated previously for human thyroid tumors (65). I found a modest, positive correlation between CYP7B1 and CD3G, a component of the T cell receptor CD3 family (**Figure 2.3I**). Likewise, there is a modest, positive correlation between CYP7B1 and both CD8 and CD4 expression (**Figure 2.3J-K**). Finally, CYP7B1 had a modest, positive correlation with both T cell effector proteins, perforin 1, granzyme B, and IFN- γ (**Figure 2.3L-N**). Even after normalizing for CD8A expression, I still observed a weak but significant positive correlation between granzyme B or perforin and CYP7B1 (**Supplemental Figure 3.3**). All breast cancer subtypes were used for these analyses, which would be expected to exhibit heterogeneity. Therefore, data from human breast tumor specimens support our pre-clinical findings that 27HC impairs T cell expansion and function.

2.3.4 27HC alters cytokine secretion in macrophages (BMDMs), potentially contributing to its suppressive effect.

Since direct contact between BMDMs and T cells was dispensable for the suppressive effects of 27HC, I next examined the involvement of potential soluble factors, focusing on those previously reported to mediate the pro-tumor or immunosuppressive effect of macrophages (144, 148). In this regard, TAMs have been shown to upregulate arginase 1 (ARG1) and indoleamine 2,3-dioxygenase 1 (IDO1) to deprive T cells of arginine and/or tryptophan, and thereby inhibit their activity (T cells being auxotrophic for these amino acids) (149). However, supplementation with either arginine or tryptophan in our BMDM-T cell co-culture failed to attenuate the immunosuppressive effect of 27HC-treated BMDMs (**Figure 2.4A, B**). CD8⁺ activity has been shown to be impaired by increased cholesterol content, and given that oxysterols would result in reverse-cholesterol transport in BMDMs, it was possible that nearby T cells were subject to elevated cholesterol (150, 151). However, expansion of T cells isolated from LDLR^{-/-} mice (and thus lacking the receptor to take up LDL-cholesterol) continued to be impaired by 27HC-treated BMDMs, although their baseline expansion was also decreased compared to wildtype T cells (**Supplemental**

Figure 3.4). It is important to note that LDLR^{-/-} T cells may still be able to internalize cholesterol via scavenger receptors, so it is still possible that cholesterol mediates these effects.

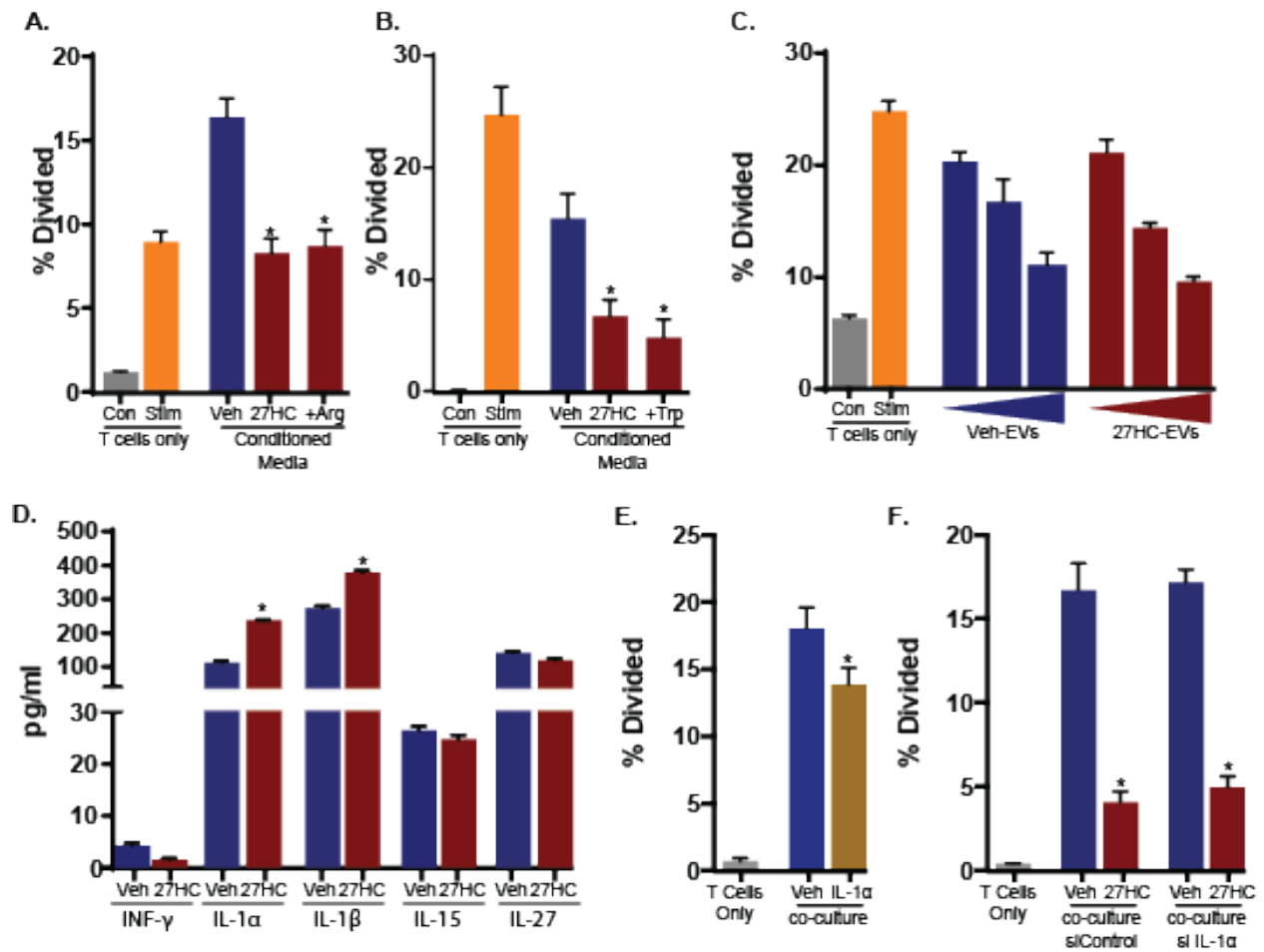


Figure 2.4: T cell suppression resulting from 27HC treated macrophages is not mediated by common mechanisms. (A) Arginine (Arg) depletion by BMDMs is not likely the cause of impaired T cell expansion as supplementation with exogenous Arg failed to rescue the phenotype (N=3 for all controls, N=6/group for co-culture conditions). BMDMs were treated with vehicle or 27HC. Those cells were then co-cultured with chemically activated wildtype T cells. In the co-culture, 0.2 g/L of Arg or equivalent volume of PBS was supplemented. T cell expansion was measured by dilution of CFSE as assessed by flow cytometry. (B) Tryptophan (Trp) depletion by BMDMs is not likely the cause of impaired T cell expansion as supplementation with 0.005 g/L of exogenous Trp failed to rescue the phenotype (N=3 for all controls, N=4/group for co-culture conditions). (C) Extracellular vesicles derived from BMDMs are not likely the cause of impaired T cell expansion. Direct treatment of T cells with extracellular vesicles decrease expansion, but there was no difference between extracellular vesicles isolated from vehicle- or 27HC-treated BMDMs (N=3). Extracellular vesicles were isolated from the conditioned media of vehicle- or 27HC-treated BMDMs at three increasing densities, using Total Exosome Isolation Reagent (Thermo Fisher, Cat no. 4478359) and then cultured with chemically activated T cells at three increasing doses. The increasing densities of starting BMDMs were 1x, 2x, or 3x the number of macrophages used in other experiments. T cell expansion was measured. (D) A cytokine array found modest changes in certain cytokines secreted from LPS-primed vehicle- or 27HC-treated BMDMs (full panels indicated in **Supplemental Table 3.1-3.2**) (N=3/group). (E) In line with previous reports, IL-1 α , at the same concentrations induced by 27HC as determined in (D), resulted in decreased T cell expansion (N=5/group, Mann-Whitney U test). (F) IL-1 α is not likely a sufficient cause of impaired T cell expansion as siRNA knockdown within BMDMs failed to reverse the effects of 27HC (N=3 for all controls, N=4-5/group for co-culture conditions). Data are presented as mean $\pm$ SEM and asterisks (*) indicate statistical significance from the respective vehicle control. (two-tailed t-test unless otherwise specified, $p < 0.05$)

There are recent reports of extracellular vesicles and their miRNA cargo being impor-

tant mediators of cholesterol homeostasis (152, 153). Extracellular vesicles, including exosomes are known to be secreted by myeloid cells, and have been implicated in immune suppression (154, 155). Therefore, it is possible that 27HC-treated BMDMs released extracellular vesicles that mediated the decreased T cell expansion and function. To test this possibility, I isolated extracellular vesicles from vehicle or 27HC-treated BMDMs and cultured them with chemically activated T cells. Although there was an inhibition of T cell expansion by extracellular vesicles isolated from increasing numbers of BMDMs, there was no difference between extracellular vesicles derived from vehicle or 27HC-treated BMDMs (**Figure 2.4C**). Therefore, it is unlikely that the inhibitory effects of 27HC-treated BMDMs are mediated through extracellular vesicles.

Several cytokines secreted by BMDMs have been described to inhibit T cell activation or proliferation. Therefore, I assessed a multiplex panel of murine cytokines at two time points: 24hrs after treatment with vehicle or 27HC and 72hrs after co-culture with T cells. The majority of changes observed with the treatment of 27HC were apparent after 24hrs (**Supplemental Table 3.1-3.2**). Under basal conditions, many cytokines were below the detection thresholds of the assay, although 13 were detected and of those, only two were significantly regulated by 27HC after 24hrs (MIP-1a and MIP-1b), but these were no longer significantly different after 72hrs in culture (**Supplemental Table 3.1**). When stimulated with LPS, several cytokines reached detectable concentrations after 24hrs, and of those, only IL-1 α and IL-1 β were significantly regulated by 27HC, although IL-6, IFN- γ , IL-18 and MIP-1b had false discovery rates <0.15 (**Figure 2.4D** and **Supplemental Table 3.2**). TGF- β was not included in the multiplex panel, but of potential relevance, and thus was assessed independently by ELISA (**Supplemental Figure 3.5**). Of those cytokines regulated by 27HC, IL-1 α was of interest as it has previously been shown to influence the growth and activity of T cells (156–159), and was consistently upregulated by 27HC under both time points. I confirmed this observation, as addition of IL-1 α at a concentration reflective of measured levels in our co-culture, resulted in reduced proliferation of T cells activated by antigen presentation (**Figure 2.4E**). However, siRNA mediated knockdown of IL-1 α in BMDMs, did not significantly impair the ability of 27HC-treated BMDMs to inhibit T cell expansion (**Figure 2.4F**). These observations indicate that while IL-1 α may play a role in suppressing T cells, it is unlikely to be only one factor among several that mediate the effects of 27HC. It is very possible that 27HC modulates the phenotype of BMDMs through several diverse mechanisms, which converge

to exhibit a strong immunosuppressive effect on T cells.

2.3.5 Reduced T cell expansion by 27HC-treated macrophages is mediated by the LXR.

Since 27HC is an abundant oxysterol, and its presence and metabolism have been clinically associated with breast, ovarian and thyroid cancers (53, 65, 77, 147), it was of importance to determine the mechanism by which 27HC alters macrophages to impair T cell expansion and function. Previously reported pathways involved in macrophage function or cholesterol signaling (PI3K, AKT, NOTCH and CaMKK2) were ruled out through the use of pharmacologic inhibitors of these enzymes (**Supplemental Figure 3.6**). Thus, to provide initial insight into potential mechanisms, I assessed the ability of 12 different oxysterols, cholesterol metabolites and synthetic LXR ligands to inhibit T cell expansion via treatment of BMDMs (**Supplemental Table 3.3**). Most oxysterols and cholesterol metabolites tested impaired T cell expansion to varying degrees (**Figure 2.5A**). Dendrogenin A at 1 μ M was a notable exception, with no noticeable effect on T cell expansion. This finding is in line with previous reports indicating that this metabolite induces tumor cell differentiation and immune cell infiltration and thus is associated with a good prognosis (74, 160). Moreover, when BMDMs were co-treated, dendrogenin A exhibited dose-dependent attenuation of the actions of 27HC (**Figure 2.5A**). This indicates that the effects of 27HC are likely receptor-mediated, and that dendrogenin A has the capacity to out-compete 27HC for binding.

27HC is known to bind to two nuclear receptors, ER and LXR, both receptors being implicated in immunomodulatory effects (102, 161). Since several oxysterols have been shown to modulate the activities of the ERs and LXRs to different extents, I first evaluated capacity of the same panel of oxysterols and cholesterol metabolites (**Supplemental Table 3.3**) to activate the ERs and LXRs. I then assessed whether there was a correlation between this receptor activation and impaired T cell expansion. T47D cells, engineered to stably express an ER-reporter (estrogen response element upstream of a luciferase construct) (162), were treated with different doses of the ligands. Several oxysterols were found to have estrogenic activity, including those not previously been reported to do so (**Figure 2.5B**, **Supplemental Figure 3.7** and **Supplemental Table 3.3**). Importantly, there was no notable correlation between the ability to activate ER-mediated

transcription and inhibition of T cell expansion (**Figure 2.5B**). Furthermore, no impact on T cell expansion was observed after treatment of BMDMs with either E2, a naturally occurring ER agonist, or ICI 182,780 (ICI, Fulvestrant) an ER antagonist and degrader (**Figure 2.5B, F**). These results indicate that impairment of T cell expansion upon 27HC treatment of BMDMs is unlikely to be ER-mediated.

In order to assess the ability of different ligands to activate the LXRs, I evaluated the extent to which they could induce ABCA1, a well-known LXR target gene, in BMDMs. As expected, 27HC and many of the oxysterols could induce ABCA1 as well as another LXR target gene, ABCG1, while E2 did alter basal expression of ABCA1 (**Supplemental Figure 3.8**). Interestingly, the ability of a ligand to induce ABCA1 expression was moderately and significantly correlated with its ability to inhibit T cell proliferation after treatment of BMDMs (**Figure 2.5C**). This suggests that modulation of the LXR is mediating the effects of 27HC and other oxysterols. To definitively test this, I used complementary strategies of RNA-interference and pharmacologic antagonists.

ER α has been reported to be the predominant subtype in macrophages (163–165). Therefore, I first evaluated the impact of knocking down ER α on the 27HC-mediated decrease in T cell expansion and function. Although siRNA-mediated knockdown resulted in an over 80% decrease in ER α mRNA, 27HC treatment of these BMDMs continued to inhibit the expansion of chemically activated T cells (**Figure 2.5D**). Similarly, siRNA against ER α in BMDMs did not impact the ability of 27HC to decrease OT-I T cell proliferation after antigen-mediated activation (**Figure 2.5E**). Furthermore, the cytotoxic capacity of T cells was reduced by 27HC-treated BMDMs, regardless of ER α expression (not shown). I confirmed these findings using the pharmacological antagonist ICI (**Figure 2.5F**) (166). ICI antagonizes both ER α and β subtypes. As expected, ICI treatment failed to rescue the inhibitory effects of 27HC-treated BMDMs on T cell proliferation or cytotoxic capacity (**Figure 2.5F** and not shown). Collectively, these data strongly indicate that the mechanism by which 27HC modulates macrophages to impair T cell expansion and function is independent of the ER.

Treatment of BMDMs with a synthetic LXR agonist, GW3965, resulted in impaired T cell expansion (**Figure 2.5A, B**), suggesting that this receptor may mediate the effects of 27HC. Both subtypes of LXRs (α and β) are expressed in BMDMs and whether they play specific roles is

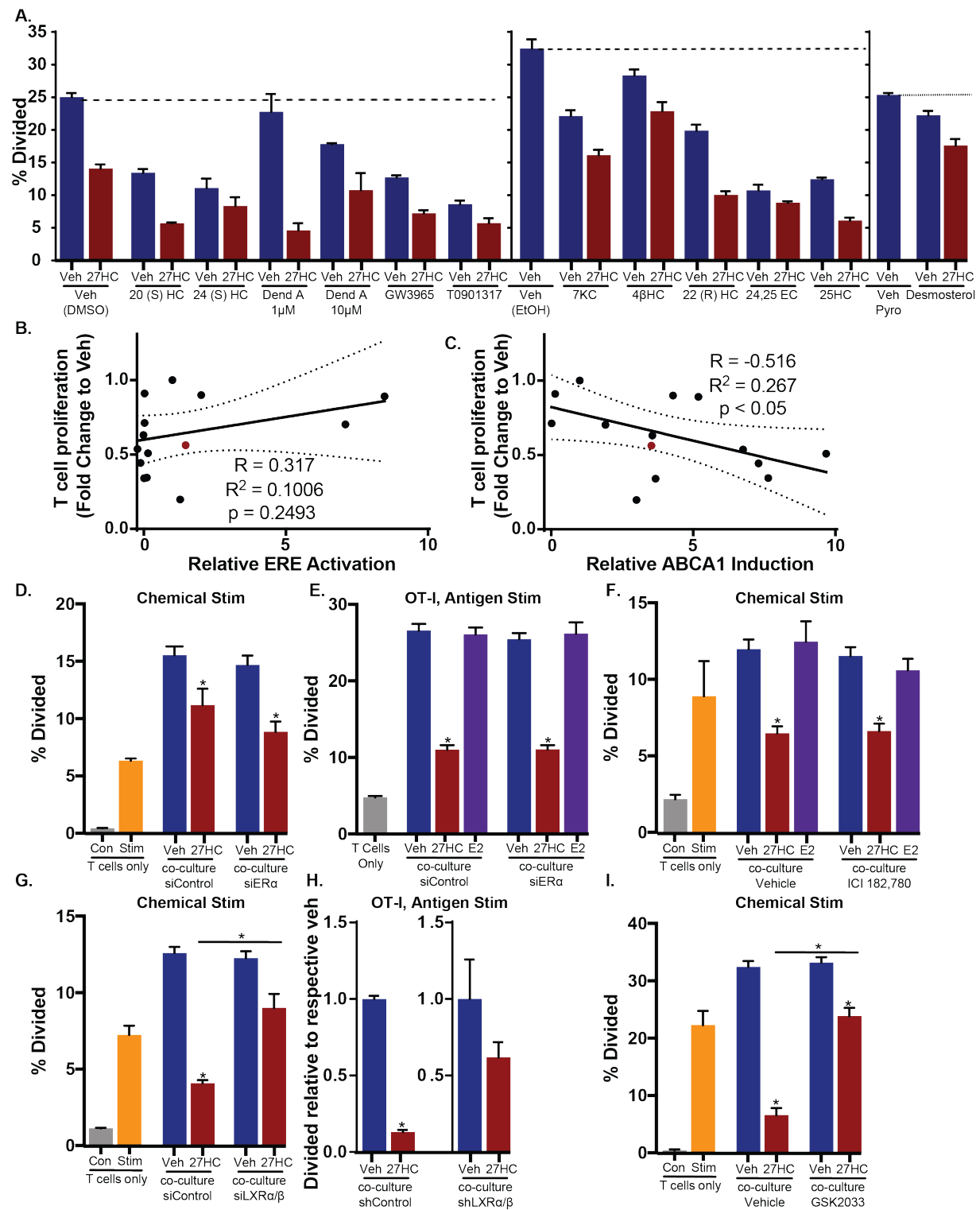


Figure 2.5

Figure 2.5: (Cont.) LXR mediates the myeloid cell immunosuppressive effect of 27HC. (A) BMDMs were treated with 12 cholesterol metabolites, LXR ligands or indicated vehicle controls for 24hrs prior to co-culture with chemically activated T cells for 72hrs, and proliferation was assessed. (B-C) Two cohorts of BMDMs were treated with ligands for 24hrs. Expression of ABCA1 mRNA was assessed in the first cohort. BMDMs from the second cohort were co-cultured with chemically activated T cells for 72hrs post treatment, at which point T cell proliferation was measured. (B) T47D cells stably transfected with a ERE luciferase reporter were treated as in (A) and the activation of ER was measured by luciferase activity. Pearson's correlation between T cell proliferation and ER activity is presented. (C) Pearson's correlation between T cell proliferation and induction of ABCA1 mRNA is presented. (D) BMDMs were transfected with siControl or siER α for 48hrs and then treated with vehicle or 27HC. After 24hrs, the treatments were washed off, and media was replaced. Those BMDMs were co-cultured with chemically activated T cells for 72hrs. (E) BMDMs transfected with siControl or siER α for 48hrs were primed with OVA and treated with vehicle or 27HC for 24hrs. BMDMs were then co-cultured with CD3+ T cells from OT-I mice for 72hrs and proliferation assessed. (F) BMDMs were treated with vehicle, 27HC, ICI (an ER antagonist), E2, ICI+27HC or E2+27HC. BMDMs were then co-cultured with chemically activated wildtype T cells as in (D). (G) BMDMs were transfected with siControl or siLXR α/β for 48hrs. Those BMDMs were then harvested, treated and co-cultured with chemically activated T cells as in (D). (H) BMDMs were transduced with lentiviral shControl or shLXR α/β plasmids for 96hrs and then treated and co-cultured as in (E). Data presented as normalized to respective control. (I) LXR antagonist (GSK2033) attenuates effects of 27HC. BMDMs were treated and co-cultured with T cells as in (D). All T cells were labeled with CFSE and the proliferation was assessed by flow cytometry. Data are presented as mean $\pm$ SEM and asterisks (*) indicate statistical significance from the respective vehicle control (N=3 for all T cell only controls. For co-culture conditions: N=4 for A, E, H and I. N=3 for B. N=5-6 for D and F. N=6 for G. (two-tailed t-test, $p < 0.05$). For luciferase activity in (C), N=4)

unclear. Therefore, I elected to knockdown both subtypes simultaneously. mRNA expression shows that siRNA-mediated knockdown of LXR α was marginal in several attempts, while LXR β knockdown was moderate (around 10% and 50%, respectively, **Supplemental Figure 3.9A**). However, even in the context of this partial knockdown of LXR, the immunosuppressive effect of 27HC-treated BMDMs on chemically activated T cells was significantly attenuated (**Figure 2.5G**). shRNA against the LXRs administered by lentivirus infection of BMDMs (**Supplemental Figure 3.9B**) also lead to the attenuation of 27HC-mediated impairment of OT-I T cell expansion after antigen-activation (**Figure 2.5H**). The involvement of the LXRs was corroborated as co-treatment with the small molecule LXR antagonist, GSK2033 (*167–171*), restored T cell proliferation when cultured with 27HC-treated BMDMs (**Figure 2.5I**). In this assay, GSK2033 was also able to rescue the inhibitory effect of BMDMs treated with a synthetic ligand of the LXRs, GW3965 (**Supplemental Figure 3.9C**). GSK2033 has been implicated in modulating the activity of other receptors (such as ROR γ and ERR α) (*172*), and this cannot be ruled out here, although the effects of siRNA against the LXRs would support the conclusion that LXRs are mediating the effects of 27HC.

2.3.6 27HC-treated macrophages impair T cell function by promoting T cell apoptosis.

Mechanistically, it was important to investigate whether the impaired T cell activity was a result of impairment of initial activation or post-activation proliferation, as myeloid-derived sup-

pressor cells and macrophages have been previously shown to inhibit T cell activation (173, 174). Consistent with this notion, I observed down-regulation of several markers of T cell activation (CD25, CD27 and CD69) 48 and 72hrs after co-culture with 27HC-treated BMDMs (**Figure 2.6A-C**). However, down-regulation of CD62L expression was also observed (**Figure 2.6D**). Shedding of CD62L is commonly observed in activated T cells (175). A lower level of CD62L in T cells cultured with 27HC-treated BMDMs would suggest that those cells were indeed being activated, despite decreased expression of early activation markers and effector proteins (**Figure 2.6A-C, Figure 2.3B-D**). While mRNA expression is not necessarily correlated to surface protein concentration, one explanation for this apparent contradiction and the consistent, unidirectional change in gene expression is that 27HC-treated BMDMs induce T cell death, thereby leading to global downregulation of gene expression (176–179).

