ABSTRACT

Title of Document: CHARACTERIZATION OF LIVER X
                        RECEPTORS IN PROSTATE CANCER
                        CHOLESTEROL METABOLISM AND
                        PULMONARY IMMUNE RESPONSE

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                        and Food Science

Liver x receptors (LXRs) are central regulators cholesterol homeostasis

and the innate immune response. As modulators of inflammation and cholesterol

metabolism LXRs might diminish dyslipidemia and inflammation related

pathologies caused by high fat (HF) diets or obesity. There is also data

demonstrating that LXRs can protect against progression of prostate cancer (PCa),

but little is known about the cholesterol modulating effects of LXRs in

transformed cells. The goal of this project is to characterize the cholesterol

modulating properties of LXRs in two models of PCa and the anti-inflammatory

properties of LXRs in swine bronchial alveolar macrophages (AMs). This project

will also examine whether the anti-inflammatory and lipid lowering properties of

the dietary probiotic bacteria *Lactobacillus casei* (*L. casei*), can interact with the

LXR axis in AMs. Studies in two PCa cell lines, LNCaP and PC-3, revealed that

LXR ligands regulate the LXR responsive genes, *ABCA1* and *ABCG1* through the

LXRβ isoform and not LXRα in PC-3 cells, but only *ABCG1* in LNCaP. LXR-

ABCA1 mediated reverse cholesterol transport (RCT) resulted in a decrease in plasma membrane lipid raft cholesterol domains in PC-3 cells, suggesting a potential anti-cancer axis for LXR activation. Studies in LNCaP and PC-3 cells also demonstrated that soy isoflavones can activate transcriptional activation of *ABCA1* and *ABCG1* in LNCaP and PC-3 cells through the LXRβ isoform, but did not lead to an increase in RCT. Metabolic and anti-inflammatory studies of LXR in AM from Ossabaw pigs fed either a control (C) diet, HF, HF plus *L casei* (HFPB) or *L. casei* alone (CPB) diet revealed that AM from HF fed pigs had significantly higher concentrations of cholesteryl-esters (CE) compared with AM from control (C) diet fed pigs. *Ex-vivo* activation of LXR with the LXR ligand T0901317 opposed LPS mediated upregulation of *IL-1β*, *IL-6*, *IL-8* and *IL-10* mRNA levels in AM from HF, HFPB and CPB fed pigs. Finally, it was observed that LPS stimulation lead to significant inhibition of LXR transcription of *LXRα, ABCA1*, *ABCG1*, cholesterol 25 hydroxylase (*CH25H*) and *PPARγ* in AM. This effect was abrogated by *L. casei* for *ABCA1*, *CH25H* and *PPARγ* mRNA expression.

CHARACTERIZATION OF LIVER X RECEPTORS IN PROSTATE CANCER
CHOLESTEROL METABOLISM AND PULMONARY IMMUNE RESPONSE.

By

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Chapter 2:

List of Abbreviations

24,24EC	24(S),25-epoxycholesterol
25HC	25-hydroxycholesterol
ABCA1	ATP-binding cassette, sub-family A1
ABCG1	ATP-binding cassette, sub-family G 1
AM	Alveolar macrophages
Apo A-I	Apolipoprotein A-I
AR	Androgen receptor
BAL	Bronchiole alveolar lavage
CH25H	Cholesterol 25- hydroxylase
CXCR4	Chemokine receptor CXC-4
CYP7A1	Cholesterol 7α-hydroxylase
DR	Direct repeat
DRMs	Detergent resistant membrane fractions
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERK1/2	Extracellular signal-regulated kinase
FAS	fatty acid synthase
HDL	High density lipoprotein
HMG-CoA	Hydroxymethyl glutaryl synthase/reductase
IL-10	Interleukin-10
IL-1β	Interleukin-1β

IL-6	Interleukin-6
IL-8	Interleukin-8
JNK	c-Jun NH2-terminal kinase
LCAT	Lecithin-cholesterol acyltransferase
LXRE	Liver x receptor responsive element
LDL	Low density lipoprotein
LXRs	Liver X receptors
NRTF	Nuclear receptor transcription factors
P38	Mitogen-activated protein kinase 14
PCa	Prostate cancer
PKA	Protein kinase A
PPARs	Peroxisome-proliferator-activated receptors
PXR	Pregnane X receptor
RARs	Retinoic acid receptors
RCT	Reverse cholesterol transport
RXRs	Retinoid x receptors
SRB1	Scavenger receptor B1
SREBP1/2	Sterol response element-binding protein 1/2
T09	T0901317
THR	Thyroid hormone receptor
TLR4	Toll like receptor -4
TNF-α	Tumor necrosis factor-α
VDR	Vitamin D receptor

Chapter 1: Literature Review of Liver X Receptors

Discovery of Liver X Receptors

Nuclear receptor transcription factors (NRTFs) are intracellular receptors that bind to small lipophylic molecules, such as retinoids, fatty acids, vitamins (vitamin D) and steroid hormones (estrogen, glucocorticoids), and can regulate transcription of target genes by direct interaction with promoter region DNA sequences known as response elements (1,2). The response element for all 48 members of the NRTF super family consists of a the DNA consensus sequence (AGGTCA) which is found as either a direct repeat (DR) or inverted repeat, separated by 3, 4 or 5 base pairs known as DR-3, -4 and -5 elements. Genes regulated by specific NRTF are identified and defined by the specific DR-X consensus sequence found in their transcription promoter region. For example, androgen receptor (AR) and vitamin D receptor-(VDR) binds to DR-3 elements in target genes, while thyroid hormone receptor (THR) and retinoic acid receptor (RAR) bind only to DR-4 and DR-5 promoter elements respectively (1,2).

NRTF are further subcategorized whether or not they reside in the cytosol (type I) or nucleus (type II). Type I NRTF translocate the nuclear as homo-dimers and bind to promoter region DR-3 elements known as hormone responsive elements (1,2) and are either recycled back to the cytosol or are marked for ubiquitination and rapid proteosomal degradation (1,2). Type II NRTF form obligate heterodimers with another type II NRTF member, retinoid x receptors (RXR), and reside in the promoter region of target genes in a transcriptionaly

repressed under the direction of numerous transcriptional co-repressor proteins (1,2). Upon ligand activation, types II NRTFs undergo conformational changes that promote the exchange of transcription co-repressors with co-activators allowing for transcriptional regulation of the target gene (1-3). The type II NRTF subfamily includes RAR, THR, VDR, peroxisome-proliferator-activated receptors (PPARs), and liver X receptors (LXR) (1-3).

Liver x receptors, NR1H3 (LXRα) and NR1H2 (LXRβ) are the newest members to be added to the type II family of NRTF. In 1994 two laboratories separately discovered LXRα, and in 1995 four reported the discovery of LXRβ, initially coined ubiquitous receptor from screens of mouse liver and colon cDNA libraries (3-5). In humans, the genes for *LXRα* and *LXRβ* are encoded on chromosomes 11 and 19 respectively (3). Molecular weight of the protein products are 45 kiloDaltons (kDa) for LXRα and 51 kDa for LXRβ (3). Both share approximately 78% protein sequence homology in their DNA and ligand binding domains (3-5). Expression patterns differ between each isoform, with LXRβ being expressed in all tissue, while LXRα is primarily expressed in liver, kidney, adrenals, adipose, small intestine and macrophages (3).

Among the members of the NRTF superfamily, LXRs show the highest sequence conservation across species with ~75% homology in the ligand binding domains between human and non-mammals (6, 7). Sequencing data of LXRs across mammalian and non-mammalian species demonstrates that mammals posses two LXR genes (LXRα and LXRβ) while non-mammalian species have

only express LXRβ, and invertebrates none (6). A single LXR gene duplication likely occurred before or in early mammalian evolution (6).

LXRs where initially coined orphan receptors until 1996 when oxygenated cholesterol metabolites, known oxysterols, where identified as endogenous ligands for LXRα and LXRβ (8). Oxysterols are generated from cholesterol either enzymatically by sterol hydroxylases or auto-oxidation (8). The majority of oxysterols are formed in the endoplasmic reticulum (ER) during cholesterol processing and are transported from the ER and golgi complex to the cytosol and nuclear compartments by a family of highly conserved proteins called oxysterol binding proteins (OSBP) and oxysterol related binding protein (OSBP-related proteins (ORPs) (9). Figure 1 shows some common structures of oxysterols synthesized from cholesterol (9).
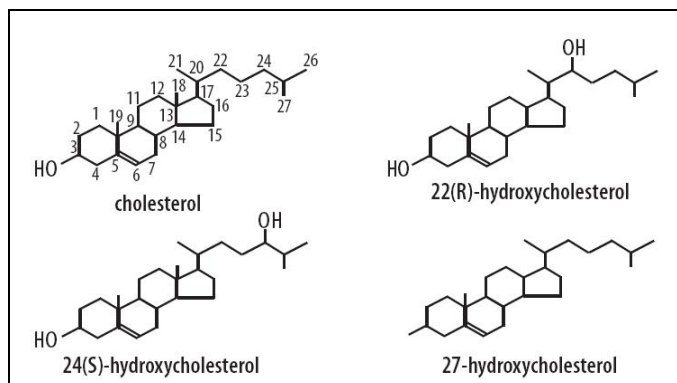
Figure 1. Oxysterols, with hydroxyl groups typically added to the sterol side-chain carbons, are robust LXR ligands. Source: J Med Biol Res 2008; 41: 545-556.

Oxysterols are found very low concentrations *in vivo*, typically at 1:1000 (~0.01μM vs. ~5000 μM) compared to cholesterol, but being more hydrophic than cholesterol, oxysterols can move more freely through cellular compartments increasing their potential as robust signaling lipids (9). Binding studies indicate

that oxysterols can activate LXRα and LXRβ at nanomolar concentrations and with similar efficiency (8, 9).

Like other members of the type II NRTF family, LXRs form obligate heterodimers with RXR and reside in the promoter region of target genes bound to a DR-4 consensus sequence, AGGTCAxxxxAGGTCA, known as a liver x receptor responsive element (LXRE) (3). LXR: RXR heterodimers form part of a protein complex of transcriptional co-repressors proteins such as silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor co-repressor (N-CoR) (10). These complexes maintain transcriptional repression of LXR responsive genes in the absence of ligand activation (10). Upon activation by oxysterols, LXRs undergo conformational changes that promote dissociation of SMRT and N-CoR, and association with co-activator proteins such as peroxisome-proliferator-activated receptor (PPAR) c co-activator 1a (PGC-1a} PPARGC1), which facilitate the transcriptional activation of the target gene (8, 10) (Fig 2). Transcriptional regulation of basal LXRα mRNA levels are self-regulated (a number of LXREs have been in the promoter of the LXRα gene) and protein levels of LXRα are dependant on phosphorylation of a number of key serine residues (11).
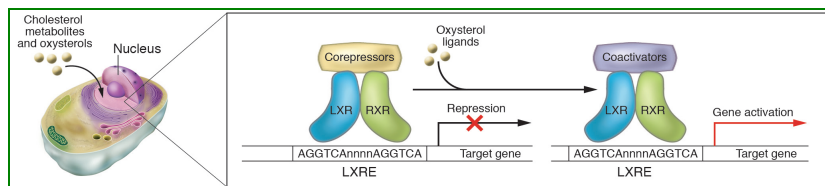
Figure 2. Model of LXR transcriptional regulation of gene expression.
Source: J Clin Invest. 2006 Mar; 116(3):607-14.

The initial discovery that LXRs are activated by cholesterol metabolites *in vitro* and not cholesterol itself, lead to speculation that LXRs may act as sensors of cellular cholesterol levels (12). Studies of lipid metabolism in LXRα and LXRβ null mice confirmed that LXRs have a central role in the regulation of cholesterol metabolism *in vivo*. Studies by Mangelsdorf et al. and Gustafson et al. both demonstrated that LXRα -/- null mice fed a chow diet enriched with 2% cholesterol presented with gross hepatic and peripheral accumulation of cholesteryl-esters and elevated serum cholesterol levels (13, 14). LXRα -/- mice had diminished hepatic expression of genes central to cholesterol and lipid metabolism including cholesterol 7α-hydroxylase (CYP7A1), hydroxymethyl glutaryl (HMG)-CoA synthase/reductase, squalene synthase (SQS), sterol response element-binding protein 1 and 2 (SREBP1 and 2), sterol CoA desaturase (SCD)-1, and fatty acid synthase (FAS) (13,14). Gustafson et. al. also demonstrated that unlike LXRα -/- mice, LXRβ -/- null mice were not prone developing hepatic cholesterol lipidosis and serum dyslipidemia on a cholesterol enriched chow diet (14). The disparity between the lipid profile of LXRα -/- and LXRβ -/- mice is explained by the dominant role that LXRα has in hepatic regulation of the bile synthesis enzyme CYP7A1 (14). LXRβ -/- null mice are able to cope with the lipid burden of a high fat diet because they retained LXRα regulation of CYP7A1 hepatic conversion of cholesterol to bile acids.

LXR Regulation of Whole Body Cholesterols Levels

By the year 2000, an expanded role for LXR regulation of cholesterol metabolism outside of the liver was established when two studies demonstrated

that the transfection of RAW 264.7 cells and THP-1 macrophages with constructs that lead to overexpression of LXRα, resulted in a strong mRNA and protein upregulation of the cholesterol efflux transporters ATP-binding cassette, sub-family A, member 1 (ABCA1) and sub-family member G 1 (ABCG1) and an increase in cholesterol efflux to lipid free apolipoprotein A-I (apo A-I). (15). Subsequent studies of cholesterol laden macrophages from low density lipoprotein (LDL) -/- null mice also demonstrated that natural and synthetic LXR ligands can increase mRNA and protein expression of ABCA1, ABCG1 transporters and increase peripheral cholesterol efflux *in vivo* (16).

These studies, coupled with previous data from LXR -/- null mice, helped construct a distinct role for LXR in regulating whole body cholesterol levels. It is now understood that among the numerous effectors involved in the regulation of whole body cholesterol levels, LXRs exists as cellular cholesterol sensors and function to limit absorption of dietary cholesterol, protect cells from the cytotoxic effects of cholesterol accumulation and permit for rapid catabolism and secretion of hepatic cholesterol and bile acids. In the intestine, post-prandial cholesterol absorption and reabsorption of hepatic cholesterol is regulated by LXR activation of cholesterol efflux pumps ABCG5 and ABCG8 (15). In peripheral tissue and cells such as smooth muscle or macrophages, excess cellular cholesterol derived from either plasma apolipoprotein B containing lipoproteins or *de novo* synthesis via the mevalonate pathway, is rapidly metabolized to oxysterols by a family or microsomal cytochrome P450 and mitochondrial hydroxylases such as CYP27A1, CYP46, cholesterol 25-hydroxylase (CH25H) (15). Binding of oxysterols to LXR

leads to increased mRNA transcription and protein translation of cholesterol

transporters ABCA1, ABCG1, which use energy from ATP hydrolysis to

transport plasma membrane free cholesterol to the outer plasma membrane leaflet

where direct interactions with lipid poor apo A-I and immature HDL particles

permit efflux and adsorption of free cholesterol (17). The formation of immature

HDL particles occurs only after ABCA1 cholesterol efflux to lipid poor apo A-1,

which is followed by esterification of apo A-I bound cholesterol by the enzyme

lecithin-cholesterol acyltransferase (LCAT) (17). Newly esterified cholesteryl-

esters partition to the interior of the apo A-I complex and results in the formation

of a more spherical, immature HDL particle (17,18). These particles remain in

circulation and continue to remove excess cholesterol from other peripheral cells

through interactions specifically with the plasma membrane cholesterol

transporter ABCG1 (17). Mature HDL particles can transport cholesteryl-esters

to the liver in a process involving hepatic scavenger receptor B1 (SRB1), or

through exchange of cholesterol for triglycerides from very low density and low

density lipoproteins (VLDL and LDL), which deliver cholesterol to steroidogenic

tissue or to the liver via LDL receptors (Fig 3). This process is collectively

referred to as reverse cholesterol transport (RCT).

Studies of mice with tissue specific knockouts of ABCA1 have

demonstrated that hepatic and intestinal expression of ABCA1 is a major

determinant of circulating apo A-I protein and HDL levels (17, 18). This is due to

a role for ABCA1 in transportation of newly synthesized apo A-I from the liver to

peripheral circulation (17, 18). Inheritance of a loss of function mutation of the

ABCA1 gene results in a rare disorder known as Tangier's disease characterized by peripheral cholesterol accumulation, severely depressed serum high density lipoprotein, enlarged spleen and liver and risk for onset of atherosclerosis before the age of 20 (19).
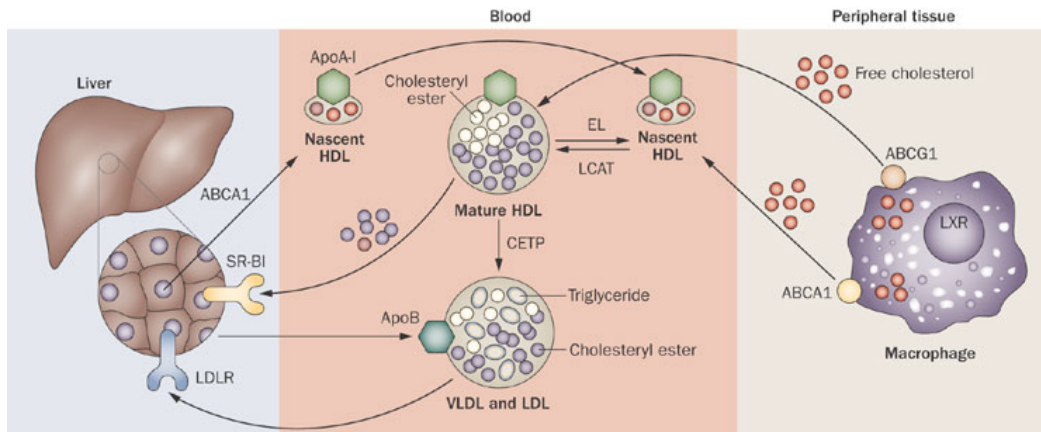
Figure 3. LXR mediates Reverse Cholesterol Transport.
Source: Nature Reviews Cardiology. 6, 455-463, 2009

In the liver activation of LXRα directs hepatic β-oxidation of cholesterol to the bile acids, cholic acid (CA) and chenodeoxcholic acid (CDCA) by the bile acid synthesis enzyme cytochrome P450, member 3A4 (CYP3A4) (CYP7A1 in mice) (8). LXRα also directs the efflux of bile and free cholesterol via ABCG5/8 from the liver, through the hepatic bile duct, to the duodenum where bile salts facilitate the absorption of post-prandial lipids and lipid soluble vitamins (e.g. vitamin A, D, K and triacylglycerol) (8). The majority of cholesterol and bile salts secreted into the small intestine are reabsorbed with little loss of cholesterol in the feces (13).

The gene duplication and evolution of both LXR isoforms as functionally redundant paralogs within the higher vertebrate and mammal kingdom occurred in parallel to appearance of the of the hepatobiliary tract and enterohepatic

circulation (6, 7). LXRs harmonize these pathways with two vital components of mammalian lipid metabolism: 1) preventing cholesterol accumulation and toxicity in peripheral tissue and 2) the need for hepatic cholesterol catabolism, synthesis of bile acids for assimilation of dietary lipids and lipid soluble vitamins. Undoubtedly, the evolution of LXRs and other hormone NRTF that are activated by cholesterol derivatives occurred in response to the increasing presence of dietary cholesterol from marine or terrestrial food sources as higher mammals evolved.

Studies have also demonstrated an emerging role for hepatic LXRα as a nutrient sensor in the regulation of carbohydrate metabolism and energy homeostasis (20). During post-prandial carbohydrate metabolism, D-glucose and glucose-6-phosphate can act as direct LXR ligands with affinity equal to oxysterols and increase expression of hepatic lipogenic enzymes such as sterol regulatory element binding protein isoform 1-c (SREBP1-c), and fatty acid synthase (FAS), as well genes involved in decreasing glucose output and increasing glucose utilization, including glucokinase, phosphoenolpyruvate carboxykinase and glucose 6-phosphatase (20).

LXRs are Anti-inflammatory and Cardio-Protective

Interest in the immuno-modulating properties of LXR grew after a number of studies demonstrated that epidermal application of LXR ligands, 22(R)-hydroxycholesterol and 24(S), 25-epoxycholesterol in a model of contact dermatitis lead to inhibition of keratinocyte proliferation, differentiation and normalization of epidermal barrier function (21). Consistent with this, another

study found that oxysterol administration to pregnant mice increased the development rate of the fetal epidermal permeability barrier (22). Both LXRα and LXRβ are expressed in epidermis, but it was demonstrated that epidermal LXRβ is responsible for the anti-inflammatory and anti-proliferative effect of LXR ligands on keratinocytes *in vivo* (21).

A more expansive role for LXR in modulating inflammation was demonstrated in 2002, when Totontoz et. al. reported that administration of the synthetic LXR ligands, GW3965 or T0901317 (T09) to apo E -/- or LDLR -/- null mice significantly diminished atherosclerotic plaques and atheroma formation (23). He went on to publish a report demonstrating that synthetic and natural LXR ligands administered to apo E -/- or LDLR -/- null atherosclerotic mice caused robust antagonism of lipopolysaccharide (LPS) mediated mRNA expression and secretion of pro-inflammatory mediators such as iNOS, TNFα, IL-6, IL-1β, MCP-1 in peritoneal macrophages (24). Atherosclerotic plaques in T09 treated mice were significantly diminished and profiling of aortic lesions revealed a decreased expression of matrix metallopeptidase 9 (MMP-9), an enzyme involved in the breakdown of extracellular matrix in normal tissue and implicated in the etiology of atheroma lesion formation (24). Numerous studies have since demonstrated a cardio-protective effect of LXR ligands in cell and animals models of obesity and cardiovascular disease (25-27). A central molecular paradigm shared by these studies is that LXR activation is cardio-protective because of its ability to: i) prevent macrophages foam cell formation by increasing cholesterol efflux via ABCA1 and ABCG1 and ii) antagonize expression of pro-inflammatory

mediators such as MCP-1, TNF-α and IL-1β, that are positively associated with atheroma formation (18).

LXR ligands can oppose LPS/toll like receptor -4 (TLR4) mediated inflammation in macrophages isolated from LXRα -/- and LXRβ -/- null mice, but not from LXRα/β -/- double knockout mice demonstrating that both LXR isoforms posses anti-inflammatory properties (24) The anti-inflammatory effect of LXR activation is not exclusive to macrophages or the dermis, but occurs systemically as it has been demonstrated that mice challenged with i.p. LPS and treated with LXR ligands have broad antagonism of inflammation in the liver, kidney, small intestine, lungs and smooth muscle (28). Inflammatory mediators opposed by LXR, such as TNF-α, IL-6, and IL-1β, do not posses LXREs in their DNA promoter regions; therefore an indirect mode of LXR opposition of these targets has been proposed. One model demonstrates that LXRs oppose pro-inflammatory mediators by nuclear trans-repression of the transcription factor NF-kB (24).

LXR ligands only suppress inflammation in LPS or toll like receptor 3 or 4 (TLR3/4) stimulated macrophages, not in naive, unstimulated cells (24). Furthermore, LXR antagonism of TLR3/4 signaling also protects macrophages from LPS mediated suppression of cholesterol efflux, as it has been demonstrated that LPS can inhibit *ABCA1*, *ABCG1* mRNA expression and lead to pathological cholesterol retention and apoptosis in macrophages in an LXR dependant manner (24). The uncoupling of the anti-inflammatory properties of LXR in the absence of an immune response likely occurs because of the high risk that phagoctytic

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cells such as macrophages have for accumulating pathological concentration of cholesterol during phagocytosis of cellular debris. Cross talk between TLR3/4 and LXR mediated cholesterol efflux may provide new insight into mechanisms through which pathogens evade immune detection and contribute to inflammatory and metabolic related disease such as atherosclerosis.