To investigate this possibility, I labeled OT-I T cells 48 or 72hrs after co-culture with Annexin V-FITC and propidium iodide. Annexin V binds to phosphatidylserine, a membrane lipid that becomes externalized and accessible during apoptosis. PI enters cells with disrupted membranes and binds DNA, indicative of cell necrosis. Through this assay, I found that at 48hr time point, there was no significant difference in live cell (Annexin V-/PI-) and necrotic cell (Annexin V-/PI+) populations (**Figure 2.6E**). However, at both 48 and 72hr time points, 27HC-treated BMDMs induced a significantly greater number of Annexin V+/PI+ (indicative of late apoptotic/necrotic) T cells (**Figure 2.6E** and **Supplemental Figure 3.10A**). It is important to note that the 27HC group also had lower levels of early apoptotic cell population (Annexin V+/PI-; **Figure 2.6E**), which could be a result of increasing number of cells moving into late apoptosis. Given that Annexin V is capable of entering ruptured membrane and binding to phosphatidylserine in necrotic cells and PI also interacts with DNA of cells during late apoptosis, I could not definitively tell whether the Annexin V+/PI+ population was a result of late apoptosis, necrosis or both (180, 181). In order to conclusively identify the type of BMDM-driven T cell death, I alternatively labeled T cells, harvested from 3 time points (24, 48, 72hrs) post co-culture with vehicle- or 27HC-treated BMDMs, with fluorescent caspase-3/7 inhibitor probe FAM-DEVD-FMK (FLICA-FAM) and propidium iodide. FAM-DEVD-FMK covalently binds to activated caspase-3/7 through the fluoromethylketone (fmk) moiety (182, 183). Because caspase-3 is an executioner in both the intrinsic and extrinsic apoptosis pathways but has no role in necrosis, presence of active caspase-3 in T cells after co-

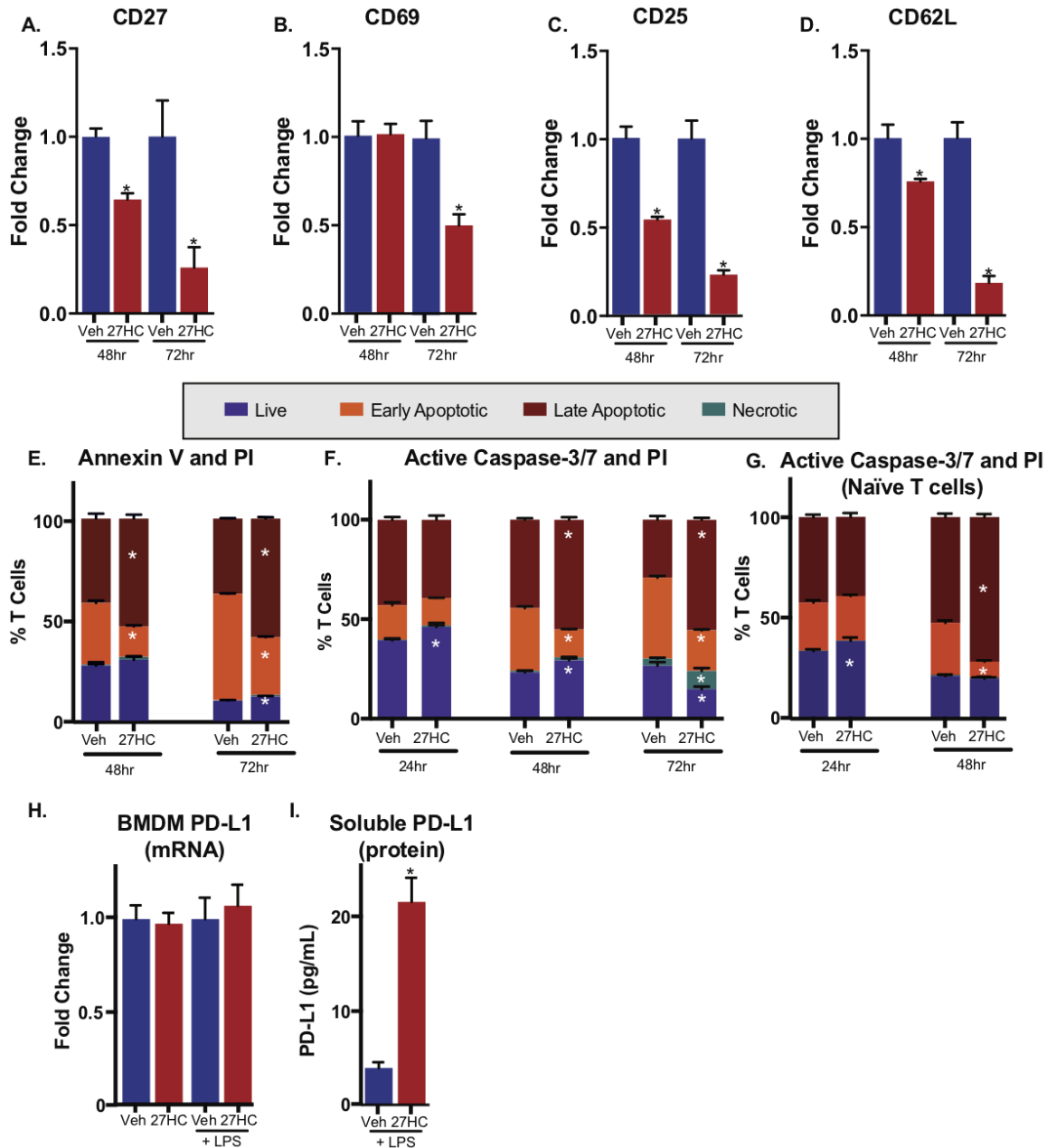


Figure 2.6: 27HC-treated macrophages induce T cell apoptosis. (A-D) Gene expression of markers of T cell activation was downregulated when T cells are co-cultured with BMDMs treated with 27HC. qPCR on T cells, 48 or 72hrs after co-culture (N=3-4/group). Data are presented as normalized to respective vehicle control. (E) OT-I T cells 48 or 72 hours after co-culture with vehicle- or 27HC-treated BMDMs exhibited enhanced apoptosis. T cells were harvested from the co-culture at two different time points and then were labeled with Annexin V-FITC and PI. Flow cytometry analysis was performed to measure the cell populations (N=4/group). (F) OT-I T cells in co-culture with 27HC-treated BMDMs exhibit increased apoptosis. T cells were harvested from the co-culture (as in E) at three different time points and then labeled with FLICA-FAM, a caspase-3/7 inhibitor that covalently binds to active forms of caspase-3/7, and PI. Flow cytometry analysis was performed to measure the cell populations (N=4/group). (G) Naïve CD4⁺ T cells were isolated from the spleen, chemically activated and cultured with conditioned media from vehicle or 27HC treated BMDMs. Subsequent analysis was similar to (F) (N=4/group). (H) PDL1 mRNA expression in BMDMs; see **Figure 2.1** for details. (I) Soluble PDL1 is increased in media from LPS-primed BMDMs treated with 27HC for 24hrs (similar experimental setup to **Figure 2.4D** (N=4/group)). Data are presented as mean $\pm$ SEM, and asterisks (*) indicate statistical significance from the respective vehicle control. (two-tailed t-test in (A-D), two-way ANOVA followed by Bonferroni's multiple comparison test in (E) and (F), $p < 0.05$)

culture with 27HC-treated BMDMs more clearly distinguishes between apoptosis and necrosis. Specifically, FLICA+/PI- and FLICA+/PI+ population indicate early and late apoptosis respectively, while FLICA-/PI+ suggests necrosis. Consistent with the Annexin V/PI assay, results from this approach support the conclusion that 27HC-treated BMDMs induce T cell apoptosis between 24 and 48 hours after co-culture (**Figure 2.6F**, **Supplemental Figure 3.10B**). Notably, T cells in contact with 27HC-treated BMDMs entered late apoptosis at a much higher rate and, 72 hours after co-culture, there were less than half the live cells when cultured with 27HC-treated BMDMs compared to control (**Figure 2.6F**). Since splenic T cells include different forms such as memory T cells and thus may not be representative of a normal T cell immune response, I isolated CD4+ naive T cells, chemically activated them, and cultured them in the presence of conditioned media from either vehicle or 27HC treated BMDMs. Under these conditions, naive T cells had increased late apoptotic cells after 48hrs of exposure to conditioned media from 27HC-treated BMDMs (**Figure 2.6G**), mirroring results from pan-splenic T cells. Therefore, our results suggest that 27HC modulated BMDMs promote accelerated T cell apoptosis, where the T cells rapidly (within 48hrs) progressed from an early into a late apoptotic stage. Admittedly, at 72hrs, a small yet significant increase in a population of necrotic T cells was observed after T cells were co-cultured with 27HC-treated macrophages, and therefore I cannot rule out the possibility that caspase-3/7-independent pathways could also play a role in mediating 27HC-driven T cell dysfunction.

The immune checkpoint molecule, PDL1 is known to induce apoptosis in effector T cells (184). Therefore, I assessed PDL1 mRNA in BMDMs treated with 27HC (same experiment as in **Figure 2.1**). No changes in PDL1 transcript were observed (**Figure 2.6H**). Intriguingly however, there was a five-fold increase in soluble PDL1 in conditioned media from 27HC-treated BMDMs, as assessed by ELISA (**Figure 2.6I**). The biological relevance of soluble PDL1 is still unclear, but emerging reports suggest that it too can induce apoptosis (185), suggesting that this may be the putative factor mediating the pro-apoptotic effects of 27HC.

2.3.7 Absence or inhibition of CYP27A1 results in increased antigen-specific T cell cytotoxicity, decreased tumor growth and enhanced efficacy of immune checkpoint inhibition.

To determine whether the impaired T cell expansion and function upon BMDM treatment with 27HC occurred within the complex in vivo microenvironment, I orthotopically grafted E0771-OVA cells into mice that had CYP27A1 genetically knocked out specifically in the myeloid immune lineage (CYP27A1^{fl/fl};LysMCre+) and respective control mice (CYP27A1^{+/+};LysMCre+) (genotyping and selective knockout demonstrated in **Supplemental Figure 3.11**). A myeloid-specific knockout approach was adopted, because introduction of exogenous 27HC would likely have direct effects on cancer cells, potentially confounding interpretation of our results (53, 77). After tumors were established, I then grafted all mice with OT-I T cells and measured resulting tumor growth; OT-I T cells were expected to specifically target OVA-expressing cells after presentation and activation by myeloid cells. Since I hypothesized that loss of myeloid cell CYP27A1 would result in increased T cell function, the OT-I T cells were grafted at a suboptimal concentration and would not be expected to fully eradicate the tumor. As expected, loss of myeloid CYP27A1 resulted in an increased number of mice whose tumors grew at a reduced rate post OT-I T cell graft compared to those grown in CYP27A1 replete mice (using a quadrupling of tumor volume as our threshold) (**Figure 2.7A** and **Supplemental Figure 3.12A-B**).

We have previously shown that 27HC robustly increases the metastatic colonization and growth of breast cancer cells in a myeloid cell dependent manner (90). Given the results of the current study indicating that 27HC-treated BMDMs significantly impair T cell expansion and anti-cancer function, we therefore wanted to determine whether loss of myeloid cell expression of CYP27A1 would itself result in altered metastatic colonization and growth. Therefore, mice were grafted intravenously with E0771 cells, and subsequent metastatic lesions were quantified 3 weeks later. Intriguingly, significantly reduced lung metastatic burden was observed in CYP27A1^{fl/fl};LysMCre+ mice compared to control mice (**Figure 2.7B** and **Supplemental Figure 3.12C**). Furthermore, the only mice exhibiting macro-metastases outside the lung were from CYP27A1 intact controls (**Figure 2.7C**). Since LysM promoter is active in all myeloid cells, one cannot ascribe the effects solely to the loss of CYP27A1 to macrophages, but rather to all types of

myeloid cells. This is likely the case, as neutrophils also displayed inhibitory effects when treated with 27HC (**Figure 2.2G**).

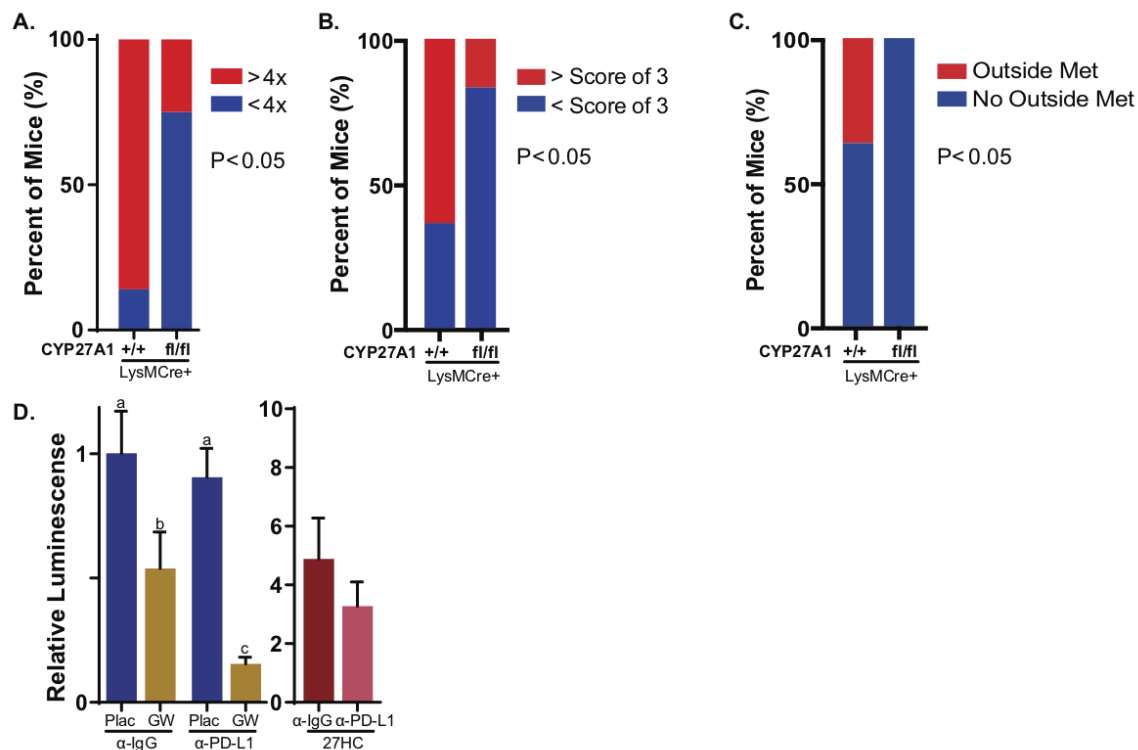


Figure 2.7: Absence or inhibition of CYP27A1 promotes T cell function, decreased tumor growth and enhanced efficacy of α PDL1. (A) CYP27A1^{fl/fl};LysMCre+ mice exhibited lower level of antigen-specific primary tumor growth. CYP27A1^{fl/fl};LysMCre+ mice (N=8) and their replete controls (N=7) were grafted orthotopically with E0771-OVA cells and tumors allowed to grow to 200 mm³. Mice then received adoptive transfer of CD3+ T cells from OT-I mice. The difference between final tumor volume and the established tumor volume at adoptive transfer was calculated as a ratio between the two. The ratio used for comparison of response between two groups was 4. (Macrophage-selective knockout in **Figure 3.11**). Growth curves are depicted in **Figure 3.12A-B**. (B) Myeloid cell CYP27A1 knockout mice exhibited lower level of metastatic burden, after intravenous graft of E0771 cells (CYP27A1^{fl/fl};LysMCre+ mice, N=12; CYP27A1^{+/+};LysMCre+ mice, N=11). The mice were euthanized 3 weeks after injection and metastatic nodules counted and then scored. Representative pictures of metastatic lungs are in **Figure 3.12C**. (C) CYP27A1^{fl/fl};LysMCre+ mice exhibited decreased extra-plural metastases. Many controls, but no myeloid cell CYP27A1 knockout mice, had metastases outside the lung (same mice as in B). (D) Small molecule inhibition of CYP27A1 reduced metastatic growth and improved checkpoint inhibitor efficacy. BALB/C mice were grafted intravenously with 4T1-luc cells, and lesions allowed to establish for 3d. At this time, mice were treated with indicated ligands, including the CYP27A1 inhibitor, GW273297X (GW). (N=10/group except for 27HC treatments which had an N of 7-8/group). Data in (A-C) are presented as contingency table bar graph and data in (D) are presented as mean $\pm$ SEM (Fishers exact test in (A-C), one-way ANOVA followed by Newman-Keuls multiple comparison test for the first 4 groups in (D), $p < 0.05$).

Immune checkpoint inhibition is, at best, modestly effective for the treatment of metastatic breast cancer, even within the patient cohort that is approved for its use. Since myeloid cells highly express CYP27A1 and are abundant in breast tumors and metastatic lesions, we speculated that inhibition of 27HC synthesis may increase the efficacy of this therapeutic strategy. Thus, we grafted mice intravenously with the highly aggressive 4T1 model of TNBC and allowed lesions to establish. At this point, mice were randomized and treated with α PDL1 (checkpoint inhibitor),

GW273297X (a small molecule inhibitor of CYP27A1), or both. As expected, treatment with α PDL1 alone had no significant effects on metastatic burden (**Figure 2.7D**). In this model, exogenous 27HC treatment robustly increased metastatic burden, regardless of whether mice were treated with α PDL1, indicating that the pro-metastatic effects of 27HC are likely a combination of enhanced colonization (90) as well as growth (**Figure 2.7D**). Interestingly however, treatment with GW273297X as a single agent modestly reduced metastatic burden (**Figure 2.7D**). Importantly, co-treatment with GW273297X and α PDL1 further reduced metastatic burden, suggesting that myeloid-cell synthesized 27HC does impede the actions of checkpoint inhibition.

2.4 Discussion

Despite recent improvements in the treatment of breast cancer, advanced-stage and metastatic disease remain major contributors to breast cancer mortality. Although success using checkpoint inhibition in advanced TNBC have been reported and is now approved in combination with nab-Paclitaxel for a subset of patients, its overall response rate in breast cancer remains low (106). Therefore, developing a clear understanding of the checkpoint-independent immune suppression biology within breast cancer is critical to the future development of curative therapies. The current work indicates that the actions of cholesterol metabolites such as 27HC on myeloid cells results in severe T cell impairment, both in terms of expansion as well as function.

Clinically, elevated cholesterol has been shown to be associated with breast cancer recurrence, while patients taking cholesterol-lowering medication (statins) have increased time to recurrence (186). In preclinical models, a high cholesterol diet increases tumor growth and metastasis of mammary cancer (186). However, our previous work has found that the pathophysiology of cholesterol on breast cancer progression is largely attributed to its primary metabolite, 27HC, as the growth and metastatic effects of a high cholesterol diet are absent in CYP27A1^{-/-} mice (53, 90). 27HC promotes primary tumor growth through a cancer-cell intrinsic mechanism by being an ER agonist and activating the ERs (53). However, exogenous 27HC does not require the expression of ERs in cancer cells to robustly increase metastatic burden in mice (53, 90). Engagement of the LXR by 27HC did result in increased EMT and modest increases in metastatic capacity of mam-

mary cancer cell lines (53, 187). However, we have shown that 27HC also acts in a cell extrinsic manner to robustly promote metastatic colonization (90). Specifically, it was found that myeloid cells were required for these effects. Immune depletion of neutrophils ablated the pro-metastatic effects of 27HC, suggesting involvement of this cell type. This is perhaps because neutrophils dominate the myeloid cell landscape at the lung metastatic site of mice (188, 189). On the other hand, the ovarian tumor microenvironment required 27HC, which appeared to mediate its effects through MDSCs (77). Thus, it is likely that while 27HC exerts its effects on all myeloid cells, the extent to which it does so is dependent on the tumoral and microenvironmental context. Therefore, we explored the biology of 27HC in the prototypical myeloid cell, the macrophage.

Here, we found that antigen-primed, 27HC-exposed macrophages not only failed to promote the expansion of T helper cells and cytotoxic T cells, likely through induction of apoptosis, but also severely impaired the cytolytic capacity of cytotoxic T cells against cancer cells. We further showed that the suppression of T cells by 27HC-treated macrophages is not limited to the antigen-presentation pathway, as unprimed macrophages could also suppress chemically activated T cell expansion. The general suppression of T cells by 27HC-modulated macrophages suggests even though T cells are already active, their proliferation and effector functions could still be severely impaired by 27HC-exposed macrophages in the tumor microenvironment. This is in line with a previous report that an oxysterol mixture could promote M2-like macrophages (190).

The ability of media-conditioned with macrophages treated with 27HC to suppress T cells indicates that certain soluble factors mediate the effects. Through our experiments, we ruled out obvious metabolic regulators (arginine and tryptophan) and extracellular vesicles. We have also identified several cytokines that are regulated by 27HC in time-dependent manners. Of those that are consistently altered by 27HC and known to result in T cell impairment, the involvement of IL-1 α (156–159) was specifically tested, with our results indicating that this cytokine alone was not mediating the effects of 27HC.

27HC-treated BMDMs were found to increase T cell apoptosis. Conditioned media from 27HC-BMDMs also increased apoptosis in nave T cells, in line with our other evidence suggesting that a soluble factor was responsible for the T cell suppressive effects of 27HC. Since the immune checkpoint molecule, PDL1 is known to induce apoptosis in effector T cells (184), we explored whether this factor was regulated by 27HC. Its transcript levels were not altered in BMDMs post-

treatment with 27HC (**Figure 2.6H**). To our surprise however, soluble PDL1 was significantly increased in conditioned media from 27HC-treated BMDMs. The soluble form of PDL1 is an active area of study, with several isoforms (splice variants) being described (*191–193*), and its precise biological functions and relevance being unclear. However, it has been found to induce apoptosis, similar to its membrane-bound form (*185*). This suggests that soluble PDL1 may be the putative factor mediating the pro-apoptotic effects of 27HC. Our results provide strong rationale for future studies exploring whether this is indeed mediating the effects of 27HC, how 27HC alters its secretion but not its mRNA expression, and whether 27HC alters different splice variants. The specific soluble factor(s) from myeloid cells responsible for mediating the suppressive effects of 27HC remain to be determined.

Our results are supported by preclinical models as metastatic lesions from mice treated with 27HC had decreased CD8+ T cells (*90*). Furthermore, human patient tissue from breast cancer tumors with decreased CYP7B1 expression and therefore expected increased 27HC concentrations (*65*) had decreased expression of CD3G, CD4, CD8, perforin and granzyme B (*90*) and **Figure 2.3I-M**). This highlights the relevance of our results to human health and provides context to a recent report indicating that the recruitment of monocytes and subsequent epigenetic silencing of CYP7B1 results in the accumulation of intratumoral 27HC in breast cancer, and corroborated by another report that breast tumors had increased 27HC compared to normal-adjacent material (*54, 194*).

There have been several reports indicating that the synthesis/secretion of oxysterols is altered upon inflammatory stimuli (*195*), and in turn, oxysterols exert modulatory activities on the immune system and/or inflammation (*150, 196*). Mechanistically, we have shown that, macrophage LXRs, but not ERs, are required for the immunosuppressive effect of 27HC, as knocking down LXRs or blocking them with a pharmacological antagonist attenuates the suppressive effects of 27HC on T cells. Further supporting this finding are revelations of a positive correlation between the ability of different oxysterols, cholesterol metabolites and synthetic LXR agonists to induce the LXR target gene ABCA1, and their ability to suppress T cell expansion. The involvement of LXR in the immunosuppressive activities of 27HC provides a contrast to certain inflammatory actions of 27HC being mediated through the ER, at least with respect to cardiovascular disease (*59*), although LXR activation by 27HC has been implicated in the protective effects of vitamin D on atheroscle-

rosis in a preclinical model (197). Estrogen has been shown to increase hematopoietic stem cell proliferation (57) and myelopoiesis (161). 27HC was also found to increase hematopoietic stem cell mobilization but not proliferation (57). Estrogen was found to enhance the immunosuppressive activities of MDSCs (161). However, here, we show that despite 27HC being an ER ligand, it is its activities through the LXRs in myeloid cells that result in T cell suppression.

Several previous studies have demonstrated that oxysterols and the LXR are involved in management of the innate immune system (102, 190, 198–200). Interestingly, in apparent contrast with previous reports indicating that macrophages lacking LXRs had increased apoptosis (198), or were more susceptible to LPS-induced liver injury and resistant to inflammatory responses caused by treatment with α -galactosylceramide or concanavalin-A (199), treatment of mice and humans with synthetic LXR agonists decreased MDSC abundance within tumors by decreasing their [MDSC] survival (95). Specifically, MDSCs isolated from tumors of LXR-treated mice were less able to suppress T cell expansion compared to those isolated from vehicle-treated mice (95). This apparent discrepancy with our work indicating that myeloid cells treated directly with LXR agonists impair T cell function may be due to either: (1) Differential kinetics and compensatory mechanisms, it is possible that MDSCs isolated from in vivo treated mice lose their LXR signal and over-compensate before returning to baseline. (2) Different myeloid cell types may behave differently. Our data indicate that 27HC induces an immunosuppressive phenotype on both macrophages and neutrophils, and as such MDSCs may not behave similarly. (3) LXR agonists may exert their effects in a selective fashion, similar to selective estrogen receptor modulators. In other words, different LXR agonists may behave differentially, in a context-dependent/specific manner. Even though the exact immunomodulatory pathway downstream of LXR is currently unknown, previous reports have shown that the LXR agonists suppress NLRP3 inflammasome activation, this down-regulation being associated with poorer clinical outcome in hepatocellular carcinoma (201–203). Regardless of mechanism, our work presented here, along with emerging evidence of cancer-cell intrinsic roles for the LXRs (53, 187), would suggest that caution should be taken prior to the development of LXR as an immunomodulatory target for the treatment of solid tumors. Furthermore, these results provide a strong rationale for studies to elucidate the precise signaling events downstream of LXR.

One potential limitation to our study is the reliance on BMDMs, which may not be reflective

of macrophages or other myeloid cells within the tumor microenvironment. Indeed, microenvironmental factors can significantly alter macrophage response. Since 27HC is likely one of these factors, it is still relevant that naive BMDMs are entrained by this oxysterol. Furthermore, bone marrow-derived neutrophils treated with 27HC also exhibited an anti-T cell response (**Figure 2.2**). Finally, our in vivo data (**Figure 2.7**) and correlational data from human tumors (**Figure 2.3**) support an immunosuppressive role for 27HC within the tumor microenvironment.

Collectively, we have shown that 27HC-treated macrophages strongly impair the expansion of T cells, suggesting that inhibition of 27HC synthesis should improve the efficacy of immune therapy. Indeed, in a preclinical model of metastatic breast cancer, blocking 27HC synthesis with a small molecule inhibitor of CYP27A1 resulted in a positive response to α PDL1. The expression profile of CYP27A1, which is abundant in myeloid cells the liver and within cancer cells of high-grade tumors (53, 54, 204) suggests that targeting this enzyme will have a low risk of unwarranted side effects. Our work also reinforces the broader strategy of reeducating myeloid cells to be pro-immune and anti-cancer, rather than ablating them altogether (90, 111, 205–207).

Note: this chapter cites heavily from a published research article, "27-Hydroxycholesterol acts on myeloid immune cells to induce T cell dysfunction, promoting breast cancer progression" (208), including large sections of text that are directly quoted.

CHAPTER 3

27-HYDROXYCHOLESTEROL ACTS AS A SELECTIVE LXR MODULATOR

3.1 Introduction

It is now well appreciated that the sex steroid receptors (estrogen receptors and androgen receptors) can be selectively modulated by different ligands (48, 49, 209–212); this pharmacology has been harnessed for the treatment of breast and prostate cancer. Selective estrogen receptor modulators are known to have unique pharmacology (i.e., agonistic or antagonistic properties), in a tissue and context dependent manner. For example, tamoxifen antagonizes the ER in breast tissue and cancer, but behaves as an ER agonist in the uterus and bone. In theory, one could design ligands that retained the desired biological properties whilst diminishing any undesired side effects. What remains to be determined though, is whether other nuclear receptors could also be selectively modulated, an important concept for the future development of these receptors for the treatment of different diseases.

The LXRs, LXR α and LXR β (NR1H3 and NR1H2), belong to the nuclear receptor superfamily of ligand-regulated transcription factors (102). One of their most well characterized physiological functions lies in the regulation of cholesterol homeostasis, as their activation induces the transcription of genes involved in cholesterol catabolism, reduced cholesterol uptake and cholesterol efflux (102). Cholesterol homeostasis plays fundamental roles in regulating cellular functionality and communication, including normal immune function. In the context of atherosclerosis, LXR activation promotes cholesterol efflux and reverse cholesterol transport of macrophages through ATP-binding cassette transporters, and inhibits an inflammatory response through NF κ B (213). Given such benefit, synthetic LXR ligands, such as GW3965, were once considered as therapeutics for atherosclerosis and related cardiovascular diseases (99). However, this effort was later stalled due to the concerted activation of hepatic fatty acid synthesis thereby

causing symptoms linked to dyslipidemia in human patients and mouse models (214).

LXR as a target for inflammation and cancer has also been an area of interest for some time. In terms of cancer, LXR activation is thought to promote cholesterol metabolism and efflux while inhibiting the uptake, thereby making cholesterol a limiting resource for rapidly proliferating cells (77, 103, 215). However, LXR agonists have been implicated in promoting epithelial to mesenchymal transition and increased capacity for colonization of distal metastatic sites (53). Likewise, there are many conflicting reports of different LXR ligands having disparate effects on inflammation and the immune system. Studies have shown that breast tumor-derived LXR ligands and the oxysterol 27HC could exert suppressive activities on macrophages, while an inverse agonist of LXR could stimulate CD8+ T-cell cytotoxic activity to promote tumor clearance (92, 208). On the contrary, synthetic LXR agonists including RGX-104 and GW3965 could also promote an anti-tumor immune response eliminating myeloid derived suppressor cells (MDSCs) (95).

These seemingly conflicting activities of LXR are difficult to explain. One possibility is that rather than being traditional agonists, antagonists or inverse agonists, LXR ligands are instead selective modulators, similar to selective estrogen receptor modulators (SERMs) or selective androgen receptor modulators (SARMs) (51, 211, 216, 217). Selective estrogen receptor modulators are the most well characterized and are defined by ligands of ER being able to induce tissue-selective agonist/antagonist activities via altering ER structures and therefore the engagement of co-regulator proteins with ER. The unique pharmacology of these receptors is characterized as (1) different ligands resulting in overlapping yet different gene expression profiles within the same cell type, and (2) the same ligand having cellular, tissue or context-specific effects. Evidence for this selective pharmacology for nuclear receptors other than the ERs and AR is sparser. However, it was reported that unlike GW3965, the cholesterol biosynthetic intermediate, desmosterol, downregulated fatty acid synthesis by suppressing SREBP pathways, while continuing to promote cholesterol efflux (218). A later study confirmed that desmosterol had properties indicative of a selective modulator of LXR and SREBP in a cell-specific manner in macrophages, since desmosterol strongly activated ABCA1 and suppressed SREBP pathways in macrophages, while having no effect in hepatocytes *in vitro* (99). Synthetic desmosterol mimetics recapitulated these cell-specific effects of desmosterol *in vitro* and *in vivo*. These studies satisfied the first part of the selective modulator definition and provides proof of principle that selective LXR modulators could be developed

with tailored biological effects, especially given that desmosterol is one of the major endogenous activators of LXR in the atherosclerotic plaques (218). However, a comprehensive testing of the "Selective LXR modulator (SLXRM)" concept has not been performed.