LXRs are a vital molecular target in treatment of cardiovascular disease because the potential for LXR ligands to increase cholesterol metabolism and oppose the inflammatory response. However, one drawback in developing LXRs as a cardio protective target is the increased serum triglycerides which accompany hepatic LXRα activation of lipogenic genes FAS and SREBP-1c (12, 20). The information gap regarding LXR as a nutrient sensor is still closing, but the observed triglyceridemia accompanying LXR activation is counterintuitive and probably underscores how little is understood about LXR biology. Development of synthetic ligands specific for LXRβ would allow for selective LXR activation of only peripheral RCT and minimize hepatic expression of lipogenic genes. Studies have already identified specific LXRβ ligands which result in cardio-protection *in vivo* without affecting LXRα hepatic lipogenic genes (26).

LXR Regulation of the Innate Immune Response

Because LXR activation negatively regulates TLR4 mediated inflammatory response, it has been suggested that LXR activation may compromise host immunity. This question was examined in two studies on the effects of LXR of pulmonary immunity. Birrell et. al. demonstrated a that administration of the LXR ligands T09 or GW3965 to male Wistar rats was

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associated with decreased pulmonary neutrophilia in response to LPS challenge

(29). Neutrophils from LXR treated rodents showed decreased chemotaxis in

response to the chemokine interleukin 8 (IL-8). Expanding on these results, a

study by Smoak et. al. again demonstrated that administration of T09 to mice for

5 days lead to robust antagonism of LPS or *Escherichia coli* mediated pulmonary

inflammation, but left LXR ligand treated mice with significantly decreased

pulmonary neutrophilia and a significantly increased susceptibility to infection by

Klebsiella pneumoniae compared to vehicle treated control mice 24 and 48 h post

infection (30). Moreover, T09 treated mice showed increased mortality post-

infection compared to vehicle treated controls. However, these studies did not

demonstrate whether the negative effect of LXR activation on pulmonary

immunity is related to inflammation or whether these observations where a

secondary effect of LXR mediated cholesterol modulation. Closer examination of

the impact of LXR mediated cholesterol metabolism on pulmonary immunity may

be warranted in light of studies demonstrating that loss of lung expression of

ABCA1 or ABCG1 negatively alters pulmonary function, alveolar macrophage

immune response and potentially impairing pulmonary host immunity (31, 32).

Nevertheless, it is still unclear what role (negative or positive) LXR

activation has on innate immunity. Further complicating the issue is data

demonstrating that loss of basal LXR gene expression negatively impacts the

innate immune response. Valledor et. al demonstrated that LXR stimulated bone-

marrow-derived macrophages (BMDM) isolated from LXRα/β -/- null mice are at

increased risk for apoptosis from infection by *Bacillus anthracis*, *Escherichia*

coli, and *Salmonella typhimurium* (33). BMDM from LXRα/β -/- null mice had altered expression of genes directly involved in modulating macrophage survival including pro-apoptotic effectors caspases 1, 4, 7 and 12 and Dnase 113, and anti-apoptotic genes AIM/CT2, Bcl-XL, and Birc1a (33). Loss of LXRα or LXRβ expression in mice also increases the total burden of infection by intracellular *Listeria Monocytogenes* (LM) and *Mycobacterium tuberculosis* (TB) compared to WT mice and impairs macrophages clearance and phagocytosis of LM (33). Moreover, peritoneal macrophages (PM) isolated from LXRα/β null mice are less resistant to anti-apoptotic factors secreted by LM due to loss of expression of the anti-apoptotic gene SPα (34). SPα is a LXR regulated gene in macrophages and prevents macrophages apoptosis during LM infection (34). An expansive model of the cross-talk between TLR3/TLR4 and LXR in regulating cholesterol metabolism and inflammation is proposed in figure 4.
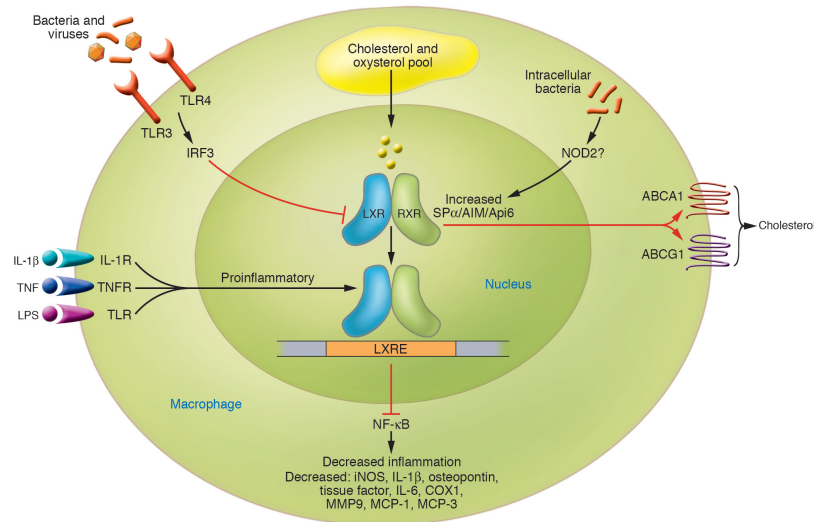
Figure 4. A model for cross talk between cholesterol metabolism and the innate immune response. Source: Clin Invest. 2006 Mar; 116(3):607-14.

Liver X Receptors and Disruption of Lipid Raft Signaling

LXR-ABCA1 mediated efflux of free cholesterol from macrophages has been vigorously studied, but broad knowledge of the mechanics involved in the active transport of free cholesterol across the plasma membrane to circulating lipoprotein particles is still growing. However there is consensus that during early steps of cholesterol efflux, ABCA1 facilitates the "flipping" of cholesterol and other phospholipids from the inner to the outer membrane leaflet in a process involving microdomains known as lipid rafts (35). Lipid rafts domains are cholesterol rich, plasma membrane microdomains that contain a comparatively higher concentration of neutral lipids (up to 50% higher) such as cholesterol and saturated phospholipids, glycosphingolipids and sphingomyelin (36). In macrophages and other metabolically active cells, there are a number of intracellular and plasma membrane kinetic pools of cholesterol which are segregated for efflux by ABCA1, and some data suggests that the largest and most active pool of membrane cholesterol is derived from lipid rafts domains (37).

Lipid rafts were first identified in electron micrographs as nanoscale invaginations in the plasma membrane (Fig 5A). Cholesterol and the saturated acyl chains of the phospholipids with these domains form strong hydrogen bonds and permit formation of condensed protein lipid microdomains that appear as "rafts" floating within the more loosely packed lipid bi-layer (38, 39). Rafts and raft proteins have been characterized by their propensity to resist extraction from membrane using conventional detergents such as triton-X 100, coining the term detergent resistant membrane fractions (DRMs) (38). Visualization of lipid raft in

living cells has been aided by fluorescent labeling of raft associated protein ganglioside m1 (Fig 5B) and fluorescence resonance energy transfer (FRET). From these techniques, rafts have been visualized as 50-70 nm structures which continually come together and dissociate with other raft domains as transitory membrane structures (38, 39). The presence of cholesterol's rigid ring structure within these domains increases "liquid order" and is a vital part of maintaining raft integrity, as disruption of membrane cholesterol diminishes the continuity of raft: raft membrane interactions (38-40).

Functionally, raft domains permit rapid, lateral co-localization of receptors with other co-receptors, kinases and other signaling machinery in complex referred to as a raft signalosome (40). Signalosomes form larger, more stable signaling platforms which facilitate the amplification of signal transduction from scattered membrane receptors (38-40). An example of a raft associated receptor is T cell antigen receptor (TCR), which, along with its co-activator CD-3, rapidly move into lipid raft domains upon binding of a major histocompatibility complex (MHC) from an antigen presenting cell (APC), where SRC protein tyrosine kinases (LCK and FYN) phosphorylate the TCR-associated CD3 complex and amplify signal transduction and T cell mediated adaptive immunity (41) (Fig 5C). Other examples of membrane receptors associated with raft domains include Immunoglobulin E (IgE) signaling, chemokine receptor CXC-4, (CXCR4), C-C chemokine receptor type 5 (CCR5), epidermal growth factor receptor (EGFR) and interleukin-6 receptor (IL-6R) (42). The signal transduction and protein trafficking occurring in cholesterol rich raft domains are not only relevant to

normal cellular physiology, but have been shown to be constitutively active in a number of transformed cells (36, 43-45).
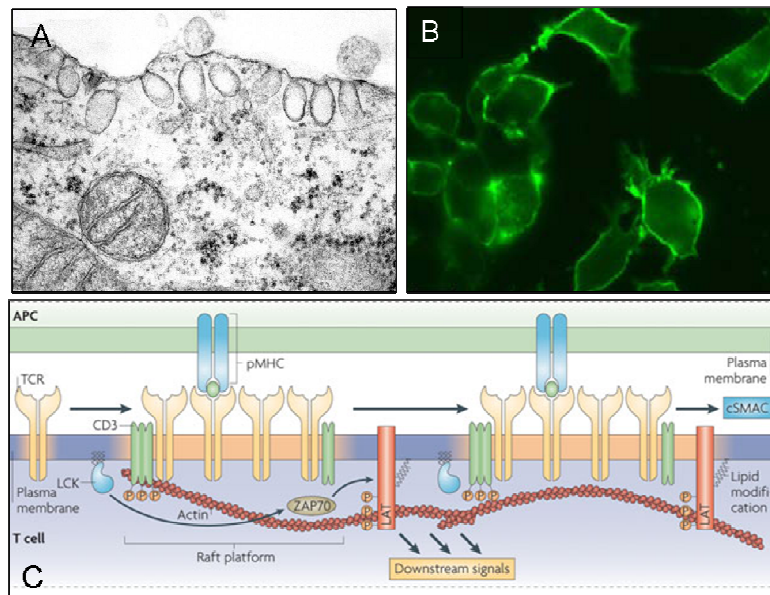
Figure 5. Lipid Rafts: A) Lipid rafts viewed under an electron microscope. B) Fluorescent micrograph of lipid raft domains. C) T-Cell receptor (TCR) activation model. Upon ligand activation, individual TCRs mobilize into larger signaling assemblies within lipid raft domains. Sources A, B: Molecular Biology of the Cell. 4th edition. Alberts B, Johnson A, Lewis J, et al.New York: Garland Science; 2002. C: Nature Reviews Molecular Cell Biology. 2010;11, 688-99.

Studies have demonstrated that LXR-ABCA1 mediated efflux lowers raft cholesterol, alters membrane dynamics and interactions of raft domains leading suppression of raft dependant receptor assembly and signal transduction (45-49). A profound example of this has been demonstrated in studies of human immuno-deficiency virus 1 (HIV-1). HIV-1 infection of T cells involves a receptor complex formed by CD4 and a lipid raft associated chemokine receptors CCR5 or CXCR4 (45-47). It was recently demonstrated that the synthetic LXR ligand T09 impairs CCR4/CXCR4 raft domain assembly with CD4 and HIV-1 entry into T-cells through its upregulation of LXR-ABCA1 mediated cholesterol efflux (48).

LXR-ABCA1 mediated cholesterol efflux has also been shown to prevent lateral assembly of TLR4 into raft domains and signal transduction to the pro-inflammatory MyD88 pathway (49). Numerous studies have demonstrated that administration of cholesterol binding agents can oppose a number of disease specific raft signaling cascades (45-49). However the discovery of that LXR-ABCA1 may serve as an endogenous negative regulator of raft signaling introduces the potential to exploit an endogenous pathway to oppose raft signal transduction that is relevant to disease or other pathological conditions.

There are two models which are thought to most closely predict the sequential order how ABCA1 disrupts cholesterol from lipid raft domains. One model asserts (Fig 6A), that ABCA1 exists on a transition region of raft domains and through its ATPase function, generates cholesterol rich export domains by lateral translocation of cholesterol from lipid raft domains to adjacent non-raft domains (35). A second model, (Fig 6B), proposes that ABCA1 effluxes free cholesterol (FC) and phospholipids (PL) in two steps. ABCA1 first interacts with circulating apo A-I particles which undergo conformational changes permitting removal free cholesterol from adjacent lipid raft domains (35).
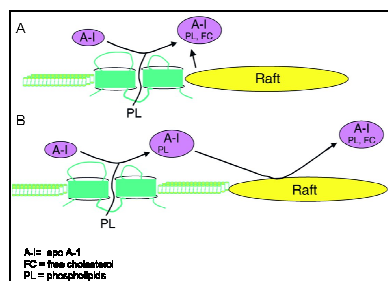
Figure 6. Two models for ABCA1 disruption of Lipid Raft Domain Cholesterol. Source: J.Clin Invest. 2002; 7:899-904.

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As modulators of lipid raft cholesterol levels, LXRs and ABCA1 represent a vital tool in the examination of raft: raft transient interactions and mobilization of membrane receptors into these domains during signal transduction. Capitalizing on the raft disrupting effects of cholesterol efflux may also reveal that the partitioning of membrane cholesterol is equally important as absolute cholesterol levels are in the pathogenesis of disease. This would make LXRs and ABCA1 an attractive clinical target for a number of diseases as our current understanding of the specific role that membrane lipid microdomains have in the etiology of disease increases.

Prostate Cancer, Cholesterol and Liver X Receptors

In recent years a number of large epidemiological studies have demonstrated an association between serum cholesterol levels and risk for advanced PCa (50-54). Prostate tumors, compared to other tumor types, appear to be specifically sensitive to growth inhibitory effect of either dietary or pharmacological cholesterol lowering (50, 53). Cholesterol levels are comparatively higher in normal prostatic tissue than other organs, which may partly explain why prostatic tumors are prone to accumulate cholesterol and why growth of PCa tumors are highly sensitive to cholesterol lowering agents (60,54). The accumulation of cholesterol in prostate and other cancers might reflect metabolic changes in transformed cells which support unchecked cell growth. The current appreciation of cholesterol as promoter of advanced PCa growth provides researchers the rationale to study the cholesterol metabolic pathways in transformed cells as novel targets for therapy. Statin therapy is associated with a

decreased risk for advanced PCa and experimental evidence suggests that this effect is associated with its cholesterol lowering properties (54). As a vital part of the cellular axis for regulation of cellular cholesterol levels LXRs also have been demonstrated to possess anti-cancer properties in models of PCa (54- 56). Studies have demonstrated that oxysterols and the synthetic LXR ligand T0901317 can inhibit growth of prostate carcinoma cells in culture and repress progression of androgen dependent tumor xenografts to a more aggressive androgen independent phenotype (54-56). The ability of LXR to inhibit PCa tumors to adopt a refractive, hormone independent phenotype experimentally is in line with population data demonstrating a specific relationship with cholesterol and advanced PCa and a cholesterol steroid axis in advanced castration resistance PCa (54- 56). A recent demonstrated that LXR ligands can reduce AKT mediated signal transduction in cholesterol rich membrane raft domains in LNCaP cells *in vivo* (57). There is growing evidence that LXR ligands can modulate growth and cancer related signalling in cancer others than prostate (58-59), however little if any information on the effects of LXRs on cholesterol metabolism in transformed cell lines has been extensively studied. Further characterization of LXR biology in transformed cell lines will contribute the current knowledge gap on this topic, and more importantly, understanding the cholesterol regulatory axis by LXR in transformed cells is vital given the current belief that serum and intra-tumor cholesterol levels positively affect tumor growth (50, 59).

Anti-Prostate Cancer and Lipid Lowering Properties of Soy

A number of population studies in East Asian males have found a correlation between consumption of soy derived foods and a reduced risk for developing prostate cancer (PCa) and other maladies (61-65). Isoflavones are a class of compounds found at high concentrations in soy derived foods, and are believed to be responsible for their anti-PCa properties (61-65). In some cohorts isoflavones can be detected a micromolar concentration in plasma, urine and prostatic fluid of Asian males consuming a soy rich diet (61-65). The most commonly found isoflavones found in soya are genistein, daidzein and glycitein (61-65). Equol, a metabolite of daidzein also found in biological specimens of individuals with high soy consumption (63). Genistein, as the most abundant isoflavone in soy, has been shown to be a robust inhibitor of protein tyrosine kinase activity, which may inhibit cancer cell proliferation and increase apoptosis (66). Animal and *in vitro* studies have identified a number of anti-PCa properties of isoflavones including, tyrosine kinase inhibition, modulators of angiogenesis and apoptosis (66, 67). Work in this laboratory has demonstrated that some isoflavones can specifically inhibit androgen receptor (AR) mediator signaling in hormone responsive LNCaP cells (66). Androgenic hormones such as dihydrotestosterone (DHT) signal their AR and are positively associated with PCA risk and progression (67). Isoflavones can retard the growth of PCa tumors experimentally, however doses experimentally administered animals could be 10 times higher than the equivalent for human consumption (61-65). Epidemiological evidence is inconsistent as to whether these properties of soy or isoflavones result

in PCa prevention in individuals with high soy consumption or to healthy men without evidence of clinical PCa (65).

In addition to the anti-PCa properties of soy isoflavones, these compounds have also been reported to posses anti-atherogenic and cholesterol lowering abilities (68-71). Animal and human studies have demonstrated that consumption of soy and soy isoflavones can reduce risk factors for cardiovascular disease including lowering low density lipoprotein (LDL) cholesterol levels, increasing high density lipoprotein (HDL) cholesterol and increase HDL/LDL cholesterol ratios (68-71). As with isoflavones and PCa, the relationship between isoflavones and cholesterol modulation are controversial (68-71). Nevertheless, the cholesterol lowering properties of soy isoflavones may provide a new paradigm to analyze their anti-PCa properties as new data suggests that cholesterol is also involved in prostate carcinogenesis (68-71).

Pulmonary Immunity, Cholesterol and Liver X Receptors

LXR is also a major pillar in the regulation of the innate immune response of pulmonary alveolar macrophages (AMs). In the lungs, LXR is expressed not only by AMs, but a number of other immune cell types including, dendritic cells, neutrophils, and type 2 alveolar cells (72). AMs are found in the alveolar spaces of the lungs and function to destroy invading bacteria or other elicitors of the innate immunity (72). The binding of bacteria, debris or immunogenic elicitors trigger AMs to engulf bacteria or foreign particles and destroyed them with the aid of peroxides and other reactive oxygen specifies (72). Activation also causes AMs to secrete pro-inflammatory cytokines and chemokines such as IL-1β, TNF-

α, IL-6 and MCP-1 which stimulate the infiltration of other inflammatory cells such as monocytes, eosinophils, neutrophils, B cells, and T cells into the lungs where they orchestrate a collective immune response (72). AMs aid in resolution of the pulmonary inflammatory response by clearing cellular debris, apoptotic immune cells and secreting anti-inflammatory mediators (72). Under circumstances of acute pulmonary infection, the inflammatory response subsides once the stimulus is removed (72). However, there are circumstances where pulmonary and systemic inflammation is chronic. Some causes are persistence of immuno resistance pathogens, continued inhalation of foreign particles, autoimmune disorders such as pulmonary fibrosis and morbid obesity (73, 74). The danger in unresolved chronic pulmonary or systemic inflammation is the onset of continuous tissue destruction and repair cycles which further damage tissue and may contribute to altered pulmonary and systemic immunity and metabolic disorders such as insulin resistance and type 2 diabetes (73). LXRs oppose TLR4/NF-kappaB mediated release of immunogenic factors from both peritoneal and AMs, such as IL-1β and TNF-α (24). These inflammatory mediators are essential to resolution of infection as they function to propel the acute phase inflammation forward. However, in light of the current paradigm that unchecked, chronic inflammation is associated with a number of metabolic diseases, the anti-inflammatory properties of LXRs propel them to the center of a debate. This relates to the role, if any, that LXRs have in stunting the inflammatory response to diminish chromic inflammation, but not to the degree which can compromise host pulmonary immunity. The data is clear that the net

effect of the short term by LXR activation during pulmonary infection is anti-inflammatory, but may compromise host immunity in the short term (72-73). Therefore, the beneficial effects of LXR activation on the innate inflammatory response may depend on whether it is in the context of the chronic inflammation or acute host infection. Examining the balance between these two extremes is vital to understanding how LXRs may fit into therapeutic approaches to mitigate inflammatory related conditions.

A second component of pulmonary immunity is the role of LXR in regulating cholesterol metabolism (72-74). An important development in our understanding of the etiology of atherosclerosis is that as a molecule, cholesterol is proinflammatory (76). The basis of this view comes from studies demonstrating that monocytes recruited and activated at endothelial cell injury engulf modified cholesterol molecules via their scavenger receptors CD36 and SCARB1 which elicits an acute inflammatory response in macrophages marked by increased secretion of inflammatory cytokines TNF-α, IL-1β and IL-6 (76, 18). Loss of CD36 auto regulation leads to continued cholesterol phagocytosis and foam cell formation (18). Experimentally, impaired LXR mediated cholesterol efflux leads to foamy macrophages which are associated with hyper secretion of inflammatory mediators and impaired phagoctytic function (77). There is now evidence that hypercholesterolemia can have a similar effect in AMs (72-75). As in peripheral macrophages, activation of LXR in AMs results in increased expression of ABCA1, ABCG1 and cholesterol efflux (72-75). However loss of cholesterol efflux capacity in mice genetically deficient in either ABCA1 or ABCG1 leads to

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abnormal pulmonary function and formation of large populations of lipid-laden AMs (72-77). Moreover mice lacking ABCG1 expression have chronic pulmonary inflammation marked by increased mRNA expression of TNF-α, Mip-1, IL-1β (72-77). Similarly, LXRα null mice show pulmonary lipidosis, inflammation and increased risk for pulmonary infection (72-77).

Collectively these studies suggest that the changes which peritoneal macrophages (PM) undergo as results of hypercholesterolemia and foam cell formation may also occur in AMs exposed to high serum cholesterol levels. As in PM foam cells, AM foam cells may exhibit an altered immune response characterized by a hypersensitivity to LPS. The risk of a heightened inflammatory response is that seen under condition of chronic inflammation where heightened tissue destruction-repair cycles ultimately impair pulmonary function and increase host susceptibility to additional infection or pulmonary insult. Therefore it should be examined whether dietary hypercholesterolemia can lead to foamy AMs and subsequent changes in response to LPS. Furthermore, LXR activation in these cells should be tested for any anti-inflammatory capacity. Such studies would be the first characterization of dietary affects on pulmonary function and may provide the basis to develop LXR ligands as anti-inflammatory agents in persons with chronic pulmonary inflammation.

Significance of the Current Project to Human Health

Chapter 2: Studies of Liver X Receptors and Prostate Cancer

The current body of data demonstrating an association between cholesterol and advanced PCa warrants the examination of cholesterol modulating therapies

that could decrease the risk for advanced PCa. Population studies have already demonstrated that long term use of the class of cholesterol lowering agents known as Statins (HMG-CoA reductase inhibitors) among men is associated with a decreased risk for advanced PCa and experimental studies suggest that the cholesterol lowering effect of statins specifically targets lipid raft domain cholesterol and associated membrane signaling (61, 62, 68). Lipid raft signaling represents a potential target in cancer and other pathologies such as HIV-1, where membrane raft signaling and integrity is linked to membrane cholesterol levels and propagation of disease specific signaling (44, 45).

In light of data demonstrating that LXR ligands can oppose PCa progression and disrupt lipid raft domains in macrophages and T-cells, chapter 2 of this project will specifically examine whether LXR ligands can modulate cholesterol metabolism in PCa cells and lead to alterations in lipid raft domain cholesterol levels. I hypothesize that LXR activation in transformed PCa epithelial cells will results in robust regulation of genes central to LXR mediated cholesterol efflux, ABCA1 and ABCG1, and that LXR-ABCA1 mediated cholesterol efflux will alter intra-cellular cholesterol levels and modulate lipid raft domains.