Downstream of cholesterol metabolism, a group of molecules called oxysterols are also key endogenous ligands of LXR specialized in regulating cholesterol homeostasis (219). Among them, 27HC is the primary circulating oxysterol in human body. Early in 2008, 27HC was identified as a SERM, displaying both estrogenic and anti-estrogenic roles in a cell-specific context (49). 27HC was later identified to mediate the high cholesterol-driven breast cancer metastasis in ER-negative mouse models, which was speculated to be facilitated through LXR, as GW3965 was identified to also promote metastasis in ER-negative mouse models, albeit to a lesser extent (53). The requirement of LXR 27HC-driven breast cancer metastasis was supported by the LXR-dependent immunosuppressive effect of 27HC on macrophages, rendering T cells dysfunctional (208). However, conflicting reports of the biological outcome of different LXR ligands roles in myeloid immune cell populations have now been described in numerous reports (92, 95). This led us to hypothesize that, like desmosterol, 27HC selectively modulates LXR, leading to the differential regulation of closely related but distinct immune populations and therefore cancer progression.

In this report, we use non-biased RNA-seq and single cell RNA-seq to explore the ability of LXRs to be selectively modulated, by comparing the effects of 27HC, an endogenous oxysterol, and GW3965, a synthetic LXR agonist on various myeloid cell populations, given the fact that 27HC and GW3965 have been shown to play pro-tumor and anti-tumor roles by interacting with the innate immune system. I show that 27HC is capable of promoting genes responsible for cholesterol efflux without upregulating *Srebf1* in macrophages. Within other subpopulations of CD11b+ myeloid populations in the metastatic breast tumor microenvironment, 27HC and GW3965 exert varied effects, including the regulation of well-known LXR target genes. These findings suggest a selective LXR modulatory nature of 27HC, distinct from a synthetic agonist. This work will serve as an important foundation for the development of novel cancer therapeutics, where the immune activities of LXR can be harnessed, and the hepatic/metabolic effects minimized.

3.2 Materials and Methods

3.2.1 Preparation of BMDMs and extraction of RNA

Bone marrow cells were collected from mouse tibia and femur. Cells were passed through 70-micron cell strainer and subsequently cultured in the presence of 25 ng/mL of M-CSF (Novo-protein). Complete RPMI media supplemented with M-CSF was replaced every 3-4 days. On day 14, BMDMs were harvested for further treatment with DMSO, 27HC, GW3965, GSK2033, E2, ICI, GW3965 + ICI, ICI + 27HC, GSK2033 + 27HC for 24hrs. Genomic DNA removal and total RNA extraction from BMDMs afterwards were performed using Qiagen RNeasy Plus Mini Kit (Cat no. 74136). Bulk RNA Sequencing Quantity of RNA was determined and then assessed for quality using AATI fragment analysis. cDNA library was subsequently prepared by the TruSeq Stranded mRNAseq Sample Prep kit (Illumina). The libraries were sequenced on three lanes for 101 cycles from single end of the fragments on a NovaSeq 6000 using a NovaSeq SP reagent kit. Fastq files were generated and demultiplexed with the bcl2fastq v2.20 Conversion Software (Illumina). RNA sequencing was performed by the DNA Services Lab at Roy J. Carver Biotechnology Center of University of Illinois Urbana-Champaign.

3.2.2 Bulk RNAseq data processing

The abundance of transcript was quantified using Kallisto (v0.44.0) pseudoalignment, using index built from a transcriptome fasta of *Mus Musculus* build GRCm38. Transcript abundance was subsequently summarized to gene-level using R package Tximport. After keeping all the genes that were expressed in at least 1 sample, 24671 genes were left for downstream analysis, and differentially expressed gene analysis was performed using R packages Limma-voom and EdgeR on the logCPM values. Multiple testing correction was made using the false discovery rate (FDR) method. Gene module discovery was performed using weighted correlation network analysis (WGCNA). GO analyses of genes presented in each module were implemented using R package TopGO.

3.2.3 Animal and animal study

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois Urbana-Champaign. Wildtype C57BL/6 and BALB/C mice were purchased from Charles River Laboratory. **Figure 3.3-3.4:** BALB/C mice were grafted intravenously with 150,000 4T1 cells stably expressing a luciferase plasmid construct (4T1-luc) per mouse. Bioluminescent signal was used to confirm the resulting metastatic burden (134). 27HC, GW3965, GW273297X treatment commenced 1d post-graft at 30mg/kg/day, 40/mg/kg/day and 100mg/kg/day, respectively, for 16 consecutive days (53, 77). 27HC was dissolved in 40% β -cyclodextrin (Sigma), and GW3965 and GW273297X were dissolved in 10% DMSO in corn oil (Sigma). 17 days after grafting, the metastatic lungs were harvested and enriched for CD45+ cells using magnetic microbeads (Miltenyi Biotec). CD45+ enriched cells were then stained with anti-CD45-PE antibody and DAPI to further flow sort for live CD45+ cells for single-cell RNA sequencing.

3.2.4 Single-cell RNA sequencing

Samples with high viability were processed for single-cell RNAseq. Single-cell libraries were prepared using the Chromium Single-Cell Gene Expression v3 kit and sequenced on an SP 2x150nt lane in a NovaSeq 6000. The single-cell libraries produced over 12 billion reads.

3.2.5 Single-cell RNAseq data processing

Fastq files were generated and demultiplexed using CellRanger v4.0. The sequenced libraries were then reference mapped and quantified using CellRanger Count v5.0 (mouse GRCm39, GENCODE M26). The resulting ambient RNA-filtered count matrices were imported into Seurat v4 (220). The cells were then filtered based on feature number > 200 (i.e., cells removed if < 200 features/cell), UMI count < 20,000 (i.e., cells removed if > 20,000 UMI count/cell), and percent mitochondrial < 7.5 (i.e., cells removed if percent mitochondrial/cell > 7.5). The filtered data matrix was then log-normalized, scaled, and clustered using the Seurat pipeline. Cell clusters were annotated based on the expression of known marker genes.

3.2.6 RNA velocity analysis

The sequenced libraries were reference mapped and quantified using kb count function from kb-python package, which is a wrapper for kallisto and bustools (mouse GRCm39, GENCODE M26) (221). Workflow lamano was chosen to generate count matrices for spliced and unspliced variants of genes. Metadata, including cell embeddings (UMAP coordinates) and cluster information, were extracted from the Seurat object. Loom files from the kb count output and the Seurat metadata were imported into the scVelo pipeline for integrated analysis to generate RNA Velocity plot based on the Seurat UMAP coordinates (222).

3.3 Results

3.3.1 Compared to a synthetic ligand, 27HC differentially regulates LXR-target genes in bone marrow-derived macrophages (BMDMs).

Oxysterols are a group of cholesterol metabolites, with 27HC being the major circulating species (103). Many of this group of compounds are endogenous ligands of LXR, which are functionally involved in the regulation of cholesterol homeostasis (103). GW3965 is a well-characterized synthetic agonist of LXR (223). Previous work has found that 27HC drives ER-negative breast cancer metastasis in a myeloid cell-dependent manner, which is likely mediated in part by the immunosuppressive effect of 27HC on myeloid immune cells including neutrophils and macrophages (53, 208, 223). On the contrary, GW3965 and another synthetic agonist RGX-104 resulted in decreased myeloid-derived suppressor cells (MDSCs) in various cancer models and therefore impeded progression of those tumors (95). In breast cancer, GW3965 was shown to promote metastasis albeit to a lesser extent than 27HC and protected the primary tumor from cytotoxic chemotherapy (53, 76). These distinct and sometimes paradoxical contributions to cancer progression of the two LXR ligands led us to hypothesize that, akin to behaving as a SERM, 27HC could also selectively modulate the LXR, differentiating the transcriptional outcome in a context- or cell-specific manner.

The first aspect of selective modulators is that they result in a unique transcriptional pro-

gram, despite both being grossly seen as agonists. It was therefore of interest to compare the regulation of LXR target genes between 27HC and GW3965 in macrophages, a major cell population within the tumor microenvironment. I first investigated a time-course expression analysis of four well-known LXR target genes in murine bone marrow-derived macrophages (BMDMs) treated with either 27HC or GW3965 (**Figure 3.1A-D**). Both 27HC and GW3965 induced the expression of ABCA1 and ABCG1, with GW3965 resulting in a much larger induction (**Figure 3.1A-B**). However, 27HC did not seem to upregulate Srebf1 or Fasn over the 24hr time course, while GW3965 increased both transcripts (**Figure 3.1C-D**). Srebf1 and Fasn are involved in lipid metabolism and are considered direct targets of LXR activation. The differential expression of these two genes indicates that the response of LXR to a synthetic ligand could be distinct from oxysterol stimulation in BMDMs - not just differences in maximum response (agonist activity).

To better understand the difference between the two ligands, I performed a non-biased, bulk RNAseq on BMDMs treated with vehicle, 27HC or GW3965. To power the detection of subtle differences between 27HC and GW3965, I included 5-6 replicates per group in this relatively homogeneous culture system. As expected, even though 27HC and GW3965 treatments have similar contribution to the transcriptional profile of macrophages on the first principal component, drastic differences present on the second principal component (**Figure 3.1E**). The loadings plot of PCA shows the top 20 genes that contribute to the second principal component, with Ldlr and Dhcr24 expression having the greatest contribution to this principal component, suggesting that the LXR may be able to differentially regulate cholesterol synthesis (e.g., Dhcr24, CYP51), uptake (e.g., Ldlr) and lipid metabolism (e.g., Insig1, Scd1) in macrophages (**Supplemental Figure 4.1A**). Similar to the PCA plot, the heatmap illustration of the 7177 differentially regulated genes (DEGs) between 27HC and vehicle or GW3965 and vehicle, clearly demonstrates that while many genes are similarly regulated by 27HC and GW3965 there are distinct sets of genes that are regulated in an opposite manner between the two LXR ligands (**Figure 3.1F**).

I next used the RNAseq data to survey six known LXR-target genes, comparing their expression between 27HC and GW3965 treatments. In support of our earlier findings, 27HC strongly induced the expression of genes related to cholesterol efflux (Abca1 and Abcg1), but not Scd1, Srebf1, Fasn or Apoe, while GW3965 strongly promoted the expression of all six genes (**Figure 3.1G-H**). As illustrated in Venn diagrams (**Figure 3.1I-J**), just under 50% of those genes differ-

entially regulated over control were similar between 27HC or GW3965 (3714 genes commonly regulated compared to 3743 not commonly regulated).

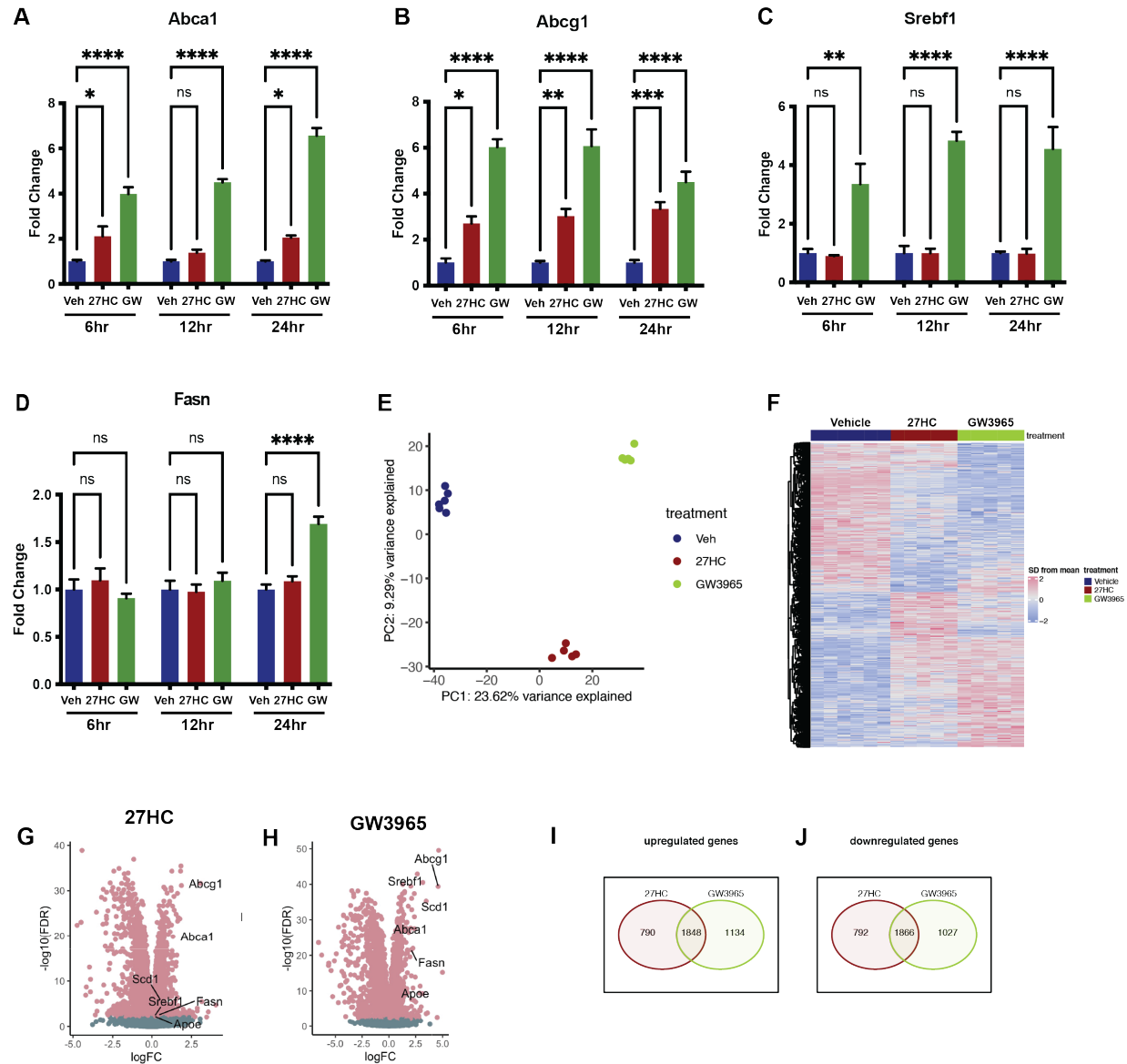


Figure 3.1: 27HC elicit differential gene expression profile in macrophages, compared to GW3965. (A-D) BMDMs were treated with vehicle, 27HC or GW3965 for 6hr, 12hr or 24hrs. Total RNA were subsequently isolated and purified. LXR target gene expression pattern on 2 dimensions was measured by RT-qPCR. (E-J) BMDMs were treated with vehicle, 27HC or GW3965 for 24hrs. Total RNA was isolated and then sequenced after genomic DNA elimination. (E) PCA analyses were then carried out on the sequenced expressions and each sample was plotted on PC1 and PC2. (F) Heatmap plotting the expression of all DEGs between 27HC and vehicle and between GW3965 and vehicle. (G-H) Volcano plot shows the fold changes and p-values of LXR-target genes under 27HC or GW3965 treatments. (I-J) Venn diagram shows the overlapping and non-overlapping DEGs between 27HC and GW3965 treatments. In A-D, data are presented as mean $\pm$ SEM and asterisks (*) indicate statistical significance (<0.05) from the respective vehicle control (N=3 in A, C-D. N=5 in B, one-way ANOVA with post-hoc Sidak's multiple comparisons test). In E-J, N=6 for vehicle, N=5 for 27HC and GW3965.

Given that 27HC is also a ligand of ER, and the differential regulation observed could be due to the input from this receptor, I included groups treated with the agonist 17 β -estradiol (E2)

and the antagonist Fulvestrant (ICI, a selective estrogen receptor degrader) (**Supplemental Figure 4.1B-C, G**). Interestingly, only a small number of DEGs are uniquely shared between 27HC and E2, and therefore the differential gene regulation between 27HC and GW3965 was unlikely driven by the engagement of ER by 27HC in BMDMs. Somewhat surprisingly, I found that antagonizing ER with ICI led to similar transcriptional outcome as modulating LXR, as represented by a large fraction of shared DEGs among 27HC, GW3965 and ICI (**Supplemental Figure 4.1B-C, G**). This observation is supported by data indicating that when ICI was co-treated with GW3965, the expression of many DEGs was not changed, especially those contributing to the first dimension of the multidimensional scaling plot (**Supplemental Figure 4.1D and G**).

Further demonstrating that LXR is selectively modulated are our observations that a ligand described as an LXR antagonist, GSK2033, and commonly used as a tool compound to implicate LXRs, in fact regulated a large number of genes in the same direction as GW3965 and fell out in its own transcriptional space on the PCA plot (**Supplemental Figure 4.1E-F, G**). When GSK2033 was added to 27HC, I found that GSK2033 was able to antagonize the upregulation of 522 shared DEGs that were upregulated by both 27HC and GW3965. GSK2033 was also able to antagonize the downregulation of 732 shared downregulated DEGs between 27HC and GW3965. However, a large number of DEGs were still shared between GSK2033 + 27HC and GW3965 as between 27HC and GW3965 (**Supplemental Figure 4.1E-F**). Collectively, these data demonstrate that GSK2033 could also be a selective LXR modulator, with more antagonistic properties than agonistic, but this has to be evaluated on a gene-by-gene basis.

Among the differentially regulated genes between 27HC and GW3965, I scanned for predicted LXR binding sites by searching for known LXREs obtained from JASPAR and HOCOMOCO databases of both isoforms in the promotor region of DNA sequences (**Supplemental Figure 4.2A-B**, respectively). For both LXR α and β , the binding motifs with model length of 19 were used. I identified 461 genes with putative LXR α binding site and 23 genes with putative LXR β binding sites (**Supplemental Table 4.1-4.2**). It is possible that this subset of genes could be differentially regulated by 27HC versus GW3965 through altered direct binding of LXR to the response element. Notably, there exists other documented ways by which LXR can regulate gene expression, such as differential binding to enhancers, tethering to another transcription factor and SUMOylation, all of which could be involved in regulating other differentially expressed genes

that did not have an LXRE in the promoter region (103).

3.3.2 In BMDMs, 27HC regulates immune-related genes both similarly and distinct to a synthetic agonist.

Previous work has described LXR as being regulating innate immunity to mediate macrophage-driven T cell suppression (102, 208). Gene expression-wise, 27HC was found to downregulate the expression of genes involved in antigen presentation, leading to reduced T cell expansion (208). Given such involvement, it was of interest to look into whether 27HC and GW3965 affect the inflammatory state of macrophages similarly or distinctly. I selected literature-documented genes involved in antigen-presentation and those upregulated in classically activated M1 macrophages (LPS-induced) to be the genes represented by inflammatory macrophages (224). Genes upregulated by IL-4 (in absence of LPS) in alternatively activated M2 macrophages were considered genes represented by resident/anti-inflammatory macrophages (224). Interestingly, for the group of genes related to inflammatory macrophages, both 27HC and GW3965 appeared to inhibit their expression, while those genes that are related to resident/anti-inflammatory macrophages did not have consistent response to 27HC and GW3965 treatments (**Supplemental Figure 4.3**). Therefore, these data indicate that 27HC and GW3965 have both shared similarities and differences in the (de)regulation of macrophages pro-inflammatory responses.

3.3.3 Specific modules of co-regulated genes exhibit differential expression patterns between 27HC and GW3965 treatments in BMDMs.

To assess the expression patterns of BMDM more generally after the treatment with different LXR ligands, I employed WGCNA algorithms on DEGs from 27HC and GW3965 treatments to identify co-regulated genes and then compared the effects of 27HC vs. GW3965 on co-regulated gene expression. Using this approach, 6 distinct modules were identified, with each of the DEGs being assigned to one module. The genes that were assigned to one module were more likely to be co-regulated given the expression patterns were similar to each other, compared to the genes in a different module (**Supplemental Figure 4.4**). The heatmaps show that modules 2 and 4 had the most pronounced differences between 27HC and GW3965 treatments. This observation is clearly

illustrated by the eigengene expression of each treatment in each module (**Figure 3.2A-F**); an eigengene is the first principal component of a particular module and describes the relative expression of all genes within that module. An eigengene with a low level of expression indicates that module genes were generally under-expressed, while a large value suggests the opposite. Specifically, in module 2, 27HC induced a large positive eigengene expression, while GW3965 was quite the opposite. In module 4, this trend was completely reversed. In module 5, 27HC considerably downregulated the expression of the module genes, while GW3965 had a very small positive effect. In contrast, both 27HC and GW3965 had the same directionality in modules 1, 3 and 6, (**Figure 2**).

To assess the functional implication of genes assigned to each module, I performed biological process (BP) gene ontology (GO) enrichment analyses on those genes. **Table 3.1** shows that 27HC and GW3965 had similar effects on the immune response and organonitrogen compound biosynthesis in BMDMs, as modules 1, 3 and 6 had significantly enriched related GO terms. On the other hand, 27HC and GW3965 seemed to elicit entirely opposite BMDM responses in terms of chemotaxis (module 2) and lipid metabolism (module 4). In more detail, module 2 genes suggest that, on the contrary to GW3965, 27HC strongly promoted the expression of genes in relation to chemotaxis. It is possible that the promotion of chemotaxis by 27HC could be mediated by LXR-independent mechanisms, involving receptors such as CXCR2 (225), though LXR-mediated chemotaxis pathways through the regulation of CD38 and CCR7 were also previously reported (89, 226). Given that both 27HC and GW3965 upregulated Cd38 and left Ccr7 unchanged, it is unlikely that this difference effect on chemotaxis was solely mediated by one of these two proteins. Nevertheless, 27HC and GW3965 also did not elicit opposite regulation of genes (Cxcr2, Mmp9, Bv8/Prok2) related to the previously reported CXCR2-dependent, LXR-independent, oxysterol-activated migrating neutrophils (**Supplemental Table 4.3**) (225). However, among the genes with predicted LXR binding site, Pdpn was assigned to module 2 and were oppositely regulated by both treatments. Previous work also reported the importance of Pdpn in mediating NK cell adhesion and tumor cell motility under tumor-hypoxic microenvironment (227). It would be therefore intriguing for future studies to further validate the involvement of LXR in this pathway. Module 5 contained genes that were enriched for cell development, and 27HC primarily downregulated these genes. Overall, these data indicate 27HC and GW3965 indeed share

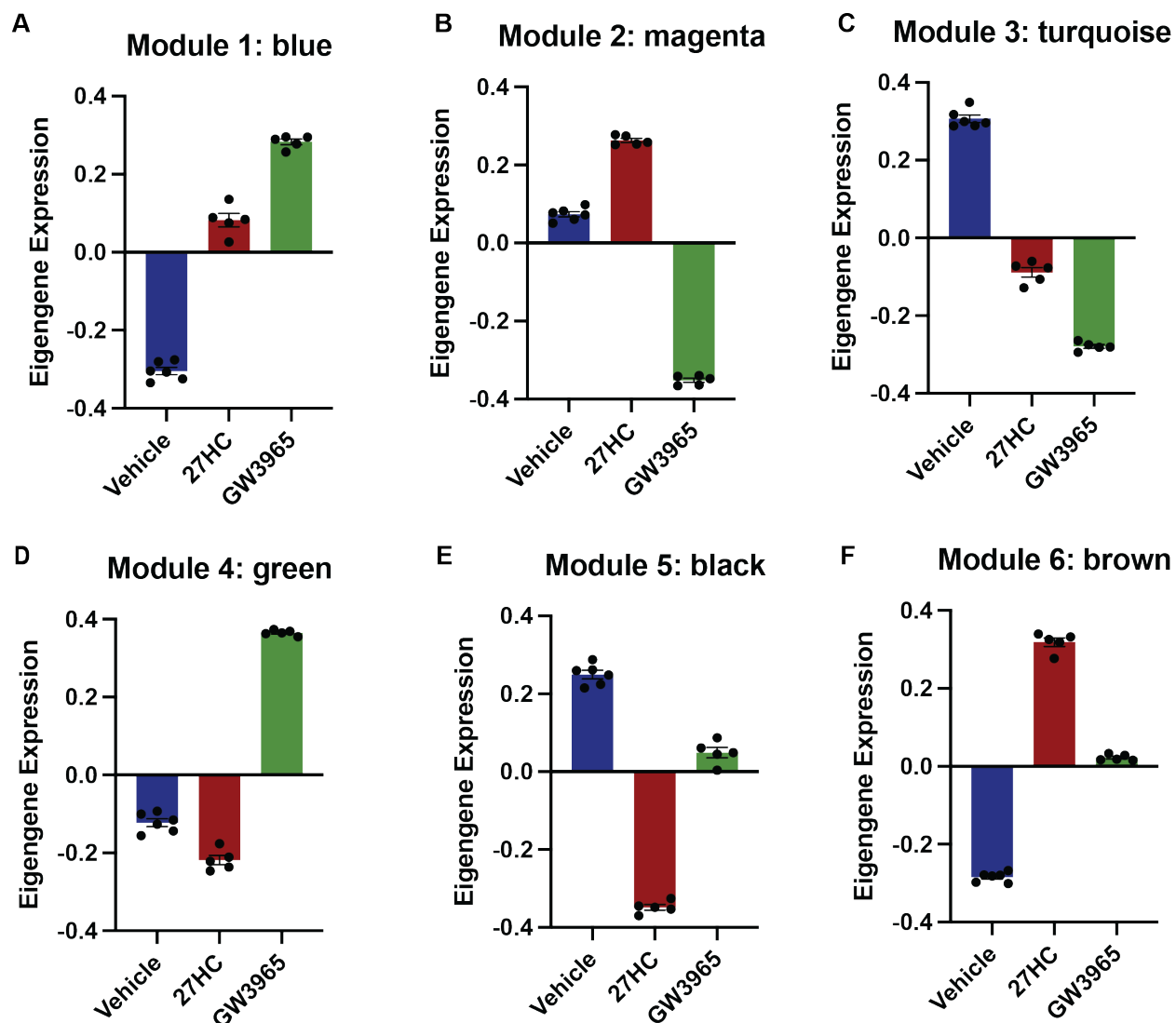


Figure 3.2: 27HC promoted differential gene expression modules in macrophages, compared to GW3965. (A-F) RNA sequenced macrophages underwent WGCNA analysis, which discovered 6 gene expression modules. The eigengene expression of each module is shown, with 27HC oppositely regulating module 2, 4 and 5 compared to GW3965.

similarities and prominent disparities which likely result in differential regulation of macrophage function, from immune function to lipid/cholesterol homeostasis, even though both have been conventionally considered LXR (partial) agonists.

Table 3.1: GO enrichment analyses on gene modules.

Module	Theme		GO ID	p-value	GO Description
1. blue	Protein Synthesis		1901566	<1e-30	organonitrogen compound biosynthetic process
			0006412	<1e-30	translation
			0043043	<1e-30	peptide biosynthetic process
2. magenta	Chemotaxis		0060326	1.30E-14	cell chemotaxis
			0070098	1.80E-14	chemokine-mediated signaling pathway
3. turquoise	Innate Immunity		0050900	8.70E-14	leukocyte migration
			0002376	<1e-30	immune system process
			0006955	<1e-30	immune response
4. green	Lipid Metabolism		0051707	<1e-30	response to other organism
			0006629	5.90E-13	lipid metabolic process
			0044281	3.20E-12	small molecule metabolic process
5. black	Cell Development		0043436	4.20E-12	oxoacid metabolic process
			0072359	6.40E-16	circulatory system development
			0030029	1.70E-15	actin filament-based process
			0009653	4.80E-15	anatomical structure morphogenesis
6. brown	Macromolecule Transport		0071840	5.30E-14	cellular component organization or biogenesis
			0042886	3.10E-12	amide transport
			0045184	3.20E-12	establishment of protein localization

3.3.4 LXR ligands differentially alter the immune cell composition of the metastatic tumor microenvironment.

Our previous work has shown that 27HC could suppress the ability of macrophages to activate T cells through antigen presentation (208). The suppression of T cells was dependent upon the macrophage expression of LXR. This finding was supported by another study, which identified TNBC-derived LXR ligands (agonists) exerting suppressive activities on macrophages (92). Similarly, Villablanca *et al.* showed that activation of LXR through tumor-derived oxysterols dampened

the maturation and therefore the anti-tumor function of dendritic cells (89). On the other hand, a handful of studies suggest immune-protective roles of LXR in myeloid cells through the use of synthetic ligands (103). For instance, the LXR agonist, GW3965, decreased MDSC abundance in murine melanoma models as well as in human samples (95). T0901317 activation of LXR down-regulated the expression of Ccl7 in macrophages, a Treg-attracting chemokine (96). Additionally, GW3965, promoted natural killer (NK) cell-mediated cytotoxicity against cancer cells (97). These contrasting reports on the effect of 27HC/LXR and synthetic ligand/LXR-mediated myeloid cell response led us to hypothesize that the activation of LXR by endogenous ligand is likely context-dependent, being tissue-type and cell-type specific. Therefore, besides macrophages, I explored the effects of 27HC versus GW3965 on the immune landscape within the microenvironment of a metastatic breast cancer model.