Data from this project could provide novel understanding of whether LXR ligands could be developed as cholesterol and lipid raft modulating agents in studies of PCa. This data would contribute to the growing body of data examining the role that cholesterol has in progression of prostate and other solid tumors.

Chapter 3: Studies of Isoflavones as LXR Modulating Agents

Studies of soy and soy isoflavones have been examined for more than a decade for their cholesterol lowering and anti-PCa properties (76). However there is a paucity of data examining whether the cholesterol lowering properties of isoflavones involves interactions with the LXR axis and whether this can occur in PCa cells. This is a vital question because of the current appreciation for the role of cholesterol in promoting PCa and for the evidence suggesting that isoflavones possess anti-PCa properties in humans (61, 72). Data from this project could provide the basis to further study isoflavones for their LXR modulating properties and for identifying dietary compounds which may interact with a particular LXR isoform. There is some evidence that soy isoflavones can increase expression of LXR responsive genes in liver (82), therefore I hypothesize that some isoflavones, which have been shown to possess anti-PCa properties, will increase expression of LXR responsive genes in PCa cells. If so, I hypothesize that isoflavones will be able to increase LXR-mediated cholesterol efflux in PCa cells.

Chapter 3 will specifically examine whether soy derived isoflavones can modulate gene expression of LXR responsive genes ABCA1 and ABCG1 and LXR mediated cholesterol efflux in PCa cells. Data from this project could provide important understanding of how dietary isoflavones might discharge their anti-cancer effect in light of the positive relationship between cholesterol and advanced PCa (61). Moreover, this project will seek to determine which LXR isoform may be involved in regulation of LXR responsive genes. This is

important as a number of studies have attempted to identify ligands which can specifically modulate ABCA1 and ABCG1 through the LXRβ isoform (83).

Chapter 4: Studies Examining the Effects of a HF Diet and Probiotics on the LXR Axis and Inflammation in Alveolar Macrophages

HF diets can alter pulmonary cholesterol levels and pulmonary function, but it is unclear if they can modulate pulmonary inflammatory response mediated by AM (84-86). Experimental evidence also demonstrates that AM that lack the capacity to efflux excess cholesterol via LXR accumulate excess cholesterol rich lipid droplets and take on a foam cell macrophage phenotype (31,32). Foam cell AM exhibit an exaggerated immune response and may provide a link between hypercholesterolemia and altered pulmonary inflammatory response (31, 32). It is unclear, however, if wild type animals on a HF diet also have evidence of AM foam cell formation and whether this associated with an altered immune response.

Therapies aimed at modulating inflammation and cholesterol metabolism in the lungs might ameliorate or diminish inflammatory and lipid related pathologies of the lung (30-32). Moreover, identifying novel therapeutic targets of lipid metabolism and inflammation may also be beneficial in cases of obesity related pulmonary lipidosis that is to poor lung compliance and increased risk for respiratory infection in some adults (88, 89). LXRs are ideal therapeutic targets for studying lipid and inflammatory related conditions due to their cholesterol lowering and anti-inflammatory properties (24). Another therapy actively studied for potential anti-inflammatory and lipid lowering properties in humans is consumption of probiotic rich foods (90-93). Probiotics are lactic acid producing

bacteria that have been demonstrated to result in a broad anti-inflammatory effect of gut mucosal mediated immunity (90-93). There is also evidence that probiotic can have systemic anti-inflammatory effects (90-93). There is little information however on whether probiotics can benefit pulmonary immunity and lipid metabolism. As with LXRs, some probiotics strains posses anti-inflammatory and lipid lowering properties (90-93) therefore also represent a valuable therapeutic target for adults with pathologies involving chronic inflammation such as asthma or dyslipidemia related to obesity, lifestyle or genetic factors.

Chapter 4 will examine whether a HF diet can lead to AM foam cells formation and affect the inflammatory response in AM. This chapter will also examine whether LXR activation can diminished the inflammatory response in stimulated AM. Moreover, this project will also examine whether the probiotic bacteria *L. casei* is associated with any anti-inflammatory or lipid lowering effects in AM. Lastly, this project will also examine whether the endotoxin LPS, can oppose LXR transcription in AM. This has been demonstrated to be a mechanism through which pathogens can alter macrophage cholesterol metabolism leading to a diminished immune response. There has been no examination of whether probiotics can modulate the LPS antagonism of LXR in AM.

I hypothesize that HF feeding will lead to evidence of foam cells formation in AM marked by CE accumulation in AM. I also hypothesize that LXR activation will result in a broad anti-inflammatory effect in stimulated AM. Lastly, I hypothesize that, as it has been shown in macrophages, that LPS will antagonize LXR transcriptional axis in AM.

Data from this project can provide a better understanding of the role that obesogenic diets, hypercholesterolemia and LXRs have on pulmonary lipid metabolism and immunity. Moreover, data from this project will increase understanding of the potential benefits of dietary probiotics on host immunity and lipid metabolism and possibly justify further examination of LXRs and probiotic bacteria as beneficial modulators of pulmonary lipid metabolism and inflammation.

Chapter 2: Characterization of Liver X Receptors in Prostate Cancer Epithelial Cells

INTRODUCTION

Experimental Rationale for LXR Studies in Prostate Cancer Cells

According to the American Cancer Society, advanced and metastatic prostate cancer (PCa) remain the most prominent cancer type and the second leading cause of all cancer deaths among males in the United States (94). Although there are no established risk factors for PCa, epidemiological and experimental studies have demonstrated a specific association between elevated cholesterol levels and high grade and advanced PCa (61-64). A number of large epidemiological studies demonstrated that men who maintain healthy cholesterol profiles, either through diet or pharmacological use of cholesterol lowering agent 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA) reductase inhibitors (statins), have lower risk for developing advanced PCa (62-64). The mechanisms surrounding the association between serum cholesterol and PCa are not well understood, but there is evidence for more than 70 years that prostate and other solid tumors accumulate higher levels of neutral lipids such as cholesterol and triglycerides compared prostatic tissue from normal men and men with benign prostatic hyperplasia (61).

Recent evidence demonstrated that cholesterol accumulation in PCa tumors increases the content of plasma membrane cholesterol rich domains known as lipid rafts (94, 95). Rafts are cholesterol rich lipid platforms in the

plasma membrane involved in amplification of extracellular signal transduction of numerous biological pathways (36). Accumulation of cholesterol in PCa tumors may be involved in a number of metabolic pathways required for unchecked growth during cancer progression and recent evidence demonstrates that statins specifically lower lipid raft cholesterol concentration and impair signal transduction relevant to PCa progression (97). Experimental studies of how cells regulate cellular and raft cholesterol levels may provide new insight into the role of cholesterol in PCa and other tumors.

Liver X receptors (LXRs: LXRα and LXRβ) are transcription factors that regulate whole body cholesterol levels through transcriptional control of a number of key cholesterol metabolizing proteins and enzymes (8). Expression patterns of LXRα and LXRβ demonstrate that LXRα is primarily expressed in liver, adipose, and enterocytes where LXRβ is expressed ubiquitously (8). The natural ligands for LXRα and LXRβ are oxygenated cholesterol intermediates such as 25-hydroxycholesterol (25HC) and 24(S), 25- epoxycholesterol (24,25EC) known as oxysterols, which are derived from both cholesterol catabolism and synthesis, respectively (8). A highly specific synthetic LXR ligand T09 has also been developed (98).

In the liver, activation of LXRα by oxysterols results in the catabolism of cholesterol by increasing expression of the rate limiting enzyme in bile acid synthesis, CYP3A4 (CYP7A1 in mice) and hepatic-biliary duct transporters ABCG5 and ABCG8 (14). LXRα activation also increases the expression of ABCA1, which is involved in hepatic export of circulating cholesterol acceptor

protein apo A-I (14). In macrophages and peripheral tissue, oxysterol activation of LXRα or LXRβ increases the mRNA and protein expression of reverse cholesterol transporters ABCA1, ABCG1 and the lipid acceptor apo E (14). Excessive cellular cholesterol, derived from either *de novo* synthesis or uptake from circulating low density lipoproteins (LDL), is removed from macrophages and peripheral cells by either non-energy dependant mechanisms or by energy dependant ABCA1, ABCG1 and circulating lipoprotein particles in a process called reverse cholesterol transport (RCT) that is chiefly regulated by LXRs (14). RCT is the major pathway involved in removal of excess cholesterol from peripheral tissue and has been shown to involve removal of cholesterol specifically from lipid raft domains.

Natural and synthetic LXR ligands also possess an anti-cancer effect on PCa and other cancers types *in vitro* and in animal studies, making LXR a potentially new therapeutic target in PCa and other tumors where plasma and intra-tumoral cholesterol levels are positively associated with advanced disease. Therefore studies examining whether RCT involving LXR and ABCA1 is functional in transformed PCa cells and whether RCT can alter cholesterol content of PCa cells and their lipid raft domains are warranted.

In light of the heterogeneity between LXRα and LXRβ signaling in other cell types, and the link between tumor cholesterol levels and advanced PCa, studies from this chapter sought to identify the relevant LXR isotypes and ligand behavior of T09, 25HC, and 24,25EC in the regulation of *ABCA1* and *ABCG1* mRNA in two models of transformed prostatic epithelial cells - LNCaP and PC-3.

This study also sought to explore whether LXR-ABCA1 driven RCT is functional in PCa cells and whether cholesterol efflux in these cells results in changes to cellular free cholesterol concentrations and to lipid raft domains.

Specific Hypotheses Tested

Studies will be performed on androgen responsive LNCaP and non-androgen responsive PC-3 prostate cancer epithelial cells to examine the following hypotheses relating to LXR biology:

a) To test the hypothesis that *ABCG1* and *LXRβ* mRNA levels are higher than *ABCA1* and *LXRα* respectively in both LNCaP and PC-3.

b) To test the hypothesis that LXRβ and not LXRα is the primary regulator of mRNA levels of the cholesterol transporters *ABCA1* and *ABCG1* in LNCaP and PC-3 cells.

c) To test the hypothesis that LXR mediated cholesterol efflux is functional in PC-3 cells.

d) To test the hypothesis that LXR mediated cholesterol efflux alters intracellular free cholesterol or cholesteryl-esters in PCa cells.

e) To test the hypothesis that LXR mediated cholesterol efflux can alter cholesterol rich plasma membrane raft domains in PCa cells.

ABSTRACT

Recent evidence suggests that the liver X receptor (LXR) is a potential anti-cancer target in prostate carcinoma. There is little characterization, however, of which of the two LXR isoforms, LXRα or LXRβ, regulates the LXR-responsive genes ATP-binding cassette sub-family members A 1 (*ABCA1*) and G 1 (*ABCG1*) in transformed prostatic epithelial cells. In this study, small interfering RNA (siRNA) was used to determine whether LXRα or LXRβ is involved in regulating *ABCA1* and *ABCG1* mRNA expression in LNCaP and PC-3 cells. Treatment of both cell lines with the synthetic LXR ligand T09 and oxysterols: 25-hydroxycholesterol (25HC) and 24(S), 25-epoxycholesterol (24,25EC), resulted in more than a 10-fold increase of *ABCA1* and *ABCG1* mRNA expression. Transfection of LNCaP cells with siRNA against either LXRβ or LXRα failed to inhibit T09 and 25HC-mediated increase of *ABCA1* mRNA. siRNA silencing of LXRβ did, however, inhibit *ABCA1* mRNA expression in 24, 25EC-treated LNCaP cells. In contrast, LXRß siRNA inhibited T09, 25HC, and 24, 25EC induction of *ABCA1* mRNA in PC-3 cells and *ABCG1* mRNA in both LNCaP and PC-3 cells. Additional experiments revealed that T09 and 25HC induction of *ABCA1* mRNA expression was significantly inhibited by the p38 stress kinase antagonist SB203580 and PKA inhibitor H89. However, siRNA studies against p38α/β and PKA revealed that p38 does not appear to be involved in T09 mediated regulation of *ABCA1* mRNA in LNCaP cells, but PKA is involved in T09 regulation of *ABCA1* mRNA in PC-3 cells. Treatment of PC-3 cells with 1 μM of T09 resulted in cholesterol efflux to apo A-I and a decrease in

cellular free cholesterol levels. This effect was not observed in LNCaP cells under the same efflux conditions. Analysis of fluorescence images of PC-3 lipid raft domains revealed that LXR mediated RCT resulted in a significant decrease in cholesterol rich lipid raft domains only in the presence of the cholesterol acceptor apo A-I.