To investigate whether 27HC modulates diverse myeloid populations in the tumor microenvironment distinctly from GW3965, we performed single-cell RNAseq on CD45+ cells isolated from metastatic lung of 4T1 tumors (**Figure 3.3A**). After preprocessing and quality control, 9 distinct populations of CD11b+ cells were identified from the CD45+ immune cell population (**Figure 3.3B-D, Supplemental Figure 4.5A-D**). Interestingly, several CD11b+ cell types were more enriched under 27HC compared to GW3965 treatments (**Figure 3.3D-E**). For example, even though both GW3965 and 27HC seem to enrich the PDL1+CD11b+ cells, compared to placebo, 27HC treatment resulted in even more of those cells. 27HC also appeared to enrich for eosinophils (defined as Adgre+CCR3+PRG2+). Prominently, among all the CD11b+ cells, there existed a large population of non-classical MHCII^{lo}CD14+Ly6C-Ly6G- monocytes, which were further characterized into 3 separate subclusters (0-2). In these subgroups of monocytes, 27HC increased the MHCII^{lo} monocyte 0 subcluster while shrinking the MHCII^{lo} monocyte 2 subcluster. On the other hand, GW3965 promoted the accumulation of MHCII^{lo} monocyte subcluster 2 the most.

Because these cell types are closely clustered together on UMAP projections, I speculated that these populations were from a common progenitor, which then differentiated into a lineage of cells within the tumor microenvironment. To understand this possible trajectory, I performed RNA velocity analysis (**Figure 3.3F**). The analysis supported our speculation and indicated two predicted terminal stages of the cell lineage: (A) MHCII^{lo} monocyte subcluster 0, and (B) PDL1+ cells. Both putative terminal subclusters were more enriched by 27HC, which is interesting given

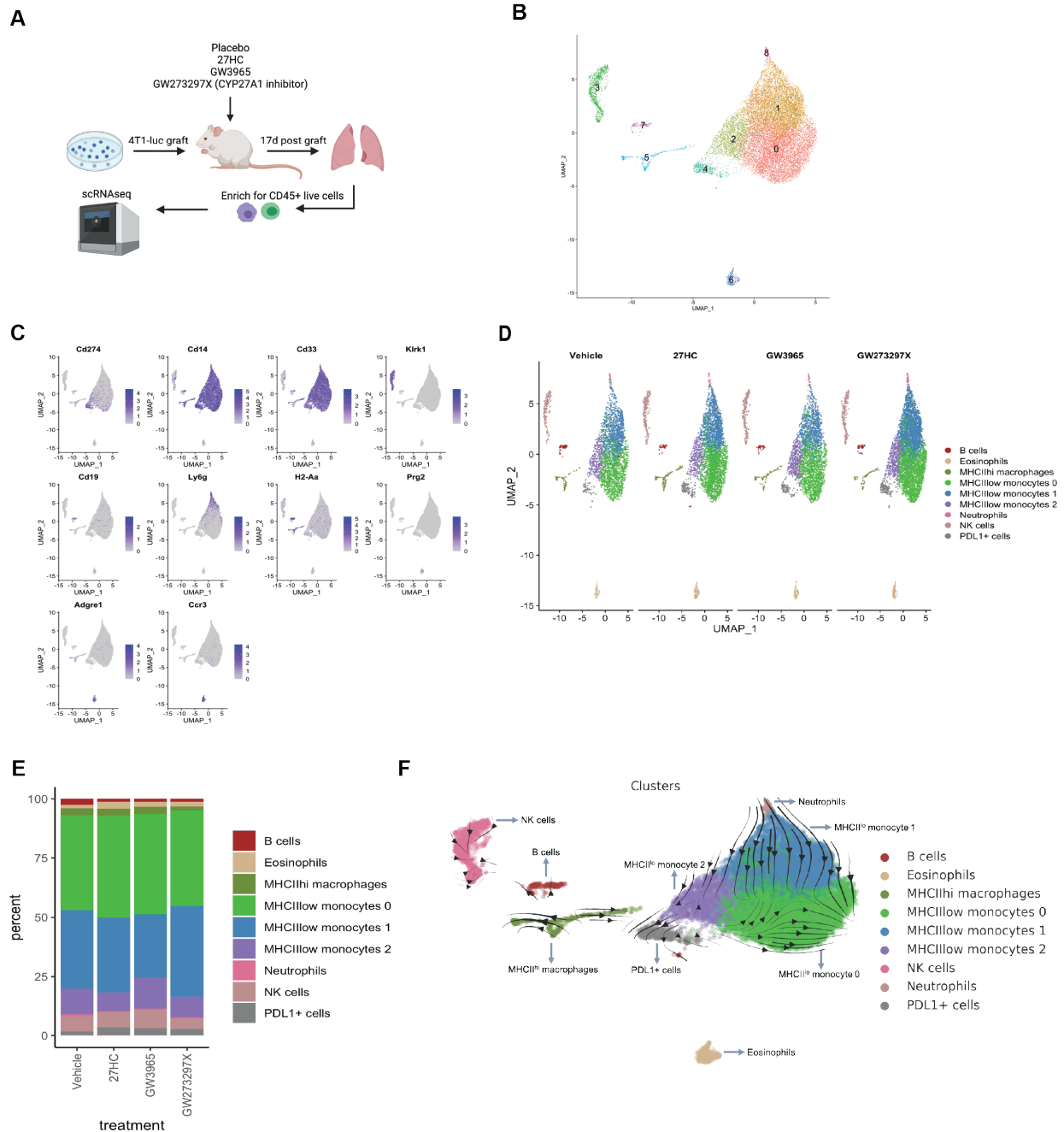


Figure 3.3: 27HC differentially changed the immune cell composition of a metastatic tumor microenvironment, compared to GW3965. (A) 4T1-luc tumor cells were grafted into BALB/c mice and the indicated treatments were given one day post graft. After 16 days of treatments, metastatic lungs were harvested and enriched for live CD45+ cells for single-cell RNA sequencing. (B-D) Post filtering, the Seurat analysis pipeline identified 9 population of CD11b+ cells, with marker gene expression highlighted in (C) and the cell populations were annotated in (D). (E) shows that each treatment enriched and diminished different subpopulations of CD11b+ cells and (F) RNA velocity analysis predicts MHCII^{lo} population 0 to be the terminal population in the closely clustered monocytic cells. N=3 for all treatment groups.

the well-known immune suppressive effects of PDL1+ cells. Additionally, PDL1+ cells, MHCII^{lo} monocyte 0 and monocyte 2 populations all significantly upregulated the expression of *Nfkbia*,

compared to MHCII^{lo} monocyte 1 population, suggesting an activation of NF- κ B signaling. Previous studies have shown the importance of IKK β to maintaining the alternative and pro-tumor phenotype of tumor-associated macrophages (228). Therefore, it is possible that NF- κ B signaling is one of the driving forces of the cell differentiation trajectory. These data together suggest that 27HC treatment could promote the recruitment and differentiation of terminal stage tumor-associated monocytes, which may provide an explanation to 27HC being more immunosuppressive and pro-metastatic in animal models than the synthetic agonists of LXR.

3.3.5 Differentially regulated genes within myeloid immune populations of the metastatic microenvironment.

Besides the effect on cell composition, I also explored the gene expression differences within the different myeloid cell populations from the metastatic lungs of mice treated with 27HC or GW3965. If the LXR is indeed being selectively modulated, we would expect the transcriptome being uniquely altered in different cell types. Intriguingly, the LXR α isoform was either not expressed or expressed at a very low level within the CD11b⁺ cells of the metastatic microenvironment, while almost all cell types expressed LXR β (**Supplemental Figure 4.6A**). This is in contrast to cultured BMDMs, where LXR isoforms were more similarly expressed (**Supplemental Figure 4.6B**). Differential expression analysis showed a large fraction of DEGs from placebo that were not shared between 27HC and GW3965 in the different myeloid subtypes (**Figure 3.4A-C**), similar to our data from homogeneous in vitro cultures of macrophages (**Figure 3.1-3.2**). Further assessing the differential gene modulation capacity of 27HC, I next scrutinized the expression of the conventional LXR target genes. Strikingly, among the myeloid cell types surveyed, differential regulation by the same ligand of well-known LXR-target genes were observed. In Ag-presenting MHCII^{hi} macrophages, GW3965 only substantially upregulated the expression of Abca1 compared to placebo, while Abcg1, Srebf1 and Apoe were not. However, in PDL1⁺ cells, Abca1, Abcg1 and Srebf1 were all upregulated by both 27HC and GW3965, with the effect of GW3965 being more pronounced (**Figure 3.4D-E, Supplemental Figure 4.7A-B**). Unlike in BMDMs, 27HC was able to modestly induce Srebf1 in this cell population, suggesting a selective modulatory effect on PDL1⁺ cells. Since LXR α was lowly expressed or not detected in these cells, the effect on

Srebf1 is likely to be mainly attributed to by LXR β . As expected from low LXR (α and β) expression in eosinophils, the select LXR target genes were not significantly altered upon treatment with either ligand (**Figure 3.4F**, **Supplemental Figure 4.7C**).

Interestingly, within the closely related MHCII^{lo} monocyte subpopulations, differential regulation of these LXR target genes was observed (**Figure 3.4G-I**, **Supplemental Figure 4.7D-F**). In one of the predicted terminal subpopulations, MHCII^{lo} monocyte 0, 27HC induced the expression of Srebf1, leaving Abca1 and Abcg1 largely unchanged, whereas GW3965 only upregulated Abca1 (**Figure 3.4G**). It is surprising to note that even though GW3965 upregulated Abca1, it quite strongly suppressed the expression of Abcg1 in this subpopulation. Changes in MHCII^{lo} monocyte 1 cell population by 27HC and GW3965 were quite similar compared to the monocyte 0 population, except for 1 gene, ABCG1, which was not strongly suppressed by GW3965. In MHCII^{lo} monocyte 2, Srebf1 was lightly upregulated by GW3965 (**Figure 3.4I**). These data would indicate that 27HC likely moderately promoted fatty acid synthesis in these three closely related nonclassical monocyte populations without upregulating cholesterol transport proteins. In fact, Srebf1 has been implicated in the degradation of Abca1 (229), and increased expression of Srebf1 without increasing Abca1 could potentially lead to a net decrease in cholesterol efflux, leading to cholesterol accumulation in those cells.

To understand the functional outcome of differential regulation among those cells, I performed overrepresentation analysis on the differentially regulated genes using the hallmark pathways. Given the overall small number of statistically significant DEGs, I focused on the largest cell population with the most DEGs, the MHCII^{lo} monocyte 0 subpopulation. In the MHCII^{lo} monocyte 0 subpopulation, the TNF- α signaling pathway was the most significantly downregulated by 27HC, while the most significantly upregulated by GW3965, even though both treatments significantly downregulated the IFN- γ pathways (**Supplemental Figure 4.6C-D**), whereas in BMDMs these pathways were concertedly downregulated by both treatments (**Supplemental Figure 4.8A-B**).

Taken together, our data suggest that GW3965 may not be a pure agonist of LXR in every cell population and support the idea that the modulation of LXR could be highly sensitive to cell/tissue types, even among highly related cell types characterized only by small changes in the expression of multiple genes. These data strongly support the notion that similar to the steroid

receptors the LXR can be selectively modulated.

3.3.6 CYP27A1 inhibitor shrinks the terminal state myeloid cell types and promotes the early stages.

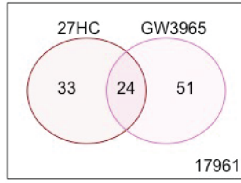
27HC is generated from cholesterol through enzymatic pathway by CYP27A1. In our previous work, we identified that removing the endogenous source of 27HC by knocking out CYP27A1 expression in BMDMs promoted their ability to induce T cell proliferation. A CYP27A1 inhibitor (CYP, GW273297X) was also shown to improve the immune checkpoint inhibition efficacy in breast and ovarian cancer models (77, 208). Therefore, I wanted to investigate the metastatic microenvironment myeloid cell composition when mice were treated with CYP27A1 inhibitor. Interestingly, unlike 27HC and GW3965, CYP27A1 inhibitor promoted the number of MHCII^{lo} monocyte 1 cells, which were predicted to be a cell type that is early in the nonclassical monocyte lineage (**Figure 3.3D-E**). CYP27A1 also reduced the proportion of PDL1+ cells, compared to 27HC. These data suggest that CYP27A1 inhibitor, contrary to the effect of 27HC, may function to reduce the differentiation or recruitment of certain late-stage myeloid cell populations, compared to 27HC. Also, expectedly, the CYP27A1 inhibitor did not obviously activate or suppress LXR-target genes in most of the myeloid cell populations, except for in the PDL1+ cells (**Figure 3.4D-I**). In actuality, treatment with the CYP27A1 inhibitor moderately promoted the expression of *Abca1*, *Abcg1* and *Srebf1* in PDL1+ cells, which may suggest a compensatory mechanism or off-target effect of CYP27A1 inhibitor in this cell type (**Figure 3.4E**).

3.4 Discussion

LXRs have been identified to play key roles in regulating immune surveillance and therefore cancer progression (92, 95, 103, 230–232). However, conflicting reports have described distinct primary effects of LXR activation on various immune cell populations. It has been widely described that LXR activation in T cells inhibit their proliferation and pro-inflammatory functions (140, 231, 233). Recent findings scrutinized the role of LXR in a subpopulation of T cells, Tregs, and suggested that a single-copy deletion of LXR β in Tregs dampened activated Treg sur-

A MHCII^{lo} Monocyte 0

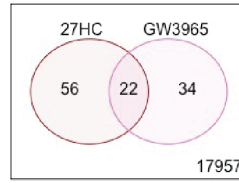
upregulated genes



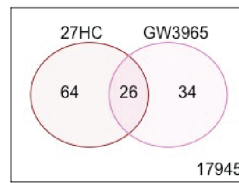
downregulated genes

**B MHCII^{lo} Monocyte 1**

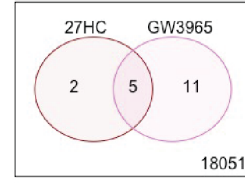
upregulated genes



downregulated genes

**C MHCII^{lo} Monocyte 2**

upregulated genes



downregulated genes

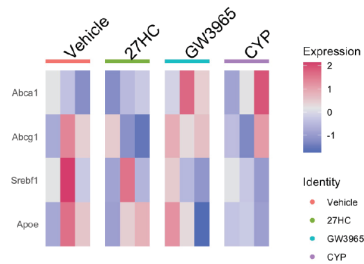
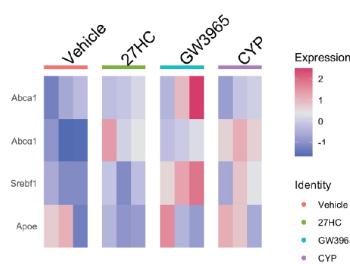
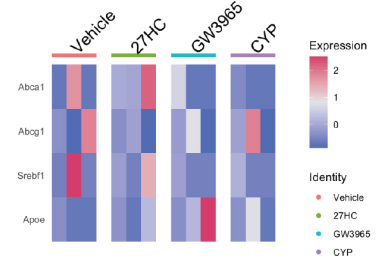
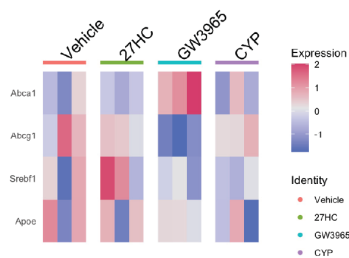
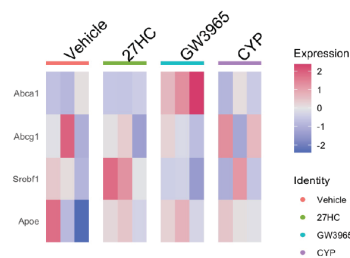
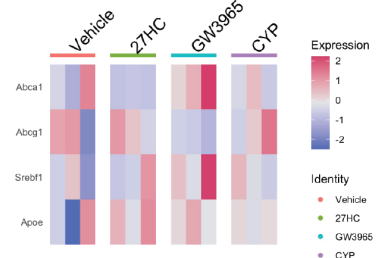
**D Ag-Presenting Cells****E PDL1+ Cells****F Eosinophils****G MHCII^{lo} Monocyte 0****H MHCII^{lo} Monocyte 1****I MHCII^{lo} Monocyte 2**

Figure 3.4: 27HC differentially regulated gene expression in distinct myeloid populations within a metastatic tumor microenvironment, compared to GW3965. (A-C) Venn diagrams show that 27HC and GW3965 share overlapping and non-overlapping DEGs. **(D-I)** Heatmaps of the expression of LXR target genes show that 27HC and GW3965 regulated LXR target genes differentially in different subpopulations of myeloid cells.

vival, which then resulted in early-onset fatal autoimmune disease (231). This report conflicted with earlier reports indicating LXR is a negative regulator of T cell proliferation and therefore

demonstrated the potential differential biology of this LXR isoform in subpopulations of T cells. LXR agonism in myeloid cell subpopulations also remains a conundrum, and its effect on cancer progression in different cancer models continues to be equivocal (92, 95, 208, 226). Such ambiguity may arise from the possibility that LXRs are subject to being selectively modulated in a ligand, tissue- and context-specific context (99, 214).

In this study, we demonstrated that the LXR satisfies the requirements of being selectively regulated: (1) different ligands (27HC, GW3965 and GSK2033) exert overlapping but distinct effects on the transcriptional landscape with the same cell type, and (2) the same ligand resulted in differential transcriptional profiles in different cell types, and even between highly related cell types.

Initial analysis in the relatively homogenous *in vitro* culture of BMDMs, found that 27HC induced the expression of genes involved in cholesterol efflux, but not those in fatty acid synthesis, while GW3965 induced genes in both pathways. This selective modulation is similar to the previously reported effect of desmosterol, another putative selective LXR modulator, which unlike 27HC being an oxysterol is a cholesterol metabolite intermediate upstream of cholesterol synthesis. RNAseq of BMDMs treated with 27HC or GW3965 additionally supported the findings that 27HC and GW3965 regulated the expression of genes responsible for cholesterol synthesis, lipid metabolism and chemotaxis differentially in macrophages, even though it still remains unclear whether the differences in chemotaxis could be solely attributed to LXR and future work would be required to uncover other possible LXR-mediated chemotaxis pathways. I also showed that a commonly used LXR antagonist, GSK2033, is likely behaving as a selective modulator, rather than a pure antagonist, and therefore assumptions made seeing this compound as a pure antagonist should be taken with caution.

In the metastatic tumor microenvironment, 27HC appeared to enrich several terminal myeloid cell populations, including PDL1+ cells and MHCII^{lo} nonclassical monocytes. A previous study showed that GW3965 depleted pro-tumorigenic M-MDSCs in various cancer models and these findings may provide insights as to why 27HC had a higher pro-metastatic potential even though it is also an LXR agonist (95). Besides the difference in proportions of myeloid cell subpopulations, 27HC and GW3965 also elicited differential expression of LXR-target genes in the subpopulations. As supported by previous findings that one of the LXR isoforms, LXR β , is more ubiqui-

tously expressed. I also found that tumor-associated myeloid cells mainly express this subtype, indicating that the differential regulation of LXR-target genes was primarily mediated by LXR β . Interestingly, MHCII^{hi} macrophages were the only cell type that highly expressed LXR α . To further validate our *in vivo* findings *in vitro*, future works could try and isolate the identified myeloid populations with defined markers. The isolated cells could then be treated and cultured to measure the LXR target gene expression. Additionally, lineage tracing could be employed to verify the trajectory prediction carried out by RNA velocity analysis and test whether 27HC promoted differentiation of the monocytic subpopulations at the tumor site.

Together, our findings indicate that LXRs are capable of being selectively modulated by another group of endogenous ligands, oxysterols, as illustrated by 27HC, and this modulation is highly cell type-specific. Even among the closely related cells, some level of differential expression of LXR-target genes was observed. This work could help explain the lack of comparable pro-metastatic effect of GW3965, compared to 27HC in breast cancer models. Our work also raises the possibility that many previous characterized LXR agonist or antagonist may actually function as selective modulators, as both GSK2033 and GW3965 exhibited selective regulation of LXR-regulated genes in macrophages and subpopulations of myeloid cells, respectively. These findings strengthen the importance to uncover the roles of LXR in distinct populations of immune cells, as LXR activation was shown to not yield a blanket outcome in even closely related cell types. This would then open up the potential of targeting LXR for precise therapeutic manipulation of immune cells.

CHAPTER 4

CONCLUSION AND DISCUSSION

Obesity and overweight have become an increasingly prevalent morbidity in the US and across the world, posing challenges to the management of chronic and noncommunicable diseases. It is estimated that, by 2030, 38% of the worlds adult population will be overweight and 20% will be obese, while in US over one third of the population is deemed obese (234, 235). Early in 2008, a link between obesity and cancer risk has been drawn by a meta-analysis of over 280,000 incident cases, which demonstrated a positive association between increase in body-mass index and various cancer types, including thyroid, colon, renal and breast (236). However, the precise mechanistic links between obesity and cancer progression once remained to be elucidated. Cholesterol as a common co-morbidity over the past decades has been studied as a possible etiology between obesity and cancer. Studies have suggested that mechanisms by which cholesterol promotes cancer progression are likely multifactorial and include both direct effects of cholesterol on cancer cells, as well as effects of downstream cholesterol metabolites on both the cancer cells and the microenvironment. 27HC is one metabolite in particular that has emerged as being important for cancer progression. A recent work found that the primary by-product of cholesterol metabolism, 27HC, mediated the elevated cholesterol-driven breast cancer metastasis (53). Additionally, 27HC required the presence of myeloid cell population to exert its pro-metastatic effect (90). I therefore hypothesized that 27HC modulates the myeloid immune cells to promote cancer progression.

Work presented in this dissertation first identified that 27HC is capable of directly modulating macrophages, which are one of the major myeloid cell populations in the tumor microenvironment, to suppress effector T cell expansion and cytotoxic activities against antigen-specific cancer cells. This suppression was also found to be dose-dependent, as increasing concentration of 27HC also rendered macrophages more suppressive. This work further demonstrated that the suppressive effect of 27HC required one of the two target nuclear receptors of 27HC, LXR. These findings

are significant because breast cancer is well-known for its immune-cold tumor microenvironment and immunotherapy such as checkpoint inhibition has only shown modest efficacy in TNBC. It is therefore possible that reducing endogenous source of 27HC could help mitigate the immunosuppressive tumor microenvironment and therefore improve the efficacy of immune checkpoint blockade. In support of this conjecture, experiments in chapter two showed that mice bearing 4T1 metastatic mammary tumor had significantly reduced tumor burden when the mice were treated with a combination regime of both CYP27A1 inhibitor and immune checkpoint inhibitor α PDL1, compared to α PDL1 alone. Interestingly, anastrozole, a marketed aromatase inhibitor, was found to have an off-target effect and inhibited CYP27A1 as a result. Such inhibition did not result in alterations in plasma and hepatic cholesterol content, which implied the possibility of developing therapeutics to remove sources of 27HC while having a minimal impact on cholesterol homeostasis. Upstream of CYP27A1, several studies explored additional potential therapeutic targets of the cholesterol-27HC-LXR axis. For instance, targeting PCSK9, which reduced cellular cholesterol levels, could also improve checkpoint inhibition efficacy (94). Prospective studies have furthermore shown statin use enhanced response rate or clinical outcome of immune checkpoint inhibitors in non-small cell lung cancer (NSCLC) (237,238). Yet, targeting cholesterol synthesis directly carries the risk of restoring cholesterol homeostasis, and therefore the beneficial effects may not be long-lasting.

Targeting LXR, on the other hand, has been more controversial. Our results indicated that LXR activation by 27HC suppressed T cell activity via the modulation of macrophages, and intervening this action may confer benefits in stalling cancer progression. In support of our findings, another work showed that LXR inverse agonist treatment advanced cytotoxic T cell activities and impeded TNBC development (92). Several other recent studies also suggested the suppression of different populations of myeloid immune cells when LXRs are activated. However, conflicting reports also exist, and especially a synthetic agonist of LXR has been under clinical trials for the treatment of solid tumors, after earlier reports demonstrating that synthetic LXR agonism by GW3965 and RGX-104 depleted MDSC population in various cancer models (95). The paradoxical contribution of LXR to cancer progression and the myeloid immune subpopulations led us to hypothesize that LXRs are capable of being selectively modulated and the effect of such modulation is highly context-specific, being tissue-specific and ligand-specific.

To test this hypothesis, I performed bulk RNA sequencing on macrophages treated with 27HC and various LXR/ER synthetic ligands. I found that 27HC had similar selective modulatory capacity to a cholesterol metabolite intermediate, desmosterol, in that 27HC was able to upregulate genes involved in cholesterol efflux, while leaving those related to fatty acid metabolism almost unregulated. The synthetic agonist of LXR, GW3965, instead highly promoted both pathways. This characteristic of GW3965 in fact delayed the effort of developing this agonist to treat atherosclerosis, because the activation of hepatic fatty acid synthesis caused symptoms as dyslipidemia in subjects tested. The potential of 27HC and desmosterol to selectively modulate LXR then lights up hopes to design ligands to promote certain beneficial effects while avoiding the adverse ones. Chemotaxis pathways, furthermore, were differentially regulated by 27HC and GW3965, as 27HC highly promoted gene networks implicated in chemotaxis of immune cells, while GW3965 did the opposite. I also identified that a commonly utilized LXR antagonist, GSK2033, is also likely a selective modulator, rather than a pure antagonist.

To further address whether 27HC modulates the myeloid subpopulations in the tumor microenvironment distinctly from GW3965, single-cell RNAseq was performed on immune cells isolated from metastatic lung of 4T1 tumors after daily injection of 27HC or injecting GW3965 every other day. Previous work has shown that pre-treating mice with 27HC for 5 consecutive days was sufficient to promote breast cancer metastasis (90). Here I present a model that mimics a prolonged and constant hypercholesterolemia/hyper-27HC state in mice. After annotating the myeloid subpopulations, I noted that 27HC enriched several CD11b⁺ subpopulations compared to GW3965, such as PDL1⁺ cells and the one of the nonclassical monocyte populations. These two cell types were both predicted to be terminal myeloid cell stages, according to RNA velocity analysis, which may help explain the difference in the treatment outcomes of 27HC and GW3965 in cancer models. It remains to be elucidated, nevertheless, whether the differentiation of myeloid subpopulation occurred at the tumor site or those cells were recruited overtime. Besides the influences on cell proportions, 27HC and GW3965 induced differential LXR-target gene expression in most of the cell populations, further supporting the selective modulatory capacity of 27HC in a tissue-specific context. More interestingly, GW3965 also did not behave as a pure agonist in many of the myeloid cells, further raising the possibility that LXRs function more similar to ER, with most of the ligands to ER being in fact selective modulators.