MATERIALS AND METHODS

Chemicals and Reagents

Dharmacon ON-TARGET*plus SMART*pool siRNA reagents targeting LXRα (Dharmacon Catalog # L-003413-00) or LXRβ (NM_007121) (Dharmacon Catalog # L-003412-00) , androgen receptor (AR) (Dharmacon Catalog # L-003400), MAPK11(p38β) (Dharmacon Catalog # L-003972), MAPK14(p38α)) (Dharmacon Catalog # L-003512), and PKA(cyclic AMP-dependent Protein Kinase A catalytic subunit a) (Dharmacon Catalog # L-004649) were purchased from Thermo Fisher Scientific (Lafayette, CO). HiPerFect Transfection Reagent was purchased from Qiagen (Santa Clarita, CA). The selective inhibitors for p38α/β (SB203580), c-Jun N-terminal kinase (JNK) (SP600125), extracellular signal-regulated kinases 1 and 2 (ERK1/2) (PD98059), cAMP dependent-protein kinase A (PKA) (H89), the LXR ligand 24(S),25-epoxycholesterol, and the synthetic pregnane x receptor (PXR) ligand (SR12813), were all purchased from BIOMOL International (Plymouth Meeting, PA). The synthetic LXR ligand T09 was purchased from Cayman Chemical Company, (Ann Arbor, MI). 25-hydroxycholesterol, dimethylsulfoxide (DMSO) and, human purified apo A-I were purchased from Sigma Chemical Co (St. Louis, MO). [1α,2α (n)-H^3] tritium

labeled cholesterol was purchased from Perkin Elmer (Waltham, MA). Amplex red cholesterol analysis kit and Vybrant ® Lipid Raft Labeling Kit were purchased from Invitrogen (Carlsbad, CA).

Cell Culture

The human prostate cancer cell lines LNCaP and PC-3 were purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 medium Invitrogen (Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 1% glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA) (culture medium). Cells were incubated in the presence of 5% carbon dioxide and air at 37 °C.

RNA Isolation and Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT- PCR)

For gene expression experiments, LNCaP and PC-3 cells were seeded in 6 well plates at a density of 0.5 x 10^6 cells/well and grown for 24 h in culture medium, then treated with various concentrations (0.1-10 μM) of 25HC, 24,25EC, T09, or vehicle (DMSO 0.05% v/v). After termination of experiments total RNA was isolated using the TRIzol® reagent (Invitrogen, Carlsbad, CA), and reverse transcribed to complementary DNA (cDNA) using StrataScript® First Strand cDNA Synthesis Kit (Stratagene, La Jolla, CA). Real time PCR was carried out using a TaqMan Universal PCR Master Mix on an ABI Prism 7000 and TaqMan Fast Master Mix on a 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The TaqMan probes and primers were purchased from Applied

Biosystems using inventoried TaqMan gene expression assays: *LXRα*, (assay ID: Hs00172885_m1), *LXRβ* (assay ID: Hs00173195_m1), *ABCG1* (assay ID: Hs00245154_m1), *ABCA1* (assay ID: Hs01059122_m1), *CYP3A4* (assay ID: Hs01546612), *CYP3A5* (assay ID: Hs00241417), *MDR1* (assay ID: Hs00184500), *PPARα* (assay ID: Hs00947537_m1), *PPARγ* (assay ID: Hs01115513_m1), *CYP26A1* (assay ID: Hs00175627_m1), *VDR* (assay ID: Hs01045840_m1), *HMGCR* (assay ID: Hs00168352_m1), *SQS*(assay ID: Hs00926054_m1), LDLR (assay ID: Hs00181192_m1), *SREBP1*(assay ID: Hs01088691_m1), *SREBP2* (assay ID:Hs01081784_m1), *CYP27A1* (assay ID: Hs01026016_m1), CH25H (assay ID:Hs02379634_s1), human glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* (assay ID: Hs99999905) was used as an endogenous control for all gene expression except for basal mRNA quantitation in Figure 2, where *18S ribosomal RNA (18S rRNA)* (assay ID: Hs99999901) was used as an additional control. The following amplification parameters were used for gene expression on the ABI 7000: 50°C for 2 min, 95°C for 10 min, followed by 46 cycles of amplification at 95°C for 15 sec and 60°C for 1 min and on the ABI 7900: 95°C 20 sec followed by 46 cycles of amplification at 95°C for 1 sec and 60°C for 20 sec. Quantitation of mRNA fold changes were derived using the delta threshold cycle (ΔCt) method (84).

Quantitation of Basal mRNA levels

The largest ΔCt value of one gene was assigned as the control and normalized the lower ΔCt value of the second gene to determine a comparative

ΔΔCt value. *LXRβ* ΔCt values were normalized to *LXRα* ΔCt values (control), and *ABCG1* ΔCt values were normalized to *ABCA1* ΔCt values (control).

Quantitation of Comparative Basal mRNA Levels

The higher LNCaP cells ΔCt values of each gene was assigned as the control and normalized the lower ΔCt value of corresponding genes in PC-3 cells to determine a comparative ΔΔCt value. ΔCt values for *LXRα* and *LXRβ* in PC-3 cells were normalized to *LXRα* and *LXRβ* ΔCt values in LNCaP cells and ΔCt values for *ABCA1* and *ABCG1* in PC-3 cells were normalized to ΔCt values for *ABCA1*, *ABCG1* in LNCaP cells.

Cell Transfection with Small Interfering RNA (siRNA)

LNCaP and PC-3 cells were seeded in 6-well plates at a density of 0.2 x 10^6 cells/well and grown for 24 h in culture medium; then cells were switched to serum free media in RPMI-1640 (without phenol red) containing 2.0 mM L-glutamine and 1% penicillin/streptomycin (serum-free culture medium). Cells were transfected with Dharmacon ON-TARGETplus SMARTpool siRNA oligonucleotides using the HiPerFect liposomal transfection reagent (Qiagen) according to the manufacturer's protocol. Preliminary experiments were conducted for each gene target to determine optimal transfection conditions without cytotoxicity. The concentrations of siRNA oligonucleotides used for each target were: 5 nM siRNA oligonucleotides for LXRα, LXRβ and androgen receptor (AR), 100 nM siRNA oligonucleotides for p38α, p38β and 25 nM for cyclic AMP-dependent PKA, catalytic subunit a (PKACa). After 48 h of siRNA

transfection, cells were switched back to culture medium and treated with various ligands as described in Materials and Methods for an additional 48 h.

Kinase Inhibitor Experiments

For kinase inhibition experiments, LNCaP and PC-3 cells were plated in 6-well plates as described above and pre-treated for 60 minutes with 10 μM of one of the following: the p38 MAP kinase inhibitor SB203580, the JNK MAP kinase inhibitor SP600125, the ERK1/2 MAP kinase inhibitor PD98059, the PKA inhibitor H89 or vehicle (DMSO 0.05% v/v). After 60 minutes cell culture media was replaced and cells were stimulated with 5 μM T09, 25HC, or 24,25EC for an additional 24 h.

Cholesterol Efflux Assay

PC-3 cells were plated in 10% fetal bovine serum (FBS) in triplicate and loaded with 1μCi/mL of [^{3}H] labeled cholesterol (Perkin Elmer) for 48 h. After 48 h PC-3 cells were washed thrice with PBS and switched to serum free conditions (RPMI 1640 (phenol free) + 0.2% fatty acid free bovine serum albumin (BSA) w/v). Cells were allowed 2 h to equilibrate cellular cholesterol in serum free conditions, washed thrice with PBS and treated for 48 h with either vehicle (DMSO 0.01% v/v) or 1 μM of the LXR ligand T09. After 48 h PC-3 cells were washed thrice with PBS + 0.2% BSA and incubated with either RPMI + 0.2% BSA plus 20 μg/mL of the cholesterol carrier apo A-I or vehicle (1X PBS) to initiate cholesterol efflux. Aliquots of 100 ul were collected at multiple time points for 24 h, mixed with scintillation liquid and measured for radioactivity on a scintillation counter. After 24 h cells were lysed with RIPA buffer (Thermo) and

protein measured using the bicinchoninic acid (BCA) assay (Thermo) according to the manufactures' protocol. Efflux of vehicle treated cells will be subtracted from efflux of all groups as background and efflux will be expressed as counts per minute (CPM) of radioactivity in the cell culture medium per milligram of cellular protein. Mean differences in efflux between T09 + apo A-I and apo A-I alone will be measured at time points within the linear range.

Gene Expression and Cellular Cholesterol Quantitation During Efflux

To analyze cellular cholesterol profile and gene expression during cholesterol efflux, PC-3 cells were plated under the same conditions as cholesterol efflux experiments except tritium cholesterol loading was excluded. After 48 h of cholesterol loading in 10% FBS, cells were washed and switched to serum free medium as previously described and treated for 48 h. with 1 uM T09. After 48 h cells were washed with PBS and treated with either; vehicle (1X PBS), 5ug/mL for 4 h or 20 μg/mL apo A-I for 4 and 12 h. At time points of interest total cellular cholesterol or total RNA were isolated as described in the materials and methods section above. For total cellular cholesterol, lipids were extracted using the Folch method as previously described (99). Briefly, after termination of experiments cells were lysed with cold RIPA buffer (Thermo) using 1 mL of RIPA per 10^7 cells. Cell homogenates were sonicated at 50% intensity for 30 seconds on ice followed by incubation for 30 minutes on ice. Lipids were then extracted from aliquots of cell homogenates using chloroform: methanol (2:1). Organic phase solvents were evaporate from lipid extracts under nitrogen gas and resuspend in 0.5% v/v Triton X-100 solution in water. Total and free cholesterol were

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determined enzymatically using Amplex red cholesterol quantitation kit (Invitrogen) according to the manufactures protocol. Cellular cholesterol content was normalized to whole cell lysate protein concentrations. Protein was determined using the acid (BCA) assay (Thermo).

Fluorescent Staining of Cholesterol Rich Membrane Domains

After treatments cells were washed three times in 1X PBS and incubated with 1μg/mL alexa flour conjugated cholera toxin subunit B (AF-CT-B) in 1X PBS for 10 minutes at 4 °C. Cells were then washed three times with 1 X PBS followed by incubation with 1 mg/mL anti-cholera toxin subunit B antibody (anti.CT-B) for 10 minutes at 4 °C. Cells were then washed thrice in 1X PBS and then counter stained with the nuclear fluorescent stain Hoechst 33258. Cells were mounted on microscopic slides and rafts visualized by a Nikon TE-2000-S fluorescence microscope. x and y coordinates for 10 random micrograph fields were derived from random numbers. Lipid raft positive cells were identified using a signal to noise ratio cut off of 7 based on the florescence intensity of control cells. Lipid raft positive cells were normalized as percent of total number of nuclei positive cells in each field. All imaged were analyzed using Nikon NIS elements imaging software version 3.2 (Nikon Inc., Hempstead, NY).

Statistics

Statistical analysis of data will be carried out with the GraphPad PRISM 4 program (GraphPad Software, Inc.). Contrasts of group means were computed using one or two factor ANOVA followed by Bonferroni post hoc tests. The unpaired Student's unpaired t test was used to compare experiments between two

groups. Gene expression results is expressed as means $\pm$ SE of comparative fold differences. Mean differences of post-hoc analyses are considered significant when p value is < 0.05.