Previously, LXR activation was found to work directly on T cells to suppress their proliferation (140). Our work ultimately uncovered a novel mechanism by which 27HC and its action on LXR influences the innate immune system to further dampen T cell activity and promote tumor progression. I then showcased the differential effects of 27HC and GW3965 on distinct myeloid subpopulations, in an effort to uncover the seemingly paradoxical roles of LXR activation in immune cells and its downstream implications. This attempt suggested a selective modulatory ability of 27HC, which in varied aspects yielded distinct transcriptional results in target cell populations compared to GW3965 (**Figure 4.1**).

Our findings innovatively point out a future direction for therapeutic development, where selective LXR modulators could be designed to augment the desired effects in a disease-specific context, while avoiding unwanted side effects. For example, desmosterol and 27HC could be beneficial to treating atherosclerosis without promoting lipid synthesis (99). While in cancer, another group of selective modulators could be developed to avoid 27HC-like pro-metastatic and immunosuppressive effect. Given the requirement of LXR and the impact of LXR activation could even be opposite in closely related immune populations (231), future works should also continue to unveil such effects and pharmacology to aid the effort of precisely targeting specific implicated immune populations.

Stemming from our current findings, it would also be interesting for future studies to continue the understanding of the functional roles of myeloid subpopulations in the tumor microenvironment. Specifically, this work have identified that 27HC promoted several putative cell populations of terminal stages, and thus it would be important to understand whether these terminal populations possess immunosuppressive properties and directly contribute to cancer progression using both *in vitro* and *in vivo* models. In the long term, finding possible targets to re-educate and re-program these immune populations shall also confer therapeutic advantage to the management of cancer.

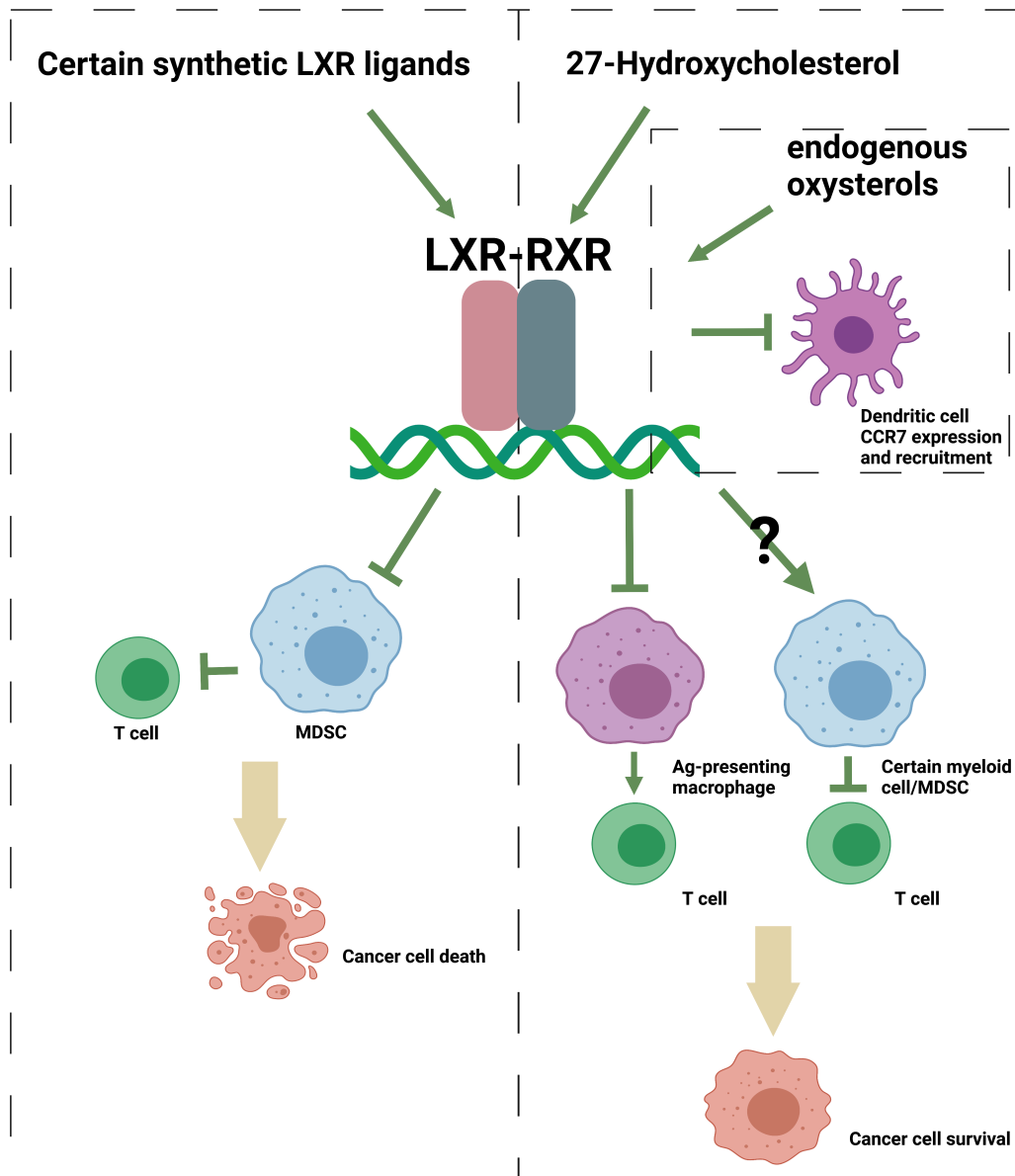


Figure 4.1: The LXRs have multifaceted contributions to cancer progression. When the LXRs were activated by certain synthetic agonists, MDSC population was depleted, leading to augmented T cell activity and cancer death in various cancer models. On the other hand, 27HC has been shown to increase the recruitment and differentiation of other types of myeloid cells including MDSCs to the tumor site, resulting in impaired T cell function. The precise role of LXR in this process has not been determined. 27HC could activate LXR in Ag-presenting macrophages and other myeloid cells such as neutrophils, resulting in a phenotype that confers suppressive effects on T cells. Additionally, endogenously produced oxysterols have been shown to decrease the expression of CCR7 within dendritic cells, impairing their ability to migrate towards tumor sites. Figure created with BioRender.

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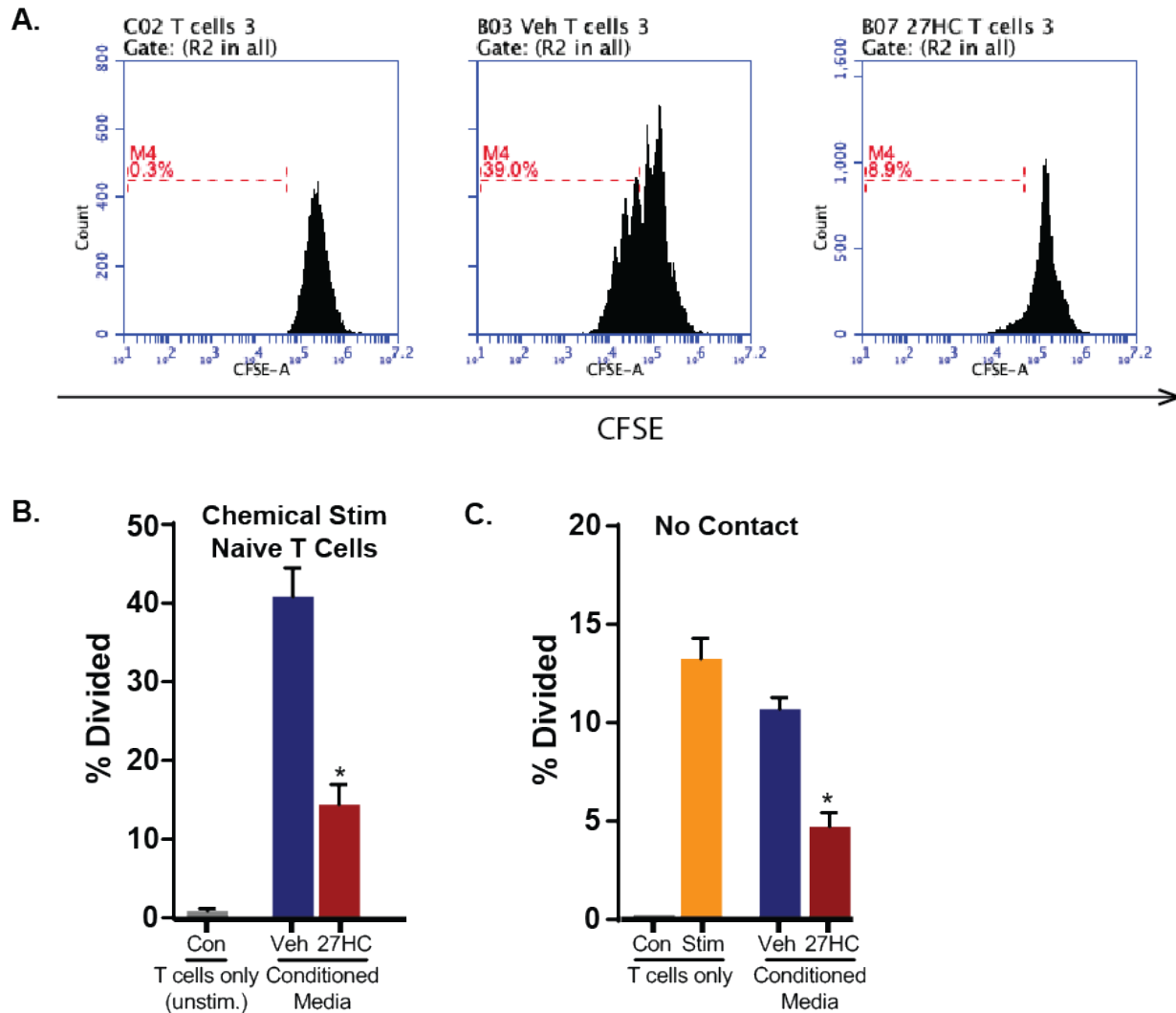
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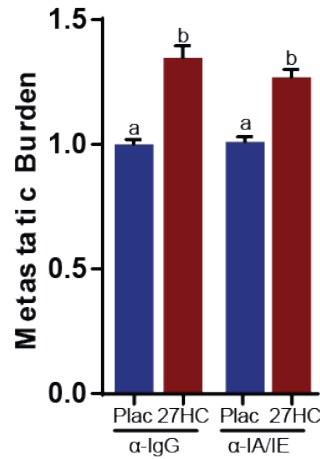
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APPENDIX A

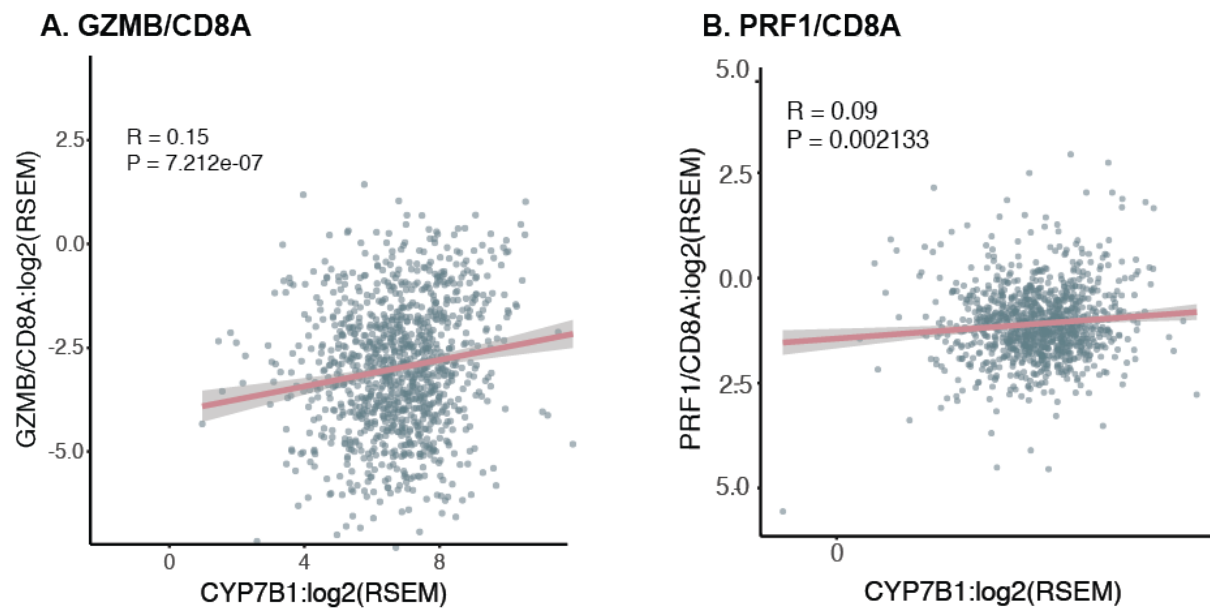
CHAPTER 3 SUPPLEMENTAL FIGURES



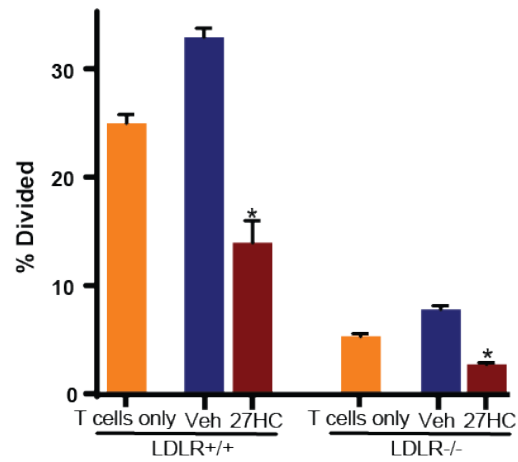
Supplemental Figure 3.1: 27HC-treated macrophages suppress T cell expansion. (A) Representative histograms from flow cytometry. CD3+ T cells were isolated from the spleen of OT-1 mice, stained with CFSE, were co-cultured with BMDMs treated with OVA₂₅₇₋₂₆₄ and either vehicle- or 27HC. 72hrs after co-culture, T cells were harvested for flow cytometry analysis. These data are from **Figure 2.3E**, and also representative of **Figure 2.2D-E**. (B) Naive T cells were isolated from the spleen of wildtype C57BL/6 mice, chemically activated and cultured in the presence of conditioned media harvested from vehicle- or 27HC-treated BMDMs. T cells were harvested for flow cytometry. (C) CD3+ T cells were isolated from the spleen of wildtype C57BL/6 mice, labeled with CFSE, chemically activated and cultured with conditioned media harvested from vehicle- or 27HC-treated BMDMs. T cells were harvested for flow cytometry. Data are presented as mean $\pm$ SEM and asterisks (*) indicate statistical significance from the respective vehicle control (N=3/group for T cell only controls, for conditioned-media conditions, N=5-6/group). (two-tailed t-test, $p < 0.05$). This sub-figure is an independent repeat of **Figure 2.1I**



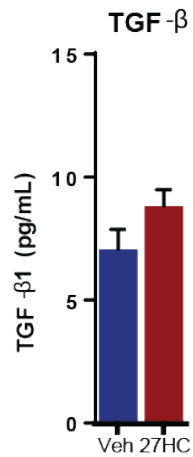
Supplemental Figure 3.2: Blockade of MHCII does not attenuate pro-colonizing effects of 27HC. Mice were pretreated with placebo or 27HC (20mg/kg/d) for 5d, as in (53). 36h and 12h prior to cell graft, mice were injected (i.p) with an isotype control antibody (IgG) or an antibody against murine I-A/I-E. Mice were then grafted with Met1 cells expressing iRFP. Resulting cancer colonies within the lung were quantified by ex vivo imaging for iRFP fluorescence. Different letters denote statistical significance, N=8/group. (one-way ANOVA followed by Neuman-Kuels multiple comparison test, $p < 0.05$).



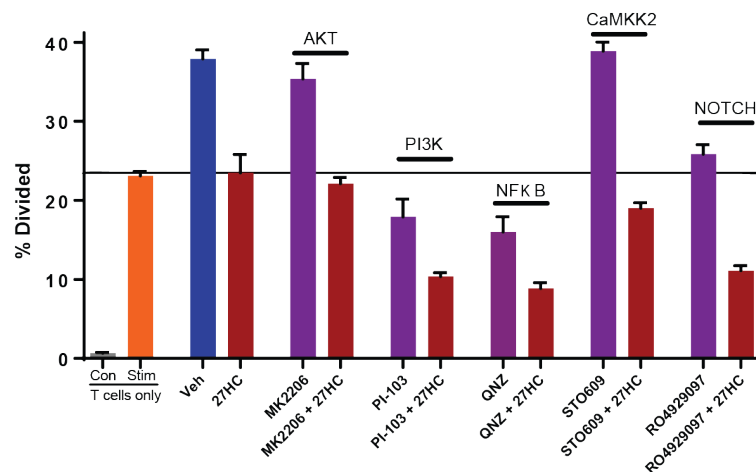
Supplemental Figure 3.3: Expression of granzyme B (GZMB) and perforin (PRF1) normalized to CD8A expression was modestly but significantly correlated with CYP7B1 expression. mRNA expressions from Human RNA sequencing data from TCGA Pan Cancer for invasive breast carcinoma were log2 transformed and Pearson's correlation was assessed (N=1077 in (A) and 1081 in (B)). These data partner with Figure 2.3L,M.



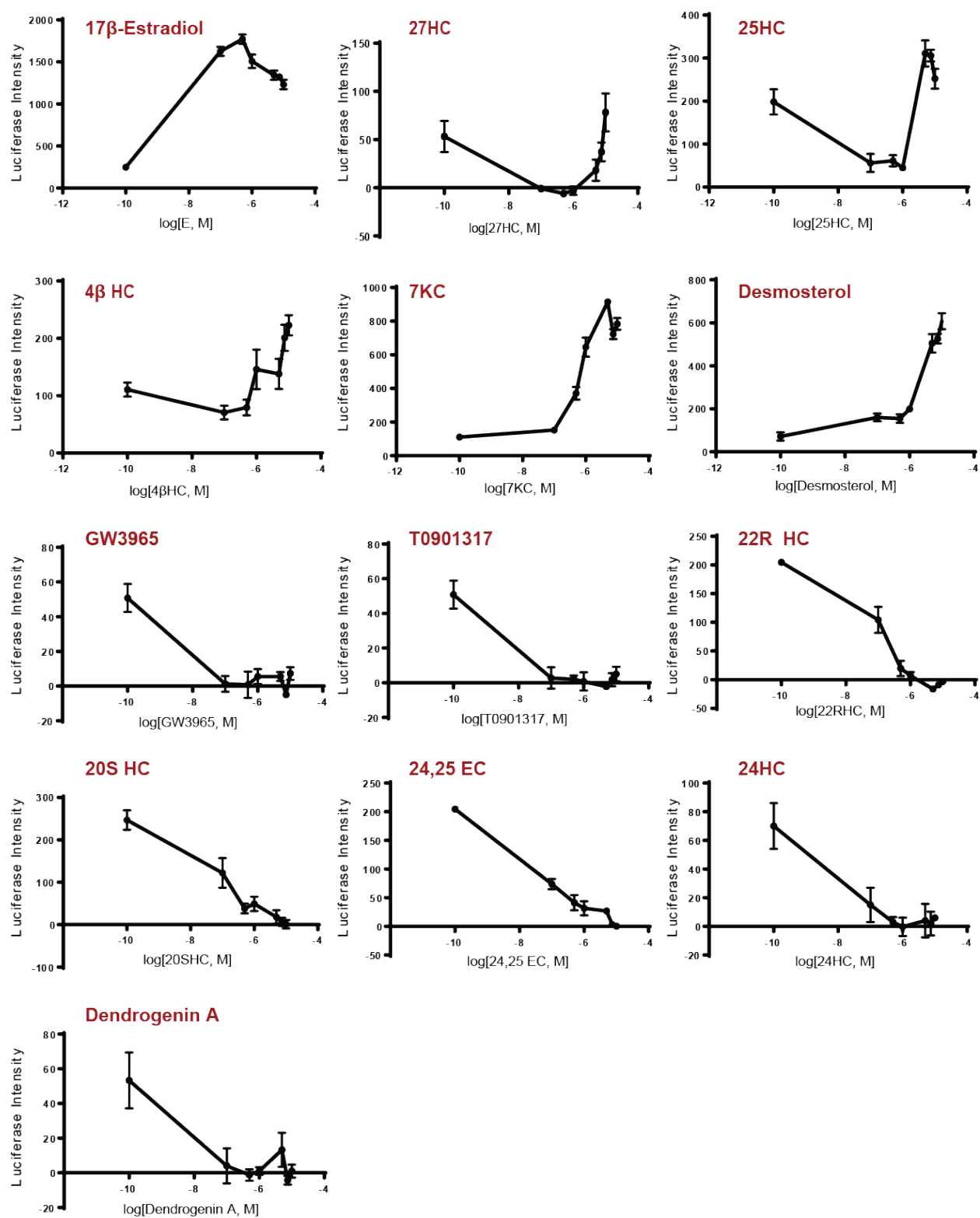
Supplemental Figure 3.4: Cholesterol efflux from macrophages unlikely to mediate the suppressive effect of 27HC on T cells. CD3⁺ T cells were isolated from wildtype C57BL/6 or LDLR^{-/-} mice, labeled with CFSE and chemically activated. They were then co-cultured with vehicle- or 27HC-treated BMDMs. 72hrs after co-culture, T cells were harvested for flow cytometry analysis. Data are presented as mean $\pm$ SEM and asterisks (*) indicate statistical significance from the respective vehicle control (N=3/group for all T cell only controls, for co-culture conditions, N=5/group). (two-tailed t-test, $p < 0.05$).



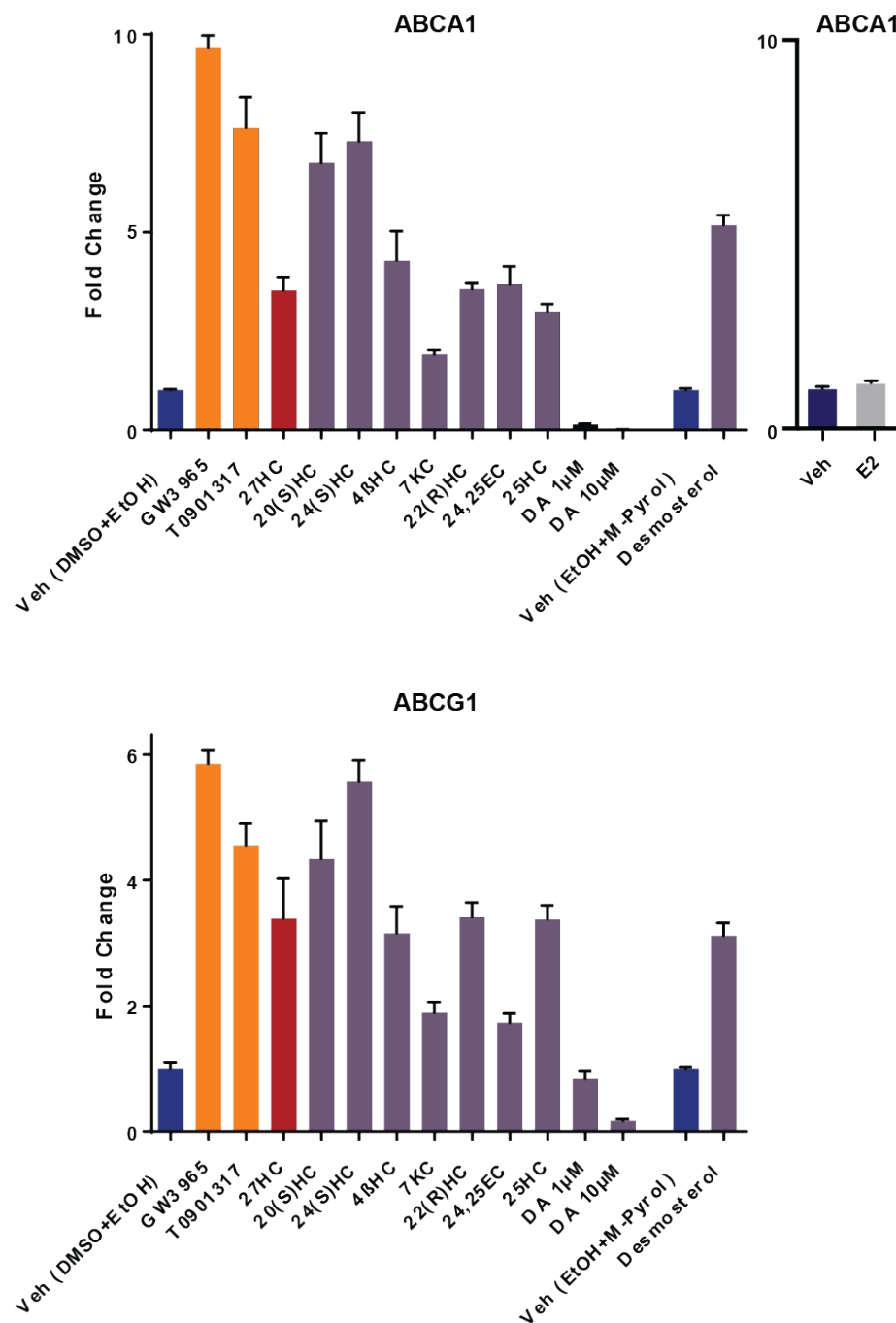
Supplemental Figure 3.5: ELISA analysis of TGF- β after macrophages were treated with LPS and either vehicle or 27HC. BMDMs were treated with LPS and either vehicle or 27HC for 24hrs. Conditioned media was harvested and TGF- β was quantified by ELISA (R&D, DY1679-05).



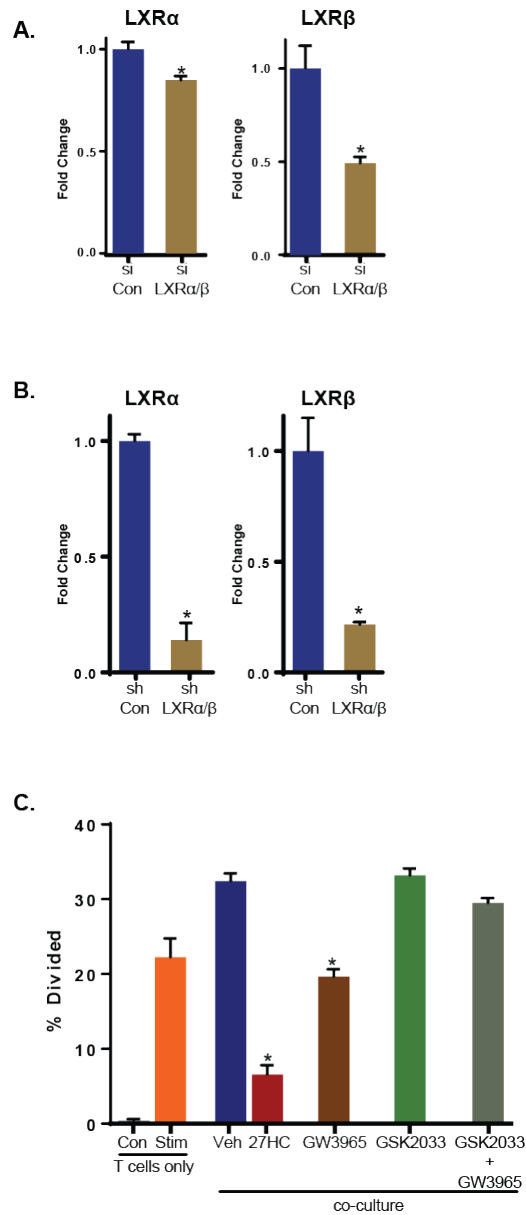
Supplemental Figure 3.6: Previously reported pathways involved in myeloid cell suppression of T cells (PI3K, AKT, NOTCH and CaMKK2) were ruled out from the suppressive mechanism of 27HC-treated macrophages. BMDMs were treated with vehicle $\pm$ indicated inhibitors or 27HC $\pm$ indicated inhibitors for 24hrs. CD3+ T cells were then isolated from wildtype C57BL/6 mouse, labeled with CFSE and chemically activated at the time of co-culture with pre-treated BMDMs. 72hrs after co-culture, T cells were harvested for flow cytometry analysis. Data are presented as mean $\pm$ SEM and asterisks (*) indicate statistical significance from the respective vehicle control (N=3/group for all T cell only controls, for co-culture conditions, N=3-4/group). (two-tailed t-test, $p < 0.05$).