RESULTS

Specificity of the Synthetic LXR ligand T09 in PCa Cells

To determine the specificity T09 in LNCaP and PC-3 cells, mRNA expression of a panel of NRs and NR responsive genes were measured in LNCaP and PC-3 cells treated with 10μM of T09 for 48 h. Figure 1 demonstrates that treatment of both cell lines with T09 resulted in specific transcriptional increases in LXR target genes *ABCA1* and *ABCG1*, while the mRNA expression of the nuclear receptors *PPARγ*, *VDR* were unchanged compared to vehicle controls (Fig 1). There was a ~80% significant decrease in mRNA expression of *PPARα*, in PC-3 cells treated with T09 compared to vehicle treated controls ($p < 0.01$). mRNA levels for the specific target genes of PXR (*CYP3A4* and *MDR-1*) and RARs (*CYP26A1*) were also unaffected by treatment with pharmacological concentrations of the LXR ligand T09 (Fig 1).

Basal Levels of LXR and Cholesterol Metabolism genes in PCa Cells

PC-3 cells are bone derived metastatic PCa cells and do not rely on steroids for maintenance of their growth program, where LNCaP cells do. PC-3 cells have been characterized as a more aggressive phenotype of PCa compared to LNCaP cells, and as bone metastasized cells, PC-3 cells represent a stage of PCa most difficult to treat in men. Human studies demonstrated that cholesterol levels are linked only to late stage advanced PCa; therefore it was examined whether

PC-3 cells express higher basal levels of LXR and cholesterol metabolism related genes compared to the less aggressive LNCaP cells. Also total levels of free cholesterol and cholesteryl-esters were compared in both cell types.

As shown in Figure 2A and 2B, the data revealed that basal mRNA levels of *LXRβ* were significantly higher than *LXRα* in both LNCaP and PC-3 cells, as were basal levels of *ABCG1* compared to *ABCA1*. Across the cell types, PC-3 cells expressed significantly higher basal levels of all genes with an exceptionally higher expression of *ABCA1* mRNA (Fig. 2C and 2D).

To determine whether PC-3 cells express higher mRNA levels of a number of other key cholesterol metabolism related genes, basal mRNA levels of genes related to cholesterol synthesis and uptake: *HMGR*, *SREBP2*, *FNTA*, *SQS* *LDLR*, triglyceride synthesis: *SREBP1*, and cholesterol degradation *CYP27A1* *CH25H* were determined. Gene expression revealed that PC-3 cells express significantly higher basal mRNA levels of *SREBP1*, *SREBP2*, *HMGR*, *LDLR* and *SQS* compared to LNCaP cells (Fig 2E). Basal levels of two key enzymes involved in cholesterol degradation and genesis of LXR ligands *CYP27A1* and *CH25H* were lower in PC-3 cells compared to LNCaP; however this difference was not significant ($p > 0.05$, Fig 2E).

Cholesterol Profile of PC-3 and LNCaP cells

Analysis of free cholesterol and cholesteryl-esters levels in PC-3 and LNCaP demonstrated that free cholesterol levels were approximately two fold higher in PC-3 cells compared to LNCaP after being cultured for 48 h in 10%

FBS ($p < 0.001$, Fig 2F). Levels of cholesteryl-esters were not detectable in either cell type (Fig 2F).

Concentration Effects of LXR Ligands on *ABCG1* and *ABCA1* mRNA Expression

To measure the concentration effects of synthetic and natural LXR ligands on mRNA expression of *ABCA1* and *ABCG1*, LNCaP and PC-3 cells were treated with increasing concentrations (0.1-10 μM) of T09, 24,25EC, and 25HC for 24 h. In both LNCaP (Fig. 3A and 3B) and PC-3 cells (Fig. 3C and 3D), treatments with T09, 25HC, and 24,25EC resulted in dose responses in *ABCA1* and *ABCG1* mRNA expression. LNCaP cells treated with the synthetic LXR ligand T09 resulted in a ~4 to 20-fold induction of *ABCA1* mRNA compared to ~4 to 10-fold inductions in 24,25EC and in 25HC treated cells (Fig. 3A). *ABCG1* mRNA fold induction in T09 treated LNCaP cells was ~9 to 20-fold compared to ~2 to 8-fold in oxysterol treated cells (Fig. 3B). PC-3 cells treated with increasing concentrations of T09 resulted in a ~12 to 25-fold induction of *ABCA1* mRNA compared to 2 to 7-fold inductions in 24,25EC and 25HC treated cells (Fig. 3C). In a similar manner to *ABCA1*, *ABCG1* mRNA fold induction in T09 treated PC-3 cells was ~8 to 15-fold compared to ~2 to 6-fold in 24,25EC and 25HC treated cells (Fig. 3D). Between PC-3 and LNCaP cells, a significantly stronger *ABCA1* mRNA induction in PC-3 cells specifically within the T09 concentration range of 0.1 and 1.0 μM was observed (~10 to 20-fold in PC-3 vs. ~3 to 5-fold in LNCaP) (Fig. 3A and 3C). In PC-3 cells, the concentration plateau for T09 induction of *ABCA1* mRNA began at 5 μM. In LNCaP cells, however, the dose- dependent

effect of T09 and all oxysterols was linear up to 10 µM. This trend was also observed for LXR ligand induction of *ABCG1* mRNA in LNCaP and PC-3 cells. Fold changes for *ABCG1* mRNA with increasing doses of all LXR ligands were similar in both LNCaP and PC-3 cells (Fig. 3B and 3D).

Temporal Effects of LXR Ligands on ABCG1 and ABCA1 mRNA Expression

Temporal changes in *ABCA1* and *ABCG1* mRNA expression were also determined by treating LNCaP and PC-3 cells with 5 µM of T09, 25HC, and 24,25 EC for 2-24 h. Fig. 4A and 4B show a significantly earlier change in mRNA response of *ABCA1* at 2 h compared to *ABCG1* in LNCaP cells (~3-fold vs. 0-fold). The opposite effect was observed in PC-3 cells, with the change in *ABCG1* mRNA increasing dramatically between 2 and 4 h by almost 10-fold, where *ABCA1* mRNA changes were approximately 5-fold less in the same time frame (Fig. 4C and 4D).

Ligand and LXR Isoform Specific Regulation of *ABCA1* and *ABCG1* mRNA Expression Varies between Cell Types

Given that both LXR isoforms are expressed in prostate cancer cells, experiments were conducted to determine whether LXRα or LXRβ are required for oxysterol- and T09- mediated induction of *ABCA1* and *ABCG1* mRNA using siRNA technology. siRNA transfection conditions resulted in ~70-80% silencing of *LXRα* and *LXRβ* mRNA expression levels in both LNCaP and PC-3 cells. In both cell lines, all LXR ligands significantly increased basal levels of *LXRβ* (Fig.

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5A and 5B, Fig. 6A and 6B), but had no significant effect on *LXRα* mRNA levels. (Fig 5A and 5B, Fig 6A and 6B).

Surprisingly, LNCaP cells transfected with either siRNA against LXRβ or LXRα could not inhibit T09 or 25HC induction of *ABCA1* mRNA (Fig.5C and 6C). Use of siRNA against LXRβ was able to inhibit *ABCA1* mRNA in LNCaP cells treated with 24,25EC only (Fig. 6C). A significant inhibition of *ABCG1* mRNA induction by all LXR ligands in LNCaP cells transfected with LXRβ siRNA was observed (Fig. 6C); however LXRα siRNA had no effect on *ABCG1* mRNA induction by any LXR ligand (Fig. 5C). In PC-3 cells, T09 and oxysterol regulation of *ABCG1* and *ABCA1* mRNA expression was more straightforward. The use of siRNA against LXRß inhibited the mRNA expression of both *ABCG1* and *ABCA1* in PC-3 cells exposed to T09, 25HC, and 24,25EC (Fig. 6D). siRNA against LXRα also had no effect on these targets in PC-3 cells (Fig. 6D).

Involvement of Kinase Signaling in LXR ligand Regulation of *ABCA1* mRNA in LNCaP and PC-3 Cells

In observing that neither LXRα nor LXRß appeared to be involved in T09 or 25HC regulation of *ABCA1* mRNA expression, a number of kinase pathways screened to determine whether T09 or 25HC can regulate *ABCA1* mRNA expression through a non-LXR mediated pathway involving kinase signal transduction. LNCaP and PC-3 cells were pre-treated for 60 minutes with 10 μM of each of the following specific kinase inhibitors: ERK1/2 (PD98059), PKA (H-89), p38α/β (SB203580) or JNK (SP600125); they were then treated with and without 5 μM of T09 and 25HC for an additional 24 h. Pre-treatment of LNCaP

cells with the p38 antagonist SB203580 caused a ~45% inhibition of both T09 and 25HC induction of *ABCA1* mRNA expression (p <0.01) (Fig. 7A). This was specific to *ABCA1* as there was no inhibition observed on *ABCG1* mRNA levels (Fig. 7B). Inhibition of p38 in PC-3 cells only affected T09-mediated induction of *ABCA1* mRNA (Fig. 7C), however unlike LNCaP cells, SB203580 also inhibited T09 mediated increase in *ABCG1* mRNA levels by ~ 36% in PC-3 cells (Fig 7D). In contrast, inhibition of JNK kinase in both LNCaP and PC-3 cells resulted in a ~6-fold induction of basal mRNA levels of *ABCA1* (Fig. 7A and 7C). Moreover, JNK inhibition produced an additive effect on T09- and 25HC-induced increase of *ABCA1* mRNA in LNCaP cells (Fig. 7A). In PC-3 cells this effect was only observed for T09 induction of *ABCA1* mRNA (Fig. 7C). In both cell lines, inhibition of ERK1/2 had no effect on mRNA expression of either *ABCA1* or *ABCG1* (Fig. 7A-D). Lastly, the PKA inhibitor H89 strongly opposed T09 induction of *ABCA1* mRNA in PC-3 cells, but had no effect on *ABCG1* (Fig 7C and 7D).

To further test whether the pharmacological inhibition of LXR transcription of *ABCA1* involved p38 in LNCaP and PKA in PC-3 cells, siRNA oligonucleotides were used to transiently silence expression of p38α, p38β in LNCaP cells and PKA in PC-3 cells treated with and without 5 μM of T09. With two isoforms of p38, LNCaP cells were transfected concomitantly with siRNA oligonucleotides against p38α, and p38β. Optimal transfection conditions were determined to yield approximately 70-80% silencing of p38α, and p38β without any cytotoxicity or off target effects (Fig 8). Contrary to the pharmacological

inhibition of LXR mediated transcription of ABCA1 that was observed with the pan p38 inhibitor SB203580, silencing expression of p38α, and p38β in LNCaP cells failed to oppose T09 mediated regulation of *ABCA1* mRNA levels (Fig. 8), suggesting that SB203580 can interact with other kinases or protein targets.

Data from experiments with PC-3 cells transfected with siRNA oligonucleotides against the cAMP dependent kinase PKA demonstrated that PKA is involved in LXR regulation of *ABCA1* in PC-3 cells. siRNA against the alpha catalytic unit of PKA significantly inhibited T09 mediated transcription of *ABCA1* mRNA (Fig 9). Also, PC-3 cells stimulated with the cAMP/PKA activator forskolin had significant increases in *ABCA1* mRNA expression which were also opposed by PKA siRNA silencing (Fig 9). The involvement of PKA in regulating LXR mediated activation of *ABCA1* mRNA does not appear to involve expression of *LXRα* or *LXRβ*, as no changes to mRNA levels of these genes were observed in cells transfected with PKA siRNA (Fig 9). Consistent with the pharmacological inhibitor studies using H89, siRNA against PKA did not opposed T09 regulation of *ABCG1* mRNA levels (Fig 9).

Cholesterol Efflux in PC-3 cells

Studies where carried out to establish whether LXR-ABCA1 mediated cholesterol efflux was functional in PC-3 cells. Studies of cholesterol efflux were also attempted in LNCaP cells, however due to the labile nature of LNCaP adhesion to the plastic cell culture plate surface, large cell losses were observed during efflux experiments due to the extensive washing steps required for the [^{3}H]

cholesterol loading and efflux assay. Due to this experimental constraint, it was not possible to objectively measure cholesterol efflux in this cells line.