Supplemental Figure 3.7: Estrogenic activity of 13 ligands. T47D cells stably expressing a ERE luciferase reporter were treated with indicated compounds and the activation of ER was measured by luciferase activity. Data are presented as mean $\pm$ SEM (N=4/treatment).

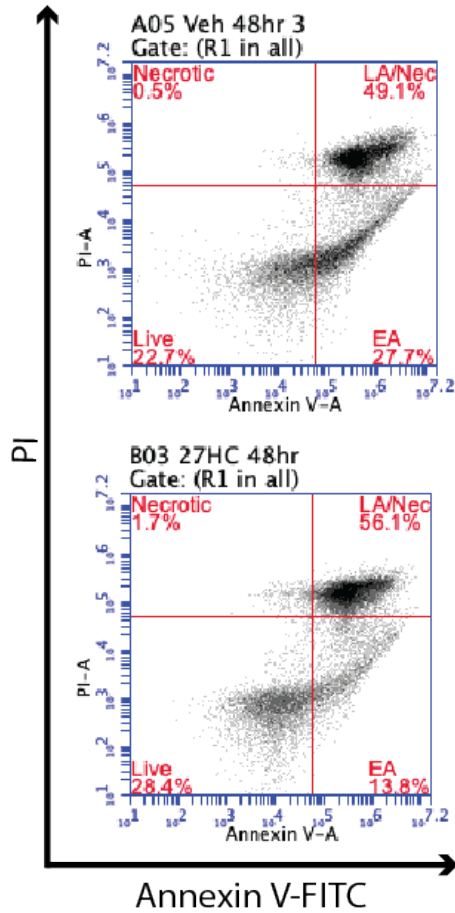


Supplemental Figure 3.8: LXR-activating activity of 12 cholesterol metabolites, synthetic LXR ligands and E2. BMDMs were treated with indicated compounds and mRNA expression of (**top**) ABCA1 (N=3/group for all cholesterol metabolites and synthetic LXR ligands, N=5 or 6/group for E2) and (**bottom**) ABCG1 (N=3/group), were quantified by qPCR. Data are presented as mean $\pm$ SEM relative to the respective vehicle control.

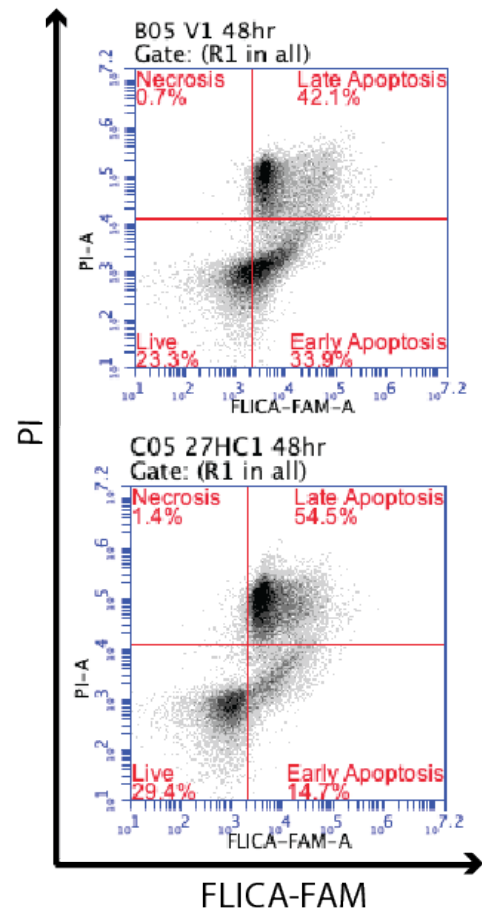


Supplemental Figure 3.9: Knockdown or inhibition of LXR isoforms attenuates immunosuppressive effects of 27HC and the synthetic LXR agonist, GW3965 (A) Representative LXRα and β expression after siRNA knockdown for 48hrs as measured by qPCR (N=3/group; This corresponds with **Figure 2.5G**). (B) LXRα and β expression after lentivirus-delivered shRNA knockdown for 96hrs as measured by qPCR (N=2/group; this corresponds with **Figure 2.5H**). (C) T cell proliferation was restored with the LXR antagonist, GSK2033. After 24hrs, the treatments were washed off and treated BMDMs were co-cultured with chemically activated wildtype T cells for 72hrs. T cells were labeled with CFSE and proliferation measured by flow cytometry (N=3/group for T cell only controls, for co-culture conditions, N=4/group). Data are presented as mean ± SEM and asterisks (*) indicate statistical significance from the respective vehicle control. (two-tailed t-test, $p < 0.05$). This corresponds with **Figure 2.5I**.

A.

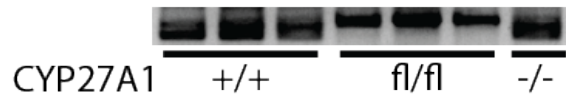


B.



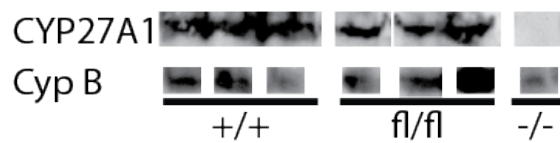
Supplemental Figure 3.10: 27HC-treated macrophages induce T cell apoptosis. (A) Representative flow cytometry dot plots of **Figure 2.6B** (48hrs), where T cells were harvested and labeled with Annexin V and PI, after co-culture with vehicle- (top) or 27HC-treated BMDMs (bottom) for 48hrs. (B) Representative flow cytometry dot plots of **Figure 2.6C** (48hrs), where T cells were harvested and labeled with FLICA-FAM and PI, after co-culture with vehicle- (top) or 27HC-treated BMDMs (bottom) for 48hrs.

DNA Genotyping:

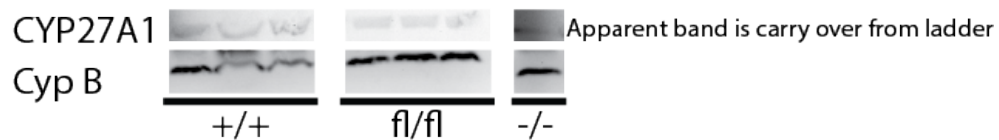


Protein by Western Analysis

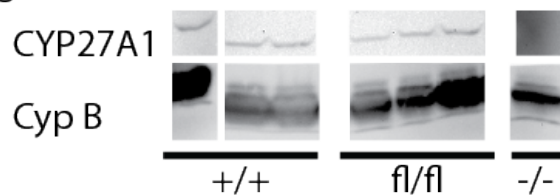
Liver



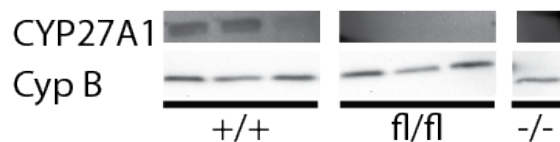
Spleen



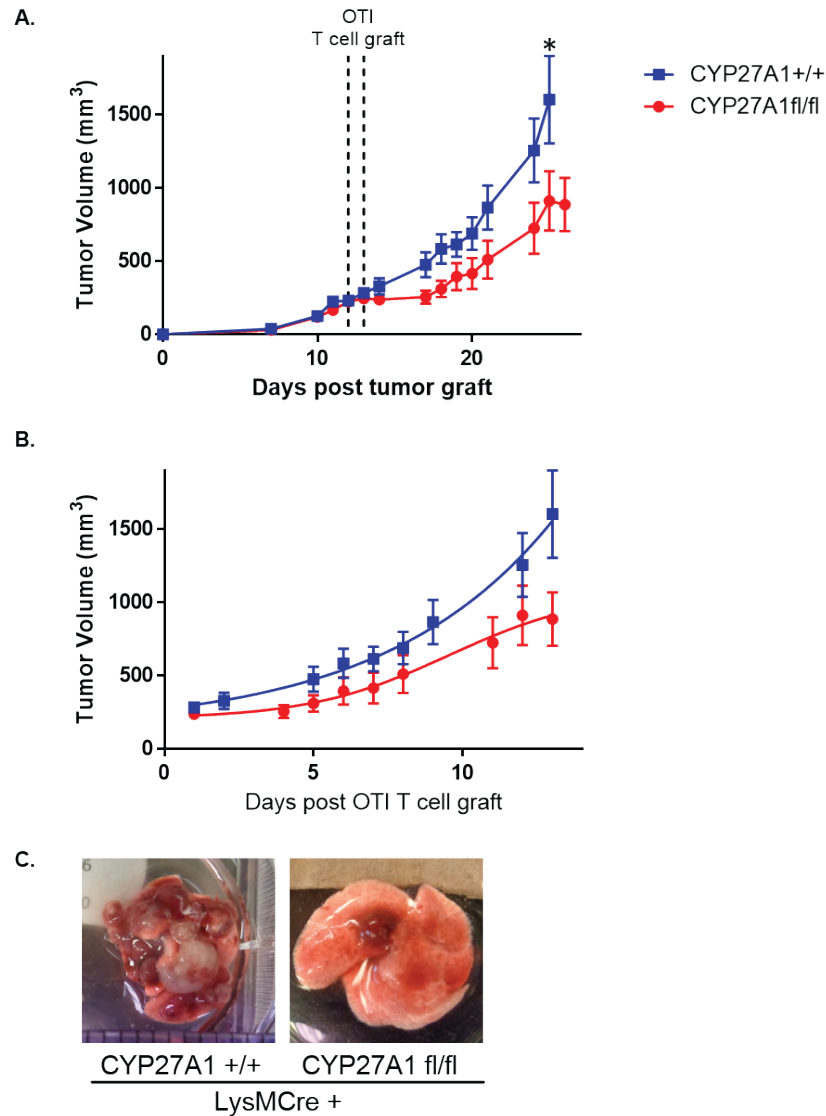
Lung



Macrophages



Supplemental Figure 3.11: CYP27A1^{fl/fl};LysMCre+ mice do not express CYP27A1 in bone marrow-derived macrophages. Tissues were harvested from 3 separate CYP27A1^{fl/fl};LysMCre+ and 3 CYP27A1^{+/+};LysMCre+ (control) male mice. Tissue was harvested from a traditional (global) CYP27A1^{-/-} mouse as a control. Bone marrow was harvested and used to derive macrophages. (A) Genotyping analysis indicating presence of floxed alleles in the CYP27A1 gene (smaller PCR amplicon). (B) Protein was extracted in RIPA buffer and run on SDS-page for Western analysis using anti-CYP27A1 (Abcam 126785) or cyclophilin B (Cyp B; Santa Cruz Biotechnology sc-517566) as an internal control. As can be noted, CYP27A1 was not expressed in macrophages, but continued to be expressed in the liver, spleen and lungs of CYP27A1^{fl/fl};LysMCre+ mice.



Supplemental Figure 3.12: Absence of myeloid CYP27A1 decreased tumor growth post T-cell graft. Mice with myeloid cell knock-out of CYP27A1 (CYP27A1^{fl/fl};LysMCre+) exhibited lower level of antigen-specific primary tumor growth. CYP27A1^{fl/fl};LysMCre+ mice (N=8) and their replete controls (N=7) were grafted orthotopically with E0771-OVA cells and tumors allowed to grow to 200 mm^3 . Control (CYP27A1^{+/+};LysMCre+) mice received an adoptive transfer of CD3+ T cells isolated from OT-I mice 12 days post-cancer graft (first dashed line), and CYP27A1^{fl/fl};LysMCre+ mice received an adoptive transfer of CD3+ T cells isolated from OT-I mice 13 days post-cancer graft. Control and CYP27A1^{fl/fl};LysMCre+ mice were euthanized 25 days after initial cancer cell graft respectively. **(A)** Tumor volumes were measured through time by digital calipers and plotted as mean $\pm$ SEM. Two way ANOVA followed by Sidaks multiple comparison test on data up to and including day 25 found a statistically significant difference between the groups on day 25 ($p < 0.05$, denoted by asterisk). **(B)** Growth of tumors post T cell transfer. This is the same data from (A), but adjusted for time post T cell transfer. Data were then fit to a curve with non-linear regression (four parameter variable slope). This corresponds with **Figure 2.7A**.

APPENDIX B

CHAPTER 3 SUPPLEMENTAL TABLES

Supplemental Table 3.1: Cytokine secretion of macrophages under two time points after Veh or 27HC treatment only.

24hr post-trt				72hr post-co-clt		
Cytokine	Treatment	Avg Conc*	FDR	Treatment	Avg Conc	FDR
ENA-78	Veh	out of range	NA	Veh	3.46	NA
ENA-78	27HC	out of range		27HC	out of range	
Eotaxin	Veh	out of range	NA	Veh	out of range	NA
Eotaxin	27HC	out of range		27HC	out of range	
G-CSF	Veh	out of range	NA	Veh	0.79	0.7898
G-CSF	27HC	0.58		27HC	0.97	
GM-CSF	Veh	out of range	NA	Veh	out of range	NA
GM-CSF	27HC	out of range		27HC	out of range	
GRO-alpha	Veh	99.19	0.982	Veh	109.46	0.0169
GRO-alpha	27HC	99.49		27HC	69.8	
IFN-a	Veh	12.48	0.5984	Veh	15.43	0.8713
IFN-a	27HC	15.64		27HC	14.17	
IFN-g	Veh	out of range	NA	Veh	out of range	NA
IFN-g	27HC	out of range		27HC	out of range	
IL-10	Veh	2.65	0.4249	Veh	1.76	0.9053
IL-10	27HC	4.89		27HC	1.63	
IL-12p70	Veh	out of range	NA	Veh	out of range	NA
IL-12p70	27HC	out of range		27HC	out of range	
IL-13	Veh	out of range	NA	Veh	out of range	NA
IL-13	27HC	out of range		27HC	out of range	
IL-15	Veh	out of range	NA	Veh	out of range	NA
IL-15	27HC	out of range		27HC	out of range	
IL-17A	Veh	0.65	0.3276	Veh	1.08	0.4097
IL-17A	27HC	1.84		27HC	1.65	
IL-18	Veh	out of range	NA	Veh	out of range	NA
IL-18	27HC	out of range		27HC	out of range	
IL-1a	Veh	out of range	NA	Veh	out of range	NA
IL-1a	27HC	out of range		27HC	out of range	
IL-1b	Veh	out of range	NA	Veh	out of range	NA
IL-1b	27HC	out of range		27HC	out of range	
IL-2	Veh	out of range	NA	Veh	out of range	NA

Supplemental Table 3.1: continued.

IL-2	27HC	out of range		27HC	out of range	
IL-22	Veh	out of range	NA	Veh	out of range	NA
IL-22	27HC	out of range		27HC	out of range	
IL-23	Veh	out of range	NA	Veh	out of range	NA
IL-23	27HC	out of range		27HC	out of range	
IL-27	Veh	0.49	NA	Veh	0.45	0.5474
IL-27	27HC	0.76		27HC	0.61	
IL-28	Veh	out of range	NA	Veh	out of range	NA
IL-28	27HC	out of range		27HC	out of range	
IL-3	Veh	out of range	NA	Veh	out of range	NA
IL-3	27HC	out of range		27HC	out of range	
IL-31	Veh	out of range	NA	Veh	6.07	NA
IL-31	27HC	out of range		27HC	6.39	
IL-4	Veh	out of range	NA	Veh	out of range	NA
IL-4	27HC	out of range		27HC	out of range	
IL-5	Veh	out of range	NA	Veh	out of range	NA
IL-5	27HC	out of range		27HC	out of range	
IL-6	Veh	out of range	NA	Veh	1.29	NA
IL-6	27HC	7.76		27HC	1.03	
IL-9	Veh	out of range	NA	Veh	out of range	NA
IL-9	27HC	out of range		27HC	out of range	
IP-10	Veh	192.42	0.5474	Veh	104.84	0.4011
IP-10	27HC	147.53		27HC	69.28	
LIF	Veh	2.13	0.7898	Veh	5.37	NA
LIF	27HC	2.6		27HC	5.29	
M-CSF	Veh	out of range	NA	Veh	out of range	NA
M-CSF	27HC	out of range		27HC	out of range	
MCP-1	Veh	91.16	0.1965	Veh	33.07	0.1306
MCP-1	27HC	196.22		27HC	16.51	
MCP-3	Veh	77.84	0.2824	Veh	152.97	0.1335
MCP-3	27HC	114.25		27HC	91.99	
MIP-1a	Veh	4.39	0.0156	Veh	3.16	0.8595
MIP-1a	27HC	6.49		27HC	3.22	
MIP-1b	Veh	38.27	0.0279	Veh	27.17	0.8392
MIP-1b	27HC	62.35		27HC	27.76	
MIP-2	Veh	519.17	0.3009	Veh	284.96	NA
MIP-2	27HC	604.1		27HC	191.19	
RANTES	Veh	23.3	0.2409	Veh	24.32	0.0634
RANTES	27HC	15.47		27HC	10.81	
TNF-a	Veh	32.95	0.6104	Veh	15.76	NA
TNF-a	27HC	39.88		27HC	15.13	

*concentration as pg/ml; trt: treatment; co-clt: co-culture

Supplemental Table 3.2: Cytokine secretion of macrophages under two time points after LPS and Veh or 27HC treatment.

24hr post-trt				72hr post-co-clt		
Cytokine	Treatment	Avg Conc*	FDR	Treatment	Avg Conc	FDR
ENA-78	Veh	174.47	0.8598	Veh	69.14	0.799
ENA-78	27HC	182.74		27HC	84.88	
Eotaxin	Veh	out of range	NA	Veh	out of range	NA
Eotaxin	27HC	out of range		27HC	out of range	
G-CSF	Veh	5.34	0.9053	Veh	3.92	0.5474
G-CSF	27HC	5.71		27HC	3.06	
GM-CSF	Veh	7.92	NA	Veh	out of range	NA
GM-CSF	27HC	8.18		27HC	out of range	
GRO-alpha	Veh	1993.92	0.2409	Veh	566.87	0.2409
GRO-alpha	27HC	1614.83		27HC	847.87	
IFN-a	Veh	15.38	0.5984	Veh	15.98	0.9105
IFN-a	27HC	21.24		27HC	16.62	
IFN-g	Veh	4.29	0.1095	Veh	0.84	0.6104
IFN-g	27HC	1.61		27HC	0.22	
IL-10	Veh	1058.73	NA	Veh	21.79	0.5984
IL-10	27HC	1052.2		27HC	18.65	
IL-12p70	Veh	6.83	NA	Veh	0.7	0.8713
IL-12p70	27HC	6.98		27HC	0.92	
IL-13	Veh	2.95	0.6104	Veh	0.92	0.6769
IL-13	27HC	3.04		27HC	1.16	
IL-15	Veh	26.45	0.5474	Veh	13.43	0.1095
IL-15	27HC	24.73		27HC	10.78	
IL-17A	Veh	14.08	0.982	Veh	2.89	NA
IL-17A	27HC	14.12		27HC	3.33	
IL-18	Veh	879.91	0.126	Veh	212.34	0.0674
IL-18	27HC	1143.92		27HC	144.8	
IL-1a	Veh	111.48	0.0279	Veh	85.53	0.126
IL-1a	27HC	238.05		27HC	105.1	
IL-1b	Veh	274.45	0.0156	Veh	30.6	0.0156
IL-1b	27HC	378.89		27HC	11.18	
IL-2	Veh	out of range	NA	Veh	out of range	NA
IL-2	27HC	out of range		27HC	out of range	
IL-22	Veh	176.97	0.4249	Veh	46.84	NA
IL-22	27HC	226.03		27HC	33.48	
IL-23	Veh	26.03	0.8303	Veh	5.04	NA
IL-23	27HC	23.87		27HC	1.16	
IL-27	Veh	141.61	NA	Veh	9.7	0.1335
IL-27	27HC	119.44		27HC	6.95	
IL-28	Veh	308.37	0.9105	Veh	127.24	0.8595

Supplemental Table 3.2: continued.

IL-28	27HC	300.85		27HC	99.97	
IL-3	Veh	out of range	NA	Veh	out of range	NA
IL-3	27HC	out of range		27HC	out of range	
IL-31	Veh	17.72	NA	Veh	10.01	0.8598
IL-31	27HC	19.49		27HC	8.96	
IL-4	Veh	out of range	NA	Veh	out of range	NA
IL-4	27HC	out of range		27HC	out of range	
IL-5	Veh	7.69	0.6769	Veh	4.16	NA
IL-5	27HC	8.43		27HC	3.26	
IL-6	Veh	7600.34	0.0674	Veh	347.71	NA
IL-6	27HC	5766.51		27HC	368.53	
IL-9	Veh	28.88	NA	Veh	out of range	NA
IL-9	27HC	29.87		27HC	out of range	
IP-10	Veh	714.98	0.8871	Veh	817.83	NA
IP-10	27HC	703.04		27HC	777.67	
LIF	Veh	17.01	0.6464	Veh	8.88	0.7898
LIF	27HC	17.97		27HC	9.67	
M-CSF	Veh	1.08	0.8713	Veh	0.83	0.4249
M-CSF	27HC	1.07		27HC	0.6	
MCP-1	Veh	10177.69	NA	Veh	1508.6	0.157
MCP-1	27HC	9294.78		27HC	1266.96	
MCP-3	Veh	472	NA	Veh	312.62	0.9053
MCP-3	27HC	483.91		27HC	315.95	
MIP-1a	Veh	39.22	NA	Veh	4.54	0.7508
MIP-1a	27HC	31.39		27HC	5.17	
MIP-1b	Veh	684.83	0.135	Veh	16.94	0.135
MIP-1b	27HC	538.14		27HC	30.42	
MIP-2	Veh	7852.33	0.667	Veh	4145.43	NA
MIP-2	27HC	7462.6		27HC	3787.83	
RANTES	Veh	679.87	0.8084	Veh	620.1	0.9187
RANTES	27HC	661.36		27HC	625.15	
TNF-a	Veh	1493.98	NA	Veh	194.18	NA
TNF-a	27HC	1472.32		27HC	196.34	

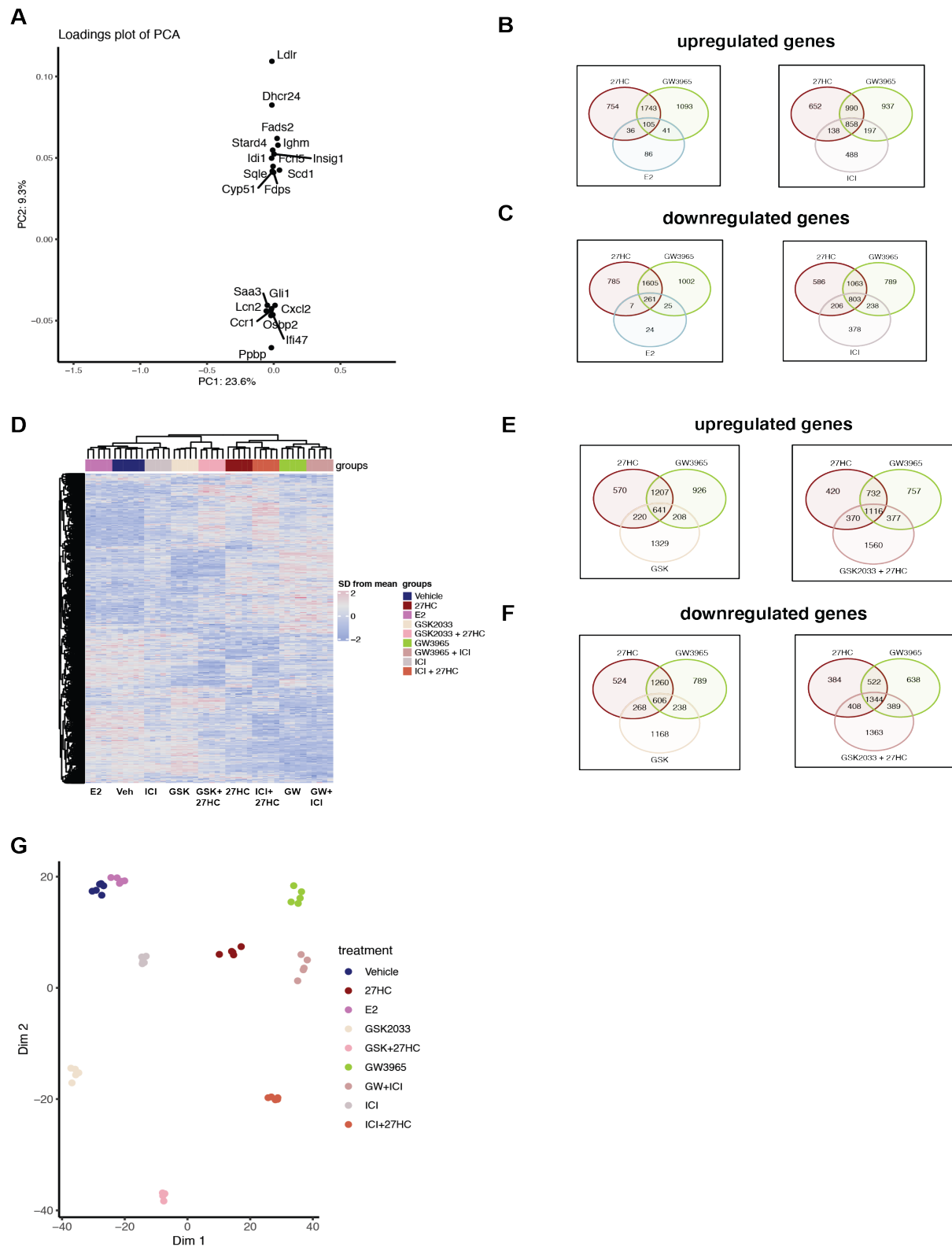
*concentration as pg/ml; trt: treatment; co-clt: co-culture

Supplemental Table 3.3: LXR and/or ER ligands

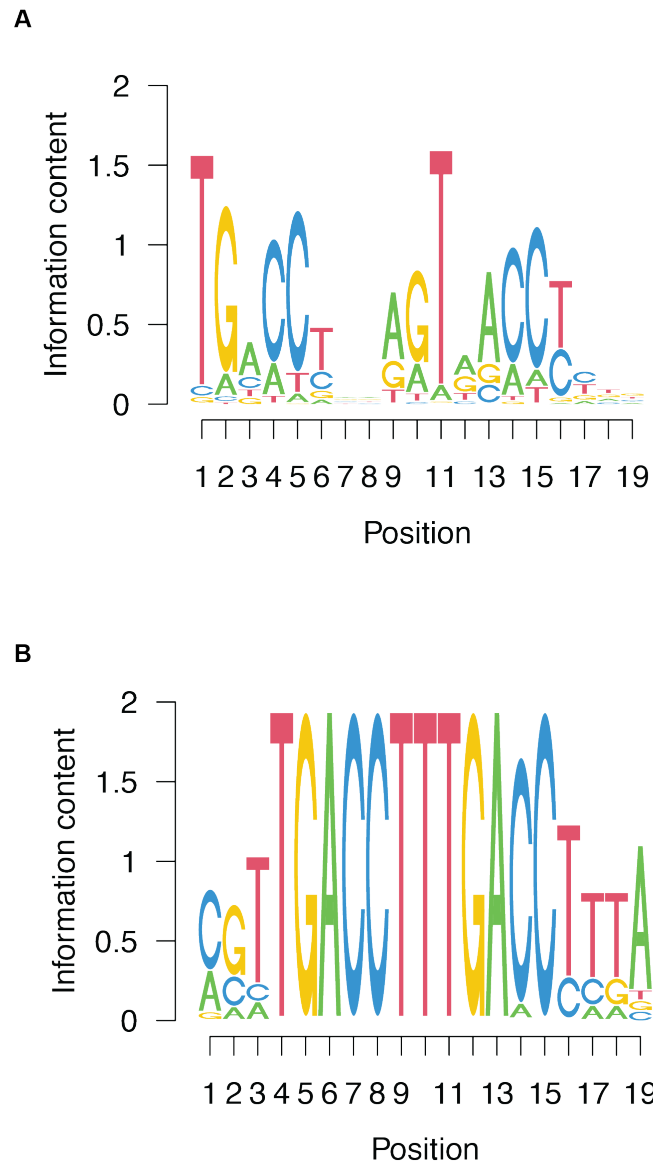
Ligand	Class	Documented Activity	Identified Activity	Literature
4 β HC	Oxysterol	LXR ligand	Putative ER ligand	(239)
20(S)HC	Oxysterol	LXR ligand		(239, 240)
22(R)HC	Oxysterol	LXR ligand		(31, 239, 241)
24(S)HC	Oxysterol	LXR ligand		(31, 239)
25HC	Oxysterol	LXR ligand, ER ligand		(31, 56, 239)
27HC	Oxysterol	LXR ligand, ER ligand		(36, 49, 53, 59, 239)
24, 25EC	Oxysterol	LXR ligand		(31)
7KC	Oxysterol	LXR ligand, ER ligand		(55, 242)
Dendrogenin A	conjugation of 5,6 α -epoxy-cholesterol and histamine cholesterol precursor	LXR ligand		(241, 243)
Desmosterol	Oxysterol	LXR ligand	Putative ER ligand	(99, 218)
GW3965	synthetic LXR agonist	LXR ligand		(241)
T0901317	synthetic LXR agonist	LXR ligand		(240, 241)

APPENDIX C

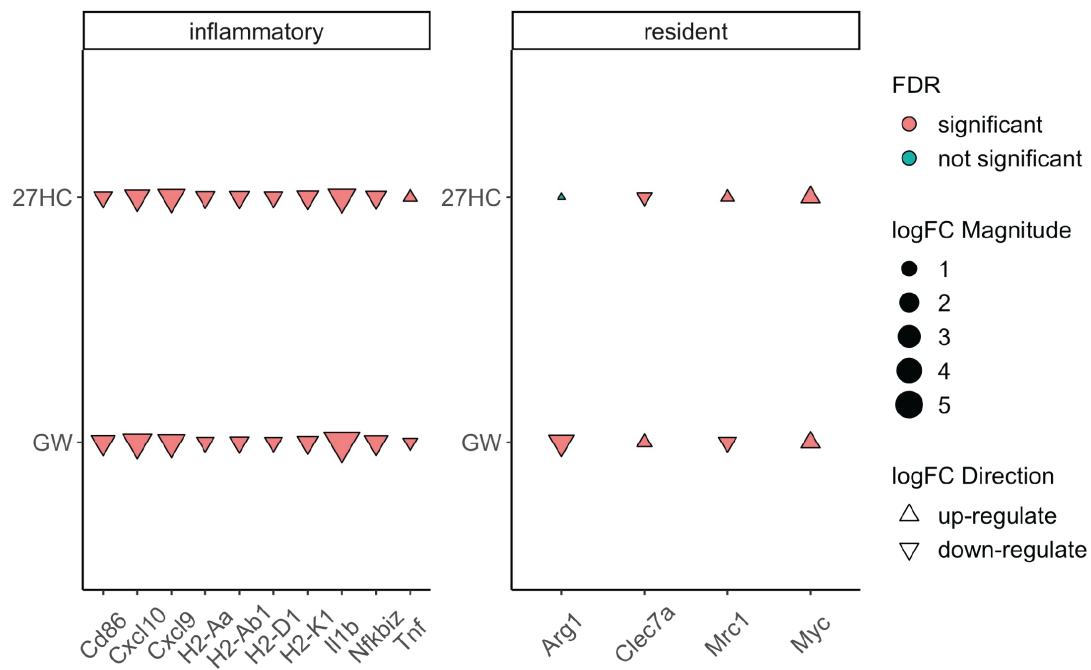
CHAPTER 4 SUPPLEMENTAL FIGURES



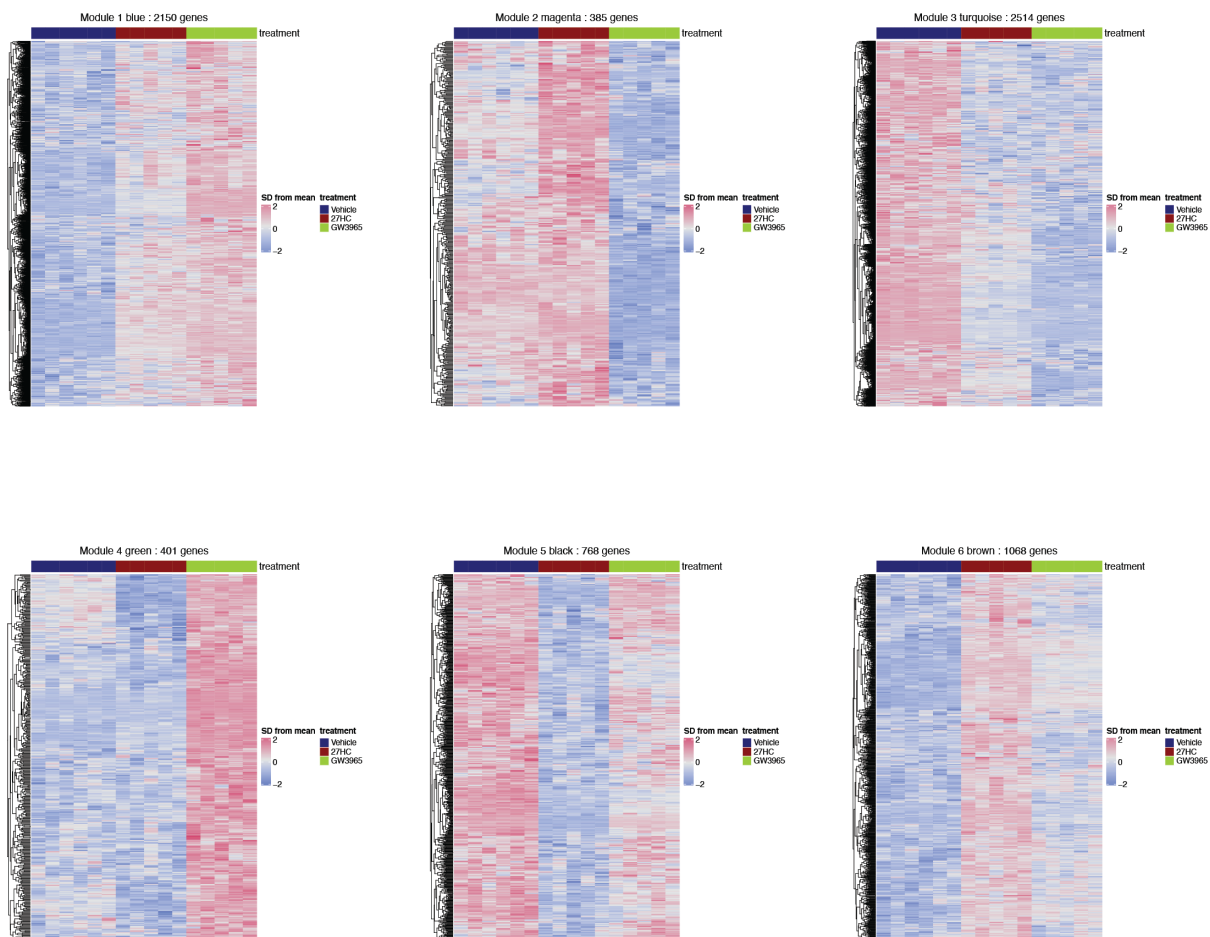
Supplemental Figure 4.1: Effects of 8 ER/LXR ligands on BMDMs. (A) PCA loadings plot of PCA analysis on sequenced genes after vehicle, 27HC and GW3965 treatments. (B-C) Venn diagrams show that E2 has minimal effect on BMDMs, while ICI had a large fraction of shared DEGs with 27HC and GW3965 (D) Heatmap of DEGs from all treatments. (E-F) Venn diagrams show that GSK2033 only antagonized a small fraction of commonly regulated genes between 27HC and GW3965. (G) Multi-dimensional scaling plot show all treatments in the first 2 dimensions.



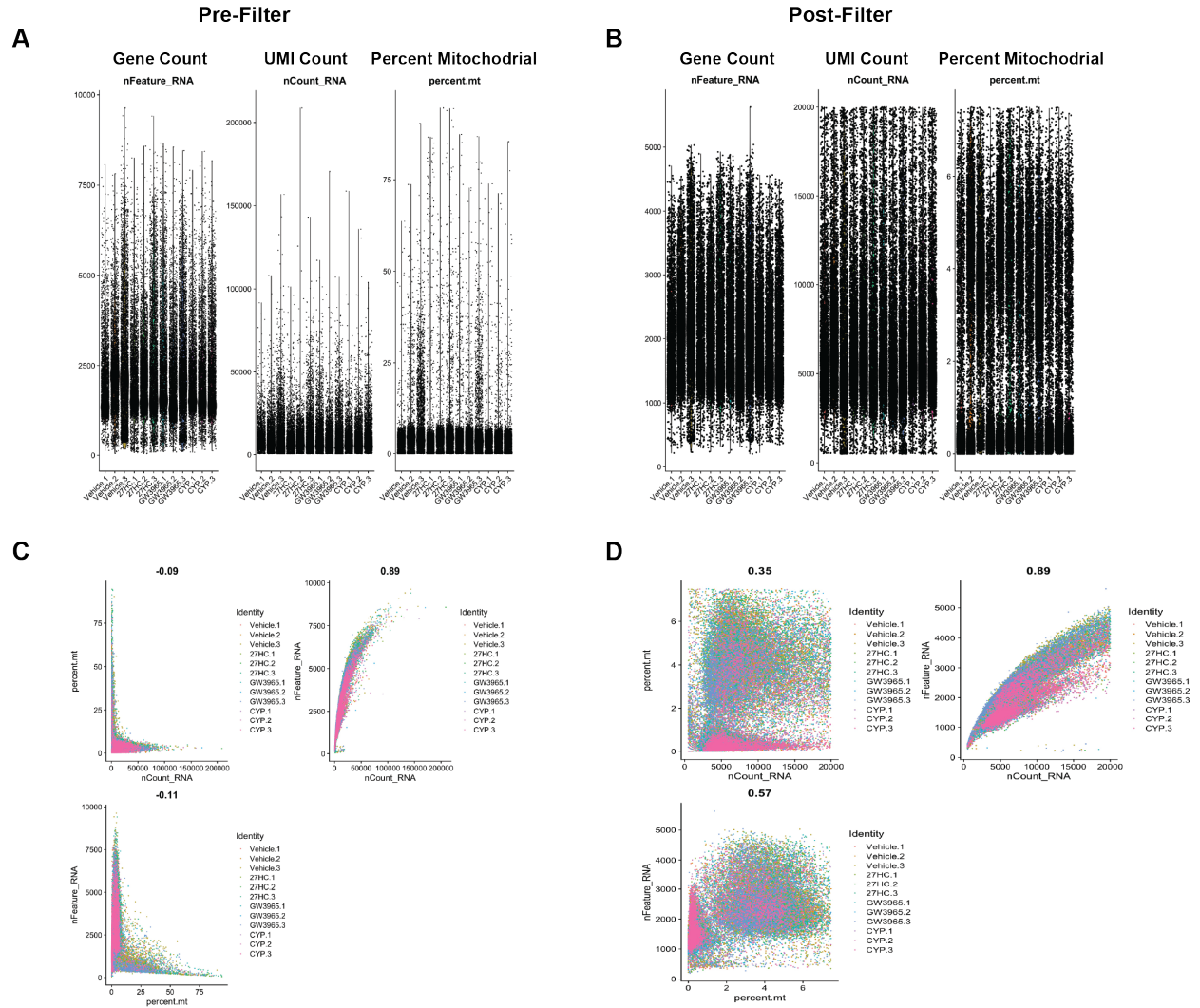
Supplemental Figure 4.2: DNA binding motifs of LXR isoforms. (A) DNA binding motif of LXR α . **(B)** DNA binding motif of LXR β .



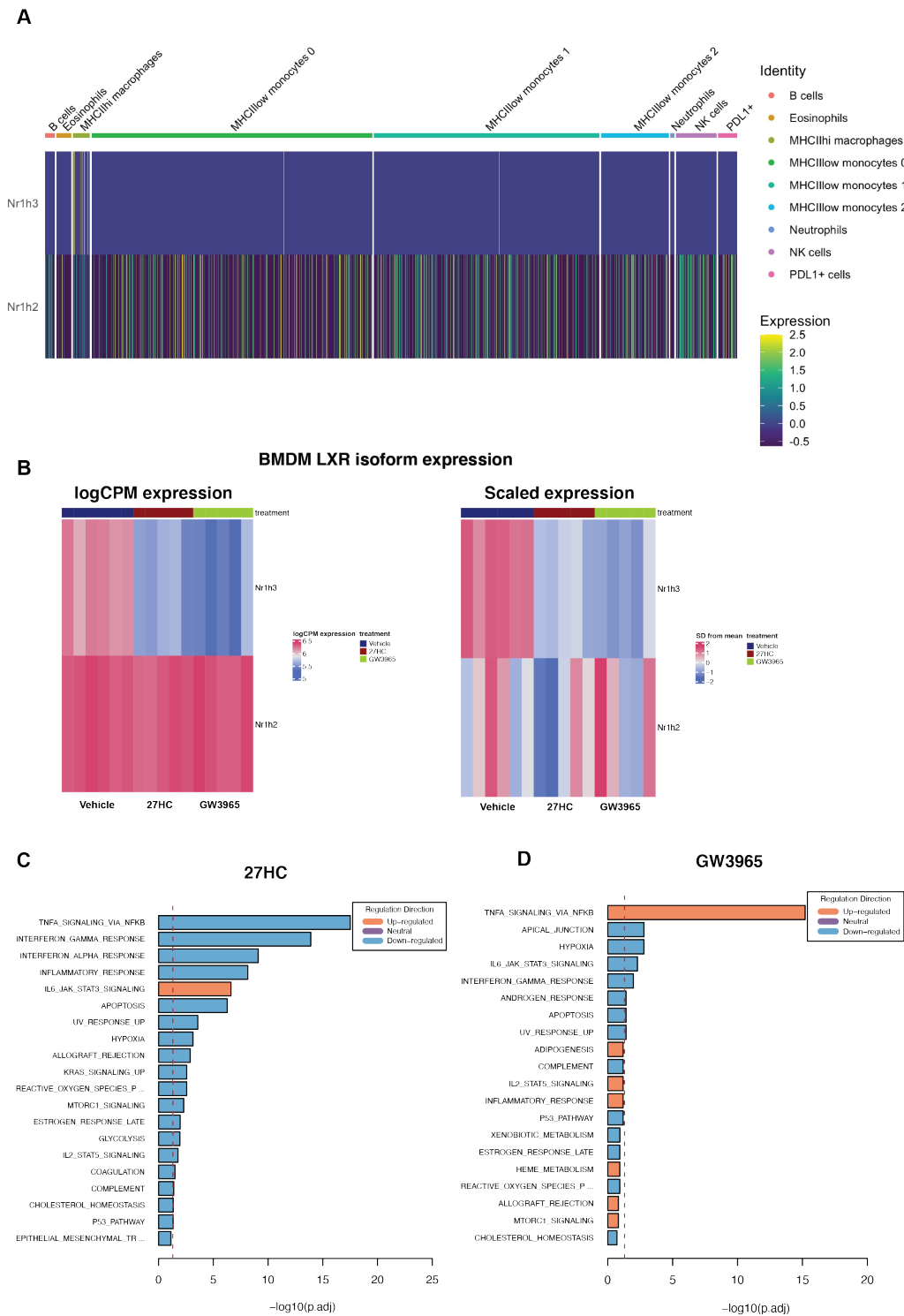
Supplemental Figure 4.3: 27HC and GW3965 similarly regulate inflammatory genes in BMDMs. 27HC and GW3965 generally downregulated the expression and pro-inflammatory genes in BMDMs, while no consistent changes were observed regarding the anti-inflammatory genes.



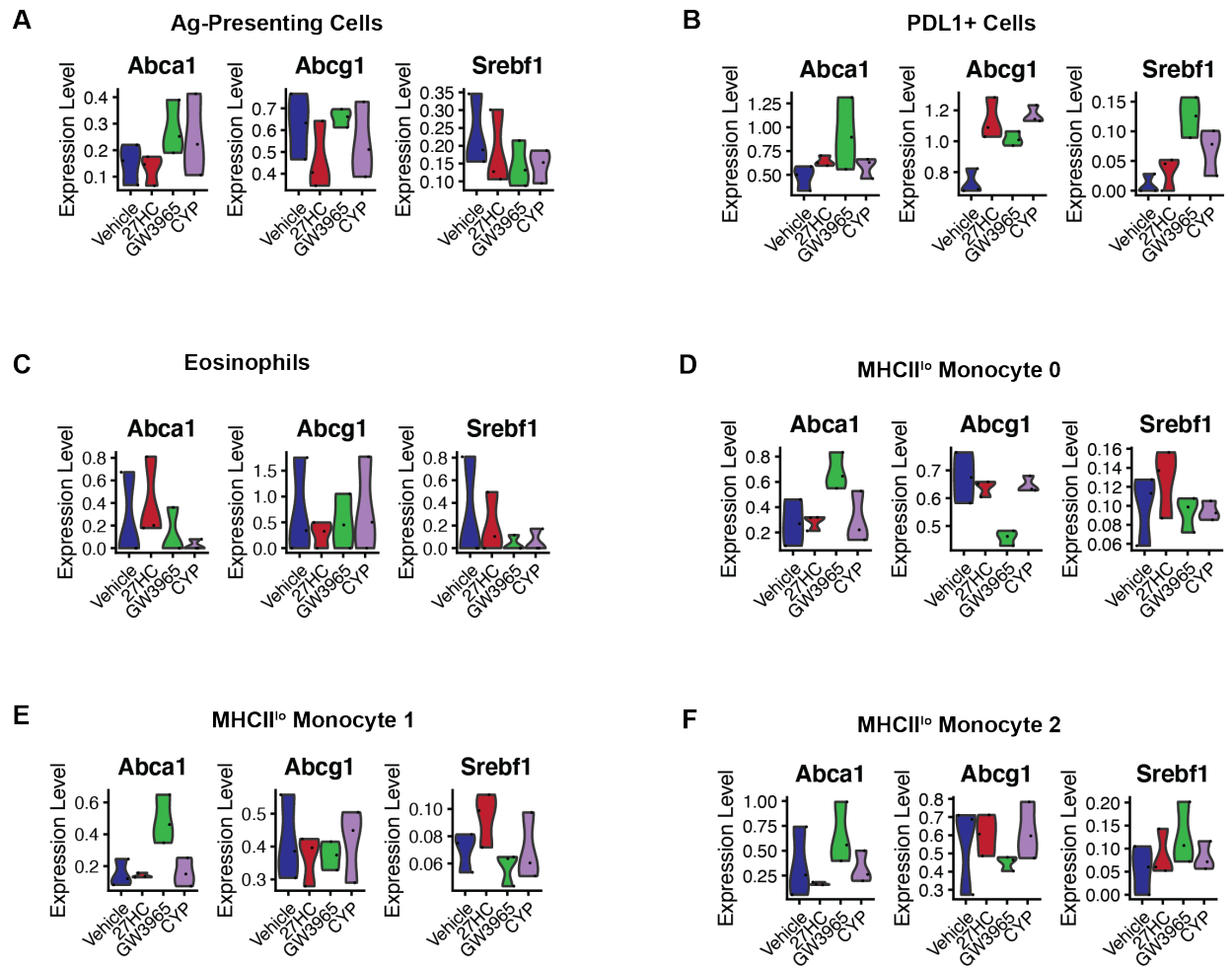
Supplemental Figure 4.4: Heatmap of WGCNA module gene expressions. Expression of genes assigned to WGCNA module 1-6.



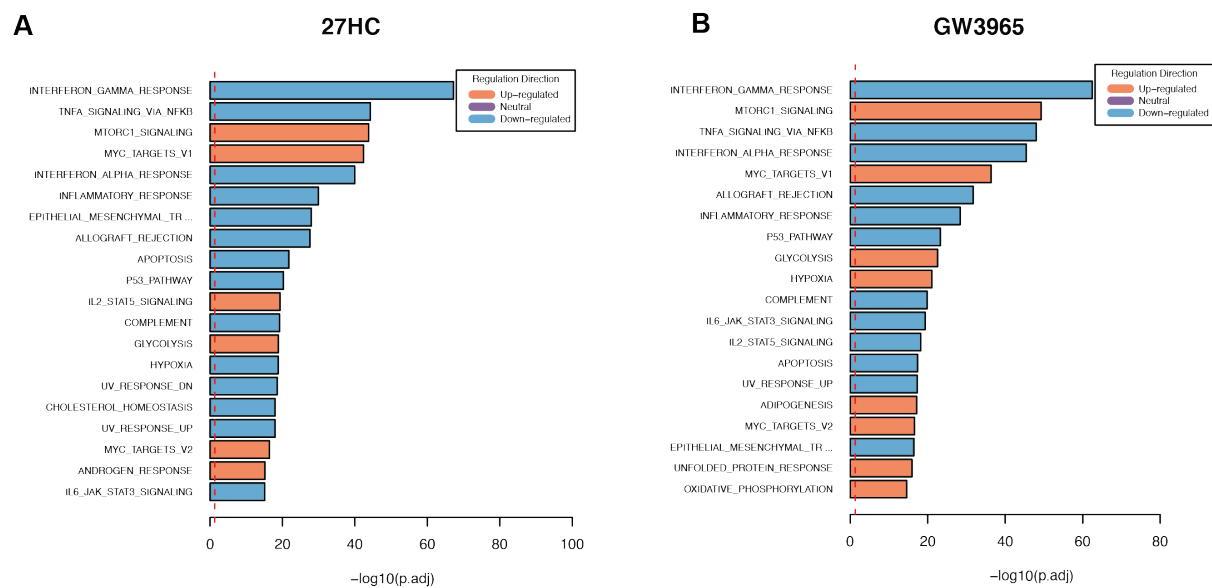
Supplemental Figure 4.5: Pre- and post-filtering metrics of single-cell RNAseq. (A-B) Gene count, UMI count and percent mitochondrial genes before and after filtering. **(C-D)** PCA plot of each treatment condition before and after filtering.



Supplemental Figure 4.6: 27HC downregulated TNF- α signaling, while GW3965 promoted it in a subpopulation of myeloid cells. (A-B) LXR isoform expression in distinct myeloid subpopulations and BMDMs. (C-D) Overrepresentation analysis of DEGs showed that 27HC downregulated TNF- α signaling, while GW3965 promoted it in MHCII^{lo} monocyte 0 population.



Supplemental Figure 4.7: Expression of LXR target genes in subpopulations of myeloid cells in the tumor microenvironment. (A-F) 27HC and GW3965 differentially regulated the expression of LXR target genes in most of the myeloid subpopulations. This figure corresponds to **Figure 3.4**



Supplemental Figure 4.8: 27HC similarly enriched the TNF- α pathway as GW3965 in BMDMs. (A-B) Overrepresentation analyses of DEGs show that 27HC similarly regulated TNF- α pathway in BMDMs, unlike in MHCII^{lo} monocyte 0.

APPENDIX D

CHAPTER 4 SUPPLEMENTAL TABLES

Supplemental Table 4.1: Genes with LXR α binding site

ensembl gene id	external gene name	change
ENSMUSG00000004609	Cd33	27HC+GW-
ENSMUSG00000005824	Tnfsf14	27HC+GW-
ENSMUSG00000017929	B4galt5	27HC+GW-
ENSMUSG00000019942	Cdk1	27HC+GW-
ENSMUSG00000021998	Lcp1	27HC+GW-
ENSMUSG00000026110	Mgat4a	27HC+GW-
ENSMUSG00000028412	Slc44a1	27HC+GW-
ENSMUSG00000028583	Pdpn	27HC+GW-
ENSMUSG00000042793	Lgr6	27HC+GW-
ENSMUSG00000046318	Ccbe1	27HC+GW-
ENSMUSG00000047250	Ptgs1	27HC+GW-
ENSMUSG00000001119	Col6a1	27HC-GW+
ENSMUSG000000031708	Tecr	27HC-GW+
ENSMUSG000000037762	Slc16a9	27HC-GW+
ENSMUSG000000037936	Scarb1	27HC-GW+
ENSMUSG000000079470	Utp14b	27HC-GW+
ENSMUSG000000000085	Scmh1	27HC0GW+
ENSMUSG000000000552	Zfp385a	27HC0GW+
ENSMUSG00000001100	Poldip2	27HC0GW+
ENSMUSG00000001225	Slc26a3	27HC0GW+
ENSMUSG00000001750	Tcirg1	27HC0GW+
ENSMUSG00000002625	Akap8l	27HC0GW+
ENSMUSG00000004071	Cdip1	27HC0GW+
ENSMUSG00000007029	Vars	27HC0GW+
ENSMUSG000000008822	Acyp1	27HC0GW+
ENSMUSG000000009207	Lnpk	27HC0GW+
ENSMUSG000000009654	Oit3	27HC0GW+
ENSMUSG00000015889	Lta4h	27HC0GW+
ENSMUSG00000015962	1700016C15Rik	27HC0GW+
ENSMUSG00000016024	Lbp	27HC0GW+
ENSMUSG00000016495	Plgrkt	27HC0GW+

Supplemental Table 4.1: continued.

ENSMUSG00000017176	Nt5c3b	27HC0GW+
ENSMUSG00000018509	Cenpv	27HC0GW+
ENSMUSG00000019437	Tlcl1	27HC0GW+
ENSMUSG00000020521	Rnft1	27HC0GW+
ENSMUSG00000020681	Ace	27HC0GW+
ENSMUSG00000020849	Ywhae	27HC0GW+
ENSMUSG00000020857	Nme2	27HC0GW+
ENSMUSG00000020889	Nr1d1	27HC0GW+
ENSMUSG00000021403	Serpinb9b	27HC0GW+
ENSMUSG00000021556	Golm1	27HC0GW+
ENSMUSG00000021559	Dapk1	27HC0GW+
ENSMUSG00000021756	Il6st	27HC0GW+
ENSMUSG00000021792	Prxl2a	27HC0GW+
ENSMUSG00000021957	Tkt	27HC0GW+
ENSMUSG00000022359	Wdyhv1	27HC0GW+
ENSMUSG00000022433	Csnk1e	27HC0GW+
ENSMUSG00000022500	Litaf	27HC0GW+
ENSMUSG00000022754	Tmem45a	27HC0GW+
ENSMUSG00000022946	Dop1b	27HC0GW+
ENSMUSG00000023571	C1qtnf12	27HC0GW+
ENSMUSG00000023905	Tnfrsf12a	27HC0GW+
ENSMUSG00000024286	Ccny	27HC0GW+
ENSMUSG00000024425	Ndfip1	27HC0GW+
ENSMUSG00000024587	Nars	27HC0GW+
ENSMUSG00000024589	Nedd4l	27HC0GW+
ENSMUSG00000024669	Cd5	27HC0GW+
ENSMUSG00000024978	Gpam	27HC0GW+
ENSMUSG00000025026	Add3	27HC0GW+
ENSMUSG00000025227	Mfsd13a	27HC0GW+
ENSMUSG00000025577	Cbx2	27HC0GW+
ENSMUSG00000025607	Copg2	27HC0GW+
ENSMUSG00000026177	Slc11a1	27HC0GW+
ENSMUSG00000026203	Dnajb2	27HC0GW+
ENSMUSG00000026915	Strbp	27HC0GW+
ENSMUSG00000026917	Wdr5	27HC0GW+
ENSMUSG00000026934	Lhx3	27HC0GW+
ENSMUSG00000027247	Arhgap1	27HC0GW+
ENSMUSG00000027455	Nsf11c	27HC0GW+
ENSMUSG00000027523	Gnas	27HC0GW+
ENSMUSG00000028910	Mecr	27HC0GW+
ENSMUSG00000028937	Acot7	27HC0GW+
ENSMUSG00000028962	Slc4a2	27HC0GW+
ENSMUSG00000029772	Ahcyl2	27HC0GW+

Supplemental Table 4.1: continued.

ENSMUSG00000030557	Mef2a	27HC0GW+
ENSMUSG00000031023	Akip1	27HC0GW+
ENSMUSG00000031871	Cdh5	27HC0GW+
ENSMUSG00000031996	Aplp2	27HC0GW+
ENSMUSG00000032051	Fdx1	27HC0GW+
ENSMUSG00000032126	Hmbs	27HC0GW+
ENSMUSG00000032172	Olfm2	27HC0GW+
ENSMUSG00000032883	Acs13	27HC0GW+
ENSMUSG00000032939	Nup93	27HC0GW+
ENSMUSG00000032946	Rasgrp2	27HC0GW+
ENSMUSG00000033701	Acbd6	27HC0GW+
ENSMUSG00000034333	Zbed4	27HC0GW+
ENSMUSG00000034522	Zfp395	27HC0GW+
ENSMUSG00000034593	Myo5a	27HC0GW+
ENSMUSG00000034826	Nup54	27HC0GW+
ENSMUSG00000034959	Rubcn1	27HC0GW+
ENSMUSG00000035329	Fbxo33	27HC0GW+
ENSMUSG00000035459	Stab2	27HC0GW+
ENSMUSG00000035969	Rusc2	27HC0GW+
ENSMUSG00000036160	Surf6	27HC0GW+
ENSMUSG00000036641	Ccdc148	27HC0GW+
ENSMUSG00000036817	Sun1	27HC0GW+
ENSMUSG00000037119	Fam91a1	27HC0GW+
ENSMUSG00000037815	Ctnna1	27HC0GW+
ENSMUSG00000037907	Ankrd13b	27HC0GW+
ENSMUSG00000037966	Ninj1	27HC0GW+
ENSMUSG00000037979	Ccdc92	27HC0GW+
ENSMUSG00000038324	Trpc4ap	27HC0GW+
ENSMUSG00000039209	Rpl39l	27HC0GW+
ENSMUSG00000039275	Foxk2	27HC0GW+
ENSMUSG00000039518	Cdsn	27HC0GW+
ENSMUSG00000040234	Tm7sf3	27HC0GW+
ENSMUSG00000040263	Klhdc4	27HC0GW+
ENSMUSG00000040464	Gtpbp10	27HC0GW+
ENSMUSG00000040883	Tmem205	27HC0GW+
ENSMUSG00000041058	Wwp1	27HC0GW+
ENSMUSG00000041143	Tmco4	27HC0GW+
ENSMUSG00000042312	S100a13	27HC0GW+
ENSMUSG00000042425	Frmpd3	27HC0GW+
ENSMUSG00000042734	Ttc9	27HC0GW+
ENSMUSG00000044197	Gpr146	27HC0GW+
ENSMUSG00000044712	Slc38a6	27HC0GW+
ENSMUSG00000046562	Unc119b	27HC0GW+

Supplemental Table 4.1: continued.

ENSMUSG00000046733	Gprc5a	27HC0GW+
ENSMUSG00000048938	Nr1h5	27HC0GW+
ENSMUSG00000049985	Ankrd55	27HC0GW+
ENSMUSG00000051506	Wdfy4	27HC0GW+
ENSMUSG00000053565	Eif3k	27HC0GW+
ENSMUSG00000055782	Abcd2	27HC0GW+
ENSMUSG00000055866	Per2	27HC0GW+
ENSMUSG00000056267	Cep70	27HC0GW+
ENSMUSG00000060152	Pop5	27HC0GW+
ENSMUSG00000061474	Mrps36	27HC0GW+
ENSMUSG00000062115	Rai1	27HC0GW+
ENSMUSG00000063253	Scoc	27HC0GW+
ENSMUSG00000066026	Dhrs3	27HC0GW+
ENSMUSG00000073468	Sft2d1	27HC0GW+
ENSMUSG00000073643	Wdfy1	27HC0GW+
ENSMUSG00000074577	Ripor3	27HC0GW+
ENSMUSG00000075704	Txnrd2	27HC0GW+
ENSMUSG00000076432	Ywhaq	27HC0GW+
ENSMUSG00000079605	Zbtb9	27HC0GW+
ENSMUSG00000091971	Hspa1a	27HC0GW+
ENSMUSG00000001632	Brpf1	27HC0GW-
ENSMUSG00000002020	Ltbp2	27HC0GW-
ENSMUSG00000002068	Ccne1	27HC0GW-
ENSMUSG00000002205	Vrk3	27HC0GW-
ENSMUSG00000002265	Peg3	27HC0GW-
ENSMUSG00000002524	Puf60	27HC0GW-
ENSMUSG00000003863	Ppfi3	27HC0GW-
ENSMUSG00000004043	Stat5a	27HC0GW-
ENSMUSG00000004677	Myo9b	27HC0GW-
ENSMUSG00000004846	Plod3	27HC0GW-
ENSMUSG00000005057	Sh2b2	27HC0GW-
ENSMUSG00000007603	Dus3l	27HC0GW-
ENSMUSG00000008855	Hdac5	27HC0GW-
ENSMUSG00000013236	Ptprs	27HC0GW-
ENSMUSG00000017132	Cyth1	27HC0GW-
ENSMUSG00000018334	Ksr1	27HC0GW-
ENSMUSG00000018474	Chd3	27HC0GW-
ENSMUSG00000018845	Unc45b	27HC0GW-
ENSMUSG00000019082	Slc25a22	27HC0GW-
ENSMUSG00000019254	Ppp1r12c	27HC0GW-
ENSMUSG00000019261	Map1s	27HC0GW-
ENSMUSG00000020220	Vps13d	27HC0GW-
ENSMUSG00000020263	Appl2	27HC0GW-

Supplemental Table 4.1: continued.

ENSMUSG00000020407	Upp1	27HC0GW-
ENSMUSG00000020437	Myo1g	27HC0GW-
ENSMUSG00000020677	Ddx52	27HC0GW-
ENSMUSG00000020893	Per1	27HC0GW-
ENSMUSG00000021488	Nsd1	27HC0GW-
ENSMUSG00000021598	Med10	27HC0GW-
ENSMUSG00000021786	Oxsm	27HC0GW-
ENSMUSG00000022103	Gfra2	27HC0GW-
ENSMUSG00000022185	Acin1	27HC0GW-
ENSMUSG00000022237	Ankrd33b	27HC0GW-
ENSMUSG00000022517	Mgrn1	27HC0GW-
ENSMUSG00000023495	Pcbp4	27HC0GW-
ENSMUSG00000023827	Agpat4	27HC0GW-
ENSMUSG00000024121	Atp6v0c	27HC0GW-
ENSMUSG00000024180	Tmem8	27HC0GW-
ENSMUSG00000024201	Kdm4b	27HC0GW-
ENSMUSG00000024236	Svil	27HC0GW-
ENSMUSG00000024426	Atat1	27HC0GW-
ENSMUSG00000024644	Cndp2	27HC0GW-
ENSMUSG00000024767	Otub1	27HC0GW-
ENSMUSG00000024833	Pola2	27HC0GW-
ENSMUSG00000024906	Mus81	27HC0GW-
ENSMUSG00000024958	Gpr137	27HC0GW-
ENSMUSG00000024960	Plcb3	27HC0GW-
ENSMUSG00000024983	Vt1a	27HC0GW-
ENSMUSG00000025128	Bhlhe22	27HC0GW-
ENSMUSG00000025159	Mms19	27HC0GW-
ENSMUSG00000025184	R3hcc1l	27HC0GW-
ENSMUSG00000025223	Ldb1	27HC0GW-
ENSMUSG00000026610	Esrrg	27HC0GW-
ENSMUSG00000026683	Nuf2	27HC0GW-
ENSMUSG00000026970	Rbms1	27HC0GW-
ENSMUSG00000026979	Psd4	27HC0GW-
ENSMUSG00000027253	Lrp4	27HC0GW-
ENSMUSG00000027333	Smox	27HC0GW-
ENSMUSG00000027940	Tpm3	27HC0GW-
ENSMUSG00000028076	Cd1d1	27HC0GW-
ENSMUSG00000028766	Alpl	27HC0GW-
ENSMUSG00000029148	Nrbp1	27HC0GW-
ENSMUSG00000029233	Srd5a3	27HC0GW-
ENSMUSG00000029409	U90926	27HC0GW-
ENSMUSG00000029726	Mepce	27HC0GW-
ENSMUSG00000029922	Mkrl1	27HC0GW-

Supplemental Table 4.1: continued.

ENSMUSG00000030213	Atf7ip	27HC0GW-
ENSMUSG00000030279	C2cd5	27HC0GW-
ENSMUSG00000030595	Nfkbib	27HC0GW-
ENSMUSG00000030605	Mfge8	27HC0GW-
ENSMUSG00000031004	Mki67	27HC0GW-
ENSMUSG00000031753	Cog4	27HC0GW-
ENSMUSG00000031833	Mast3	27HC0GW-
ENSMUSG00000032280	Tle3	27HC0GW-
ENSMUSG00000032295	Man2c1	27HC0GW-
ENSMUSG00000032554	Trf	27HC0GW-
ENSMUSG00000032796	Lama1	27HC0GW-
ENSMUSG00000032965	Ift57	27HC0GW-
ENSMUSG00000033705	Stard9	27HC0GW-
ENSMUSG00000033819	Ppp1r16a	27HC0GW-
ENSMUSG00000033965	Slc16a2	27HC0GW-
ENSMUSG00000033970	Rfc3	27HC0GW-
ENSMUSG00000034037	Fgd5	27HC0GW-
ENSMUSG00000034064	Poglut1	27HC0GW-
ENSMUSG00000034300	Fam53c	27HC0GW-
ENSMUSG00000034673	Pbx2	27HC0GW-
ENSMUSG00000035093	Secisbp2l	27HC0GW-
ENSMUSG00000035203	Epn1	27HC0GW-
ENSMUSG00000035382	Pcsk7	27HC0GW-
ENSMUSG00000035385	Ccl2	27HC0GW-
ENSMUSG00000036646	Man1b1	27HC0GW-
ENSMUSG00000036918	Ttc7	27HC0GW-
ENSMUSG00000037020	Wdr62	27HC0GW-
ENSMUSG00000037791	Phf12	27HC0GW-
ENSMUSG00000037989	Wnk2	27HC0GW-
ENSMUSG00000037993	Dhx38	27HC0GW-
ENSMUSG00000038195	Rilp	27HC0GW-
ENSMUSG00000038518	Jarid2	27HC0GW-
ENSMUSG00000038668	Lpar1	27HC0GW-
ENSMUSG00000038705	Gmeb2	27HC0GW-
ENSMUSG00000038718	Pbx3	27HC0GW-
ENSMUSG00000038762	Abcf1	27HC0GW-
ENSMUSG00000038774	Ascc3	27HC0GW-
ENSMUSG00000038806	Sde2	27HC0GW-
ENSMUSG00000039308	Ndst2	27HC0GW-
ENSMUSG00000039354	Smarca1l	27HC0GW-
ENSMUSG00000039934	Gsap	27HC0GW-
ENSMUSG00000041164	Zmiz2	27HC0GW-
ENSMUSG00000041238	Rbbp8	27HC0GW-

Supplemental Table 4.1: continued.

ENSMUSG00000041440	Gk5	27HC0GW-
ENSMUSG00000041870	Ankrd13a	27HC0GW-
ENSMUSG00000042404	Dennd4b	27HC0GW-
ENSMUSG00000042772	Smg7	27HC0GW-
ENSMUSG00000042978	Sbk1	27HC0GW-
ENSMUSG00000043587	Pxylp1	27HC0GW-
ENSMUSG00000048960	Prex2	27HC0GW-
ENSMUSG00000049076	Acap2	27HC0GW-
ENSMUSG00000049811	Fam161a	27HC0GW-
ENSMUSG00000051007	Gatd1	27HC0GW-
ENSMUSG00000051435	Fhad1	27HC0GW-
ENSMUSG00000052406	Rexo4	27HC0GW-
ENSMUSG00000052516	Robo2	27HC0GW-
ENSMUSG00000054008	Ndst1	27HC0GW-
ENSMUSG00000054823	Nsd3	27HC0GW-
ENSMUSG00000055632	Hmcn2	27HC0GW-
ENSMUSG00000056185	Snx32	27HC0GW-
ENSMUSG00000056296	Synpr	27HC0GW-
ENSMUSG00000056917	Sipa1	27HC0GW-
ENSMUSG00000057342	Sphk2	27HC0GW-
ENSMUSG00000057375	Yipf1	27HC0GW-
ENSMUSG00000059439	Bcas3	27HC0GW-
ENSMUSG00000061665	Cd2ap	27HC0GW-
ENSMUSG00000064147	Rab44	27HC0GW-
ENSMUSG00000068040	Tm9sf4	27HC0GW-
ENSMUSG00000069631	Strada	27HC0GW-
ENSMUSG00000071266	Zfp946	27HC0GW-
ENSMUSG00000074643	Cpne1	27HC0GW-
ENSMUSG00000080316	Spaca6	27HC0GW-
ENSMUSG00000092586	Ly6g6c	27HC0GW-
ENSMUSG00000001518	Itfg2	27HC+GW0
ENSMUSG00000002661	Alkbh7	27HC+GW0
ENSMUSG00000004099	Dnmt1	27HC+GW0
ENSMUSG00000005233	Spc25	27HC+GW0
ENSMUSG00000006219	Fblim1	27HC+GW0
ENSMUSG00000007050	Lsm2	27HC+GW0
ENSMUSG00000008373	Prpf31	27HC+GW0
ENSMUSG00000010554	Mettl16	27HC+GW0
ENSMUSG00000013846	St3gal1	27HC+GW0
ENSMUSG00000016319	Slc25a5	27HC+GW0
ENSMUSG00000016664	Pacsin2	27HC+GW0
ENSMUSG00000017499	Cdc6	27HC+GW0
ENSMUSG00000017765	Slc12a4	27HC+GW0

Supplemental Table 4.1: continued.

ENSMUSG00000019487	Trip10	27HC+GW0
ENSMUSG00000020260	Pofut2	27HC+GW0
ENSMUSG00000020534	Shmt1	27HC+GW0
ENSMUSG00000020689	Itgb3	27HC+GW0
ENSMUSG00000020914	Top2a	27HC+GW0
ENSMUSG00000021929	Kpna3	27HC+GW0
ENSMUSG00000021967	Mrpl57	27HC+GW0
ENSMUSG00000022102	Dok2	27HC+GW0
ENSMUSG00000022422	Dscc1	27HC+GW0
ENSMUSG00000022710	Usp7	27HC+GW0
ENSMUSG00000023951	Vegfa	27HC+GW0
ENSMUSG00000024190	Dusp1	27HC+GW0
ENSMUSG00000024222	Fkbp5	27HC+GW0
ENSMUSG00000024404	Riok3	27HC+GW0
ENSMUSG00000024451	Arap3	27HC+GW0
ENSMUSG00000024581	Napg	27HC+GW0
ENSMUSG00000024590	Lmnbl	27HC+GW0
ENSMUSG00000025001	Hells	27HC+GW0
ENSMUSG00000025155	Dus1l	27HC+GW0
ENSMUSG00000025198	Erlin1	27HC+GW0
ENSMUSG00000025574	Tk1	27HC+GW0
ENSMUSG00000025583	Rptor	27HC+GW0
ENSMUSG00000026028	Trak2	27HC+GW0
ENSMUSG00000026159	Agfg1	27HC+GW0
ENSMUSG00000026343	Gpr39	27HC+GW0
ENSMUSG00000026819	Slc25a25	27HC+GW0
ENSMUSG00000027282	Mtch2	27HC+GW0
ENSMUSG00000027379	Bub1	27HC+GW0
ENSMUSG00000027715	Ccna2	27HC+GW0
ENSMUSG00000028690	Mmachc	27HC+GW0
ENSMUSG00000029283	Cdc7	27HC+GW0
ENSMUSG00000029462	Vps29	27HC+GW0
ENSMUSG00000029475	Kdm2b	27HC+GW0
ENSMUSG00000029916	Agk	27HC+GW0
ENSMUSG00000030045	Mrpl19	27HC+GW0
ENSMUSG00000030347	D6Wsu163e	27HC+GW0
ENSMUSG00000030410	Dmwd	27HC+GW0
ENSMUSG00000030704	Rab6a	27HC+GW0
ENSMUSG00000030761	Myo7a	27HC+GW0
ENSMUSG00000030846	Tial1	27HC+GW0
ENSMUSG00000030852	Tacc2	27HC+GW0
ENSMUSG00000031387	Renbp	27HC+GW0
ENSMUSG00000031826	Usp10	27HC+GW0

Supplemental Table 4.1: continued.

ENSMUSG00000031885	Cbfb	27HC+GW0
ENSMUSG00000032589	Bsn	27HC+GW0
ENSMUSG00000032786	Alas1	27HC+GW0
ENSMUSG00000034254	Agpat1	27HC+GW0
ENSMUSG00000035455	Figl1	27HC+GW0
ENSMUSG00000035769	Xylb	27HC+GW0
ENSMUSG00000035877	Zhx3	27HC+GW0
ENSMUSG00000036323	Srp72	27HC+GW0
ENSMUSG00000036591	Arhgap21	27HC+GW0
ENSMUSG00000036768	Kif15	27HC+GW0
ENSMUSG00000037300	Ttc13	27HC+GW0
ENSMUSG00000037583	Nr0b2	27HC+GW0
ENSMUSG00000038611	Phrf1	27HC+GW0
ENSMUSG00000039031	Arhgap18	27HC+GW0
ENSMUSG00000039137	Whrn	27HC+GW0
ENSMUSG00000039842	Mcp1	27HC+GW0
ENSMUSG00000039917	Rhbdd2	27HC+GW0
ENSMUSG00000040532	Abhd11	27HC+GW0
ENSMUSG00000040945	Rcc2	27HC+GW0
ENSMUSG00000041891	Lman1	27HC+GW0
ENSMUSG00000044934	Zfp367	27HC+GW0
ENSMUSG00000045751	Mms22l	27HC+GW0
ENSMUSG00000049422	Chchd10	27HC+GW0
ENSMUSG00000053716	Dusp7	27HC+GW0
ENSMUSG00000056211	R3hdm1	27HC+GW0
ENSMUSG00000056394	Lig1	27HC+GW0
ENSMUSG00000059013	Sh2d3c	27HC+GW0
ENSMUSG00000060600	Eno3	27HC+GW0
ENSMUSG00000061244	Exoc5	27HC+GW0
ENSMUSG00000061536	Sec22c	27HC+GW0
ENSMUSG00000063511	Snrnp70	27HC+GW0
ENSMUSG00000072872	Rybp	27HC+GW0
ENSMUSG00000073889	Il11ra1	27HC+GW0
ENSMUSG00000074064	Mlycd	27HC+GW0
ENSMUSG00000074305	Peak1	27HC+GW0
ENSMUSG00000074340	Ovgp1	27HC+GW0
ENSMUSG00000096054	Syne1	27HC+GW0
ENSMUSG00000107283	Mpv17	27HC+GW0
ENSMUSG00000001127	Araf	27HC-GW0
ENSMUSG00000001300	Efnb2	27HC-GW0
ENSMUSG00000001870	Ltbp1	27HC-GW0
ENSMUSG00000002602	Axl	27HC-GW0
ENSMUSG00000003345	Csnk1g2	27HC-GW0

Supplemental Table 4.1: continued.

ENSMUSG00000004044	Cavin1	27HC-GW0
ENSMUSG00000005417	Mprp	27HC-GW0
ENSMUSG00000014158	Trpv4	27HC-GW0
ENSMUSG00000015143	Actn1	27HC-GW0
ENSMUSG00000015652	Steap1	27HC-GW0
ENSMUSG00000016382	Pls3	27HC-GW0
ENSMUSG00000019851	Perp	27HC-GW0
ENSMUSG00000020176	Grb10	27HC-GW0
ENSMUSG00000020325	Fstl3	27HC-GW0
ENSMUSG00000021384	Susd3	27HC-GW0
ENSMUSG00000021589	Rhobtb3	27HC-GW0
ENSMUSG00000021662	Arhgef28	27HC-GW0
ENSMUSG00000021880	Rnase6	27HC-GW0
ENSMUSG00000022269	March11	27HC-GW0
ENSMUSG00000022389	Tef	27HC-GW0
ENSMUSG00000022504	Ciita	27HC-GW0
ENSMUSG00000022836	Mylk	27HC-GW0
ENSMUSG00000022849	Hspbap1	27HC-GW0
ENSMUSG00000024013	Fgd2	27HC-GW0
ENSMUSG00000024044	Epb41l3	27HC-GW0
ENSMUSG00000024395	Lims2	27HC-GW0
ENSMUSG00000025006	Sorbs1	27HC-GW0
ENSMUSG00000025154	Arhgap19	27HC-GW0
ENSMUSG00000025410	Dctn2	27HC-GW0
ENSMUSG00000025420	Katnal2	27HC-GW0
ENSMUSG00000025875	Tspan17	27HC-GW0
ENSMUSG00000026604	Ptpn14	27HC-GW0
ENSMUSG00000027210	Meis2	27HC-GW0
ENSMUSG00000027215	Cd82	27HC-GW0
ENSMUSG00000027589	Pcmtd2	27HC-GW0
ENSMUSG00000027695	Pld1	27HC-GW0
ENSMUSG00000027806	Tsc22d2	27HC-GW0
ENSMUSG00000028064	Sema4a	27HC-GW0
ENSMUSG00000028706	Nsun4	27HC-GW0
ENSMUSG00000029060	Mib2	27HC-GW0
ENSMUSG00000029482	Aacs	27HC-GW0
ENSMUSG00000030170	Wnt5b	27HC-GW0
ENSMUSG00000030499	Kctd15	27HC-GW0
ENSMUSG00000030513	Pcsk6	27HC-GW0
ENSMUSG00000030638	Sh3gl3	27HC-GW0
ENSMUSG00000030796	Tead2	27HC-GW0
ENSMUSG00000031024	St5	27HC-GW0
ENSMUSG00000031078	Ctnn	27HC-GW0

Supplemental Table 4.1: continued.

ENSMUSG00000031196	F8	27HC-GW0
ENSMUSG00000031586	Rbpms	27HC-GW0
ENSMUSG00000031906	Smpd3	27HC-GW0
ENSMUSG00000032118	Fez1	27HC-GW0
ENSMUSG00000032216	Nedd4	27HC-GW0
ENSMUSG00000032869	Psmf1	27HC-GW0
ENSMUSG00000033256	Shf	27HC-GW0
ENSMUSG00000033740	St18	27HC-GW0
ENSMUSG00000033768	Nrxn2	27HC-GW0
ENSMUSG00000033955	Tnks1bp1	27HC-GW0
ENSMUSG00000034028	Cd226	27HC-GW0
ENSMUSG00000034116	Vav1	27HC-GW0
ENSMUSG00000035390	Brsk1	27HC-GW0
ENSMUSG00000036225	Kctd1	27HC-GW0
ENSMUSG00000036377	C530008M17Rik	27HC-GW0
ENSMUSG00000036564	Ndr4	27HC-GW0
ENSMUSG00000037143	Cfap61	27HC-GW0
ENSMUSG00000037652	Phc3	27HC-GW0
ENSMUSG00000038143	Stox2	27HC-GW0
ENSMUSG00000038304	Cd160	27HC-GW0
ENSMUSG00000039103	Nexn	27HC-GW0
ENSMUSG00000039323	Igfbp2	27HC-GW0
ENSMUSG00000039476	Prrx2	27HC-GW0
ENSMUSG00000039477	Tnrc18	27HC-GW0
ENSMUSG00000039497	Dse	27HC-GW0
ENSMUSG00000039536	Stau1	27HC-GW0
ENSMUSG00000039765	Cc2d2a	27HC-GW0
ENSMUSG00000039831	Arhgap29	27HC-GW0
ENSMUSG00000039943	Plcb4	27HC-GW0
ENSMUSG00000040850	Psme4	27HC-GW0
ENSMUSG00000042032	Mat2b	27HC-GW0
ENSMUSG00000042686	Jph1	27HC-GW0
ENSMUSG00000047777	Phf13	27HC-GW0
ENSMUSG00000050541	Adra1b	27HC-GW0
ENSMUSG00000052752	Traf7	27HC-GW0
ENSMUSG00000053347	Zfp943	27HC-GW0
ENSMUSG00000059409	Ppp2r5d	27HC-GW0
ENSMUSG00000061689	Dlgap4	27HC-GW0
ENSMUSG00000066036	Ubr4	27HC-GW0
ENSMUSG00000069170	Adgrv1	27HC-GW0
ENSMUSG00000070733	Fryl	27HC-GW0
ENSMUSG00000074457	S100a16	27HC-GW0

Supplemental Table 4.1: continued.

ENSMUSG00000105096	Gbp10	27HC-GW0
-: significantly downregulated, 0: unchanged, +: significantly upregulated		

Supplemental Table 4.2: Genes with LXR β binding sites

ensembl gene id	gene name	change
ENSMUSG00000010663	Fads1	27HC-GW+
ENSMUSG00000021957	Tkt	27HC0GW+
ENSMUSG00000025950	Idh1	27HC0GW+
ENSMUSG00000049985	Ankrd55	27HC0GW+
ENSMUSG00000063531	Sema3e	27HC0GW+
ENSMUSG00000068740	Celsr2	27HC0GW+
ENSMUSG00000024426	Atat1	27HC0GW-
ENSMUSG00000036561	Ppp6r2	27HC0GW-
ENSMUSG00000045625	Pigz	27HC0GW-
ENSMUSG00000052516	Robo2	27HC0GW-
ENSMUSG00000068040	Tm9sf4	27HC0GW-
ENSMUSG00000002147	Stat6	27HC+GW0
ENSMUSG00000008398	Elk3	27HC+GW0
ENSMUSG00000030410	Dmwd	27HC+GW0
ENSMUSG00000053153	Spag16	27HC+GW0
ENSMUSG00000060600	Eno3	27HC+GW0
ENSMUSG00000061244	Exoc5	27HC+GW0
ENSMUSG00000005417	Mprip	27HC-GW0
ENSMUSG00000025812	Pard3	27HC-GW0
ENSMUSG00000028464	Tpm2	27HC-GW0
ENSMUSG00000038143	Stox2	27HC-GW0
ENSMUSG00000055720	Ubl7	27HC-GW0
ENSMUSG00000056824	Zfp663	27HC-GW0

-: significantly downregulated, 0: unchanged, +: significantly upregulated

Supplemental Table 4.3: Select genes involved in LXR-dependent/independent chemotaxis

gene name	logFC.HCvsVeh	FDR.HCvsVeh	logFC.GWvsVeh	FDR.GWvsVeh
Ccr7	-1.1243274	0.22640442	-1.4086927	0.08057211
Cd38	1.29348797	3.36E-13	1.97608681	2.61E-20
Cxcr2	-1.3846782	0.10853454	-0.5411718	0.63391076
Mmp9	-0.4613963	1.67E-06	-2.0379436	5.18E-22
Prok2	0.00719125	0.99463523	0.06075877	0.90098006