PC-3 cells loaded with 1μCi/mL of tritium labeled cholesterol for 48 h followed by 48 h treatment with 1 uM of the LXR ligand T09 displayed a significant rate of cholesterol efflux at 2 h of upon exposure to apo A-I (Fig. 10B). Cells pre-treated with vehicle and apo A-I were able to efflux cholesterol, however this was not significantly higher than cells pre-treated with T09 without apo A-I. Under the present experimental conditions cholesterol efflux was initiated by 2 h and was linear up to ~6 h of exposure to apo A-I (Fig. 10A). Cells pre-treated with T09 alone did not show significant rates of cholesterol efflux demonstrating that efflux was dependant on the presence of apo A-I and therefore specific to LXR-ABCA1 mediated efflux. siRNA against LXRα or LXRβ were performed to determine which receptor isoform was involved in mediating cholesterol efflux from PC-3 cells. Optimal conditions for siRNA transfection of LXRα or LXRβ were determined in previous experiments and produced 80-90% silencing of these targets, however it was determined that the experimental conditions of cholesterol efflux, i.e. loading of cells with 1μCi/mL of tritiated cholesterol for 48 h in 10% FBS, was not sufficient to permit successful PC-3 transfection with siRNA oligos against LXRα or LXRβ.

Cholesterol Quantitation Prostate Cancer Epithelial Cells

To determine if LXR-ABCA1 mediated cholesterol efflux can leads to a decrease in cellular cholesterol levels in PC-3 cells during cholesterol efflux, PC-3 cells were plated for RCT as described in material and methods and RCT

initiated with the addition of 5 and 20 μg/mL of apo A-I for 4 h and 12h. After 4h and 12 h total lipids were isolated and cholesteryl-esters and free cholesterol determined enzymatically. Figure 11A demonstrates that after 4 h of RCT, PC-3 cells chased with 5 and 20 μg/mL apo A-I have significantly lower free cholesterol levels compared to cells only pre-treated with T09 alone. (Fig 11A). After 12 h of incubation with 20 μg/mL apo A-I free cholesterol levels in PC-3 cells significantly increased compared to cells treated with apo A-I for 4 h (checked bars vs. solid black bar) suggesting a compensatory increase in cellular cholesterol after RCT (Fig 11A). Cholesteryl-esters were not detectable in PC-3 cells under any of the experimental conditions (Fig 11 A). Treatment with 1 μM of T09 for 48 h was unable to alter cellular cholesterol levels in PC-3 cells cultured with 10% fetal bovine serum (FBS) (Fig 11B); demonstrating that LXR-ABCA1 mediated RCT result in changes in cellular free cholesterol levels in PC-3 cells only in the presence of lipid free apo A-I.

Cholesterol Metabolism Gene Signature of PC-3 cells during Efflux

To determine the gene expression changes during RCT a panel of genes related to LXR-mediated efflux, *de novo* cholesterol synthesis and cholesterol uptake were measured at 4h and 12 h after RCT conditions. After 4 h of cholesterol efflux, as expected, mRNA levels of *ABCA1* and *ABCG1* increased significantly in cells pre-treated with T09 compared to vehicle treated cell (Fig. 11C and 11D), however in T09 + apo A-I chased cells the mRNA levels of *ABCA1* and *ABCG1* were significantly higher than in cells treated with T09 alone ($p < 0.001$ and $p < 0.05$ respectively). After 12 h of RCT *ABCA1* mRNA levels

were significantly lower than at 4h of RCT, where *ABCG1* mRNA levels were unchanged (Fig 11C and 11D). Three genes central de novo cholesterol synthesis *SREBP2*, *HMGR*, and uptake of circulating cholesterol levels, low density lipoprotein receptor (*LDLR*), were also measured during cholesterol efflux at 4h and 12h. After 4 h of RCT there were no significant changes in *SREBP2*, *HMGR* and *LDLR* mRNA levels (Fig 11E-G). After 12 h of RCT mRNA levels of *SREBP2* and *HMGR* remained unchanged, however mRNA expression of *LDLR* significantly increased in T09 + apo A-I treated cells compared to vehicle and T09 treated cells ($p < 0.01$, Fig 11G).

Alexa Fluor Staining of Plasma Membrane Lipid Rafts

To determine whether LXR-ABCA1 mediated cholesterol efflux can alter plasma membrane lipid raft domains, PC-3 cells were labeled with the beta subunit of cholera toxin (Ct-b) conjugated to Alexa fluor. Ct-b binds with high affinity to lipid raft domains protein ganglioside gm1 (gm1) and anti-cholera antibodies induce co-patching of raft positive domains (100). This process is attenuated with perturbation to raft cholesterol level and is reflected in decreased fluorescence intensity of Alexa fluor (100). Fluorescent micrographs of PC-3 cells demonstrated that formation of lipid raft domains were significantly diminished in T09 + apo A-I treated cells compared to T09 alone treated cells ($p < 0.01$, Fig 12), suggesting that that LXR-ABCA1 mediated cholesterol efflux can disrupt lipid raft formation. T09 pre-treatment alone also appeared to decrease the number of lipid raft positive cells, but this effect was not significant (*$p > 0.05$ Fig 12*)**.** Collectively these findings suggest that increased ABCA1 gene expression, in

itself, can lead to early partitioning of cholesterol from raft to non-raft domains, but initiation of cholesterol efflux is necessary for significant disruption of raft domains in PC-3 cells.

DISCUSSION

The recent emergence of data suggesting that LXR ligands activated an anti-cancer effect in models of prostate carcinoma prompted us to examine the regulation of LXR-mediated pathways in LNCaP and PC-3 human prostate cancer cells (65, 66). This study specifically sought to characterize in these transformed epithelial cells which LXR isoform is involved in the transcriptional regulation of the LXR responsive genes *ABCA1* and *ABCG1*. I hypothesized that the LXR regulatory mechanisms in these models of prostatic carcinoma would be distinct from other cell types and that a cell-specific response would have to be considered in any future studies of the anti-cancer effect of LXR ligands.

Specificity of Synthetic LXR ligand T09 in PCa cells

There is data demonstrating cross talk between NR with the type 2 family of NRTF (1). It was recently demonstrated that the synthetic LXR ligand T09 can activate PXR in other cell types (101). In the cell types used in the current project, there was no evidence that the LXR ligand T09 activates PXR based on the absence of any mRNA changes to two known PXR responsive genes *CYP3A4* and *MDR-1*. However, there was evidence that T09 can significantly reduce mRNA levels *PPARα* in PC-3 cells. There were no other NR targets affected by T09 treatment in either LNCaP or PC-3. Nevertheless, despite the lack of any apparent interaction between T09/LXR and PXR in these cell types. Results from

experiments conducted using T09 in PC-3 cells should be weighed against any possible involvement of PPARα and the current experiment does reveal a lack of specificity of T09 in PC-3 cells when used at 10μM.

PC-3 cells express higher levels of LXR and Cholesterol Metabolism Related Genes and Cholesterol Levels than LNCaP cells.

LXR isoform mRNA profiling determined that relative *LXRβ* mRNA expression is significantly higher than *LXRα* in both LNCaP and PC-3 cells. This did not come as a surprise as most expression profiling has determined that *LXRα* is primarily expressed in liver, adipose and enterocytes, where *LXRβ* is expressed ubiquitously (6, 7). Our *LXRα* and *LXRβ* mRNA profiling does conflict with a previous study which reported that LXRα is the predominant isoform in LNCaP cells and the ratio of *LXRα* to *LXRβ* in PC-3 cells is approximately one (102). We used a similar normalization method as this previous report (102), but found *LXRβ* to have higher relative mRNA levels in both LNCaP and PC-3 cells.

Our basal mRNA measurements revealed disproportionally lower *ABCA1* mRNA expression compared to *ABCG1* in both LNCaP and PC-3 cells. There is evidence in some cell types that without functional ABCA1, that ABCG1 alone is incapable of regulating the cholesterol efflux machinery due to the cooperative requirement of ABCA1 and apo A-I in formation of pre-HDL particles (18). Further support for a critical role of ABCA1 in HDL formation and cholesterol efflux come from observations of severe HDL deficiency and pathological cholesterol retention in persons with functional mutations of the *ABCA1* gene (19). Carriers of *ABCA1* mutations, such as those seen in Tangier disease, are

largely incapable of normal reverse cholesterol transport despite carrying normal copies of the *ABCG1* gene (19). If ABCA1 synergizes with ABCG1 for cholesterol removal in LNCaP and PC-3 cells as it does in other cell types, then our data may have revealed a propensity for impaired cholesterol efflux in these transformed cell models. This would support a body of data demonstrating abnormal prostatic cholesterol retention in men with advanced PCa (61-64).

A number of epidemiological studies have demonstrated that the association between cholesterol and PCa exists only for aggressive forms of PCa (61-64). It is not clear why the association between cholesterol and PCa only appears in late stage and metastatic disease, but some data suggests that late stage, hormone refractory PCa is associated with increase the rate of cholesterol synthesis, possibly to support increased prostatic steroidogenesis (103). This may suggest that some metabolic genes involved in energy and lipid metabolism would be a poor predictor of those at risk for advanced disease, but therapies aimed at shifting the balance of cholesterol metabolism towards degradation and removal may have a positive outcome of these aggressive stages of disease. There is already epidemiological data supporting that either drug therapy through use of statins or lifestyle to control serum cholesterol levels protects against aggressive, late stage PCa (61-64). In the current study, the two cell models used, LNCaP and PC-3 have origins which link them to two clinically discrete stages and grades of PCa. PC-3 cells are derived from grade IV, hormone refractive PCa bone metastasis and display a more aggressive phenotype (33 h doubling time in nude mice), and LNCaP cells isolated from lymph nodes of metastatic hormone

sensitive PCa are relatively less aggressive (88 h doubling time in nude mice)

(104). Expression patterns of genes central to the LXR axis and *de novo*

cholesterol synthesis revealed that PC-3 cells expression disproportionately higher

levels of *LXRα*, *LXRβ*, *ABCA1* and *ABCG1* compared to LNCaP cells. The

relative difference was most striking for *ABCA1* which had almost a 20 fold

greater mRNA level. Expression of genes related to cholesterol *SREBP2*, *HMGR*

and *SQS* and triglyceride synthesis *SREBP1* where also significantly higher in

PC-3 cells. Expression of two genes central to oxysterol synthesis, *CYP27A1* and

CH25H, were lower in PC-3 cells, but this was not significant. Cholesterol

analysis also revealed that PC-3 cells contain significantly higher levels of free

cholesterol compared to LNCaP. This observation supports data demonstrating

that PC-3 cell basal cholesterol synthesis is higher than LNCaP cells and are more

sensitively to sterol deprivation that LNCaP cells (105). Despite having

significantly higher levels of LXR responsive genes, *ABCA1* and *ABCG1* than

LNCaP, free cholesterol levels in PC-3 cells still remain higher than LNCaP,

suggesting that the net cholesterol turnover remains lower in the more aggressive

cell type. Nevertheless, it is not clear if the higher aggrieves phenotype of PC-3

cells and its higher rate of cholesterol synthesis are related, and studies to

determine this relationship are needed.

Expression Data from NCBI Gene Expression Omnibus Database

To further examine whether the *ABCA1, ABCG1* mRNA ratio profile in

these transformed cell lines differs to those found in normal prostate epithelial

cells, a query was performed using the National Center for Biotechnology

Information's (NCBI) Gene Expression Omnibus database www.ncbi.nlm.nih.gov/projects/geo (106). We found in two separate array profiles of normal prostate epithelial cells that *ABCA1* mRNA expression was approximately 2 to 3-times greater than *ABCG1* (GEO accessions: GDS1746 (37), GDS1973 (38). We also found expression profile data in GEO accession: GDS1746 which demonstrated that, similar to our findings, a low *ABCA1, ABCG1* mRNA ratio exists in LNCaP cells. Collectively, these data strongly suggested that the *ABCA1, ABCG1* mRNA expression ratio is decreased in transformed prostatic epithelial cells and supported data demonstrating abnormal prostatic cholesterol retention in PCa (61). Due to the integral role that LXR and ABCA1 have in cholesterol efflux, it is warranted to determine whether suppression of LXR-ABCA1 function is another mechanism through which transformed prostatic cells can increase their cholesterol pool.

Involvement of LXRβ is dependant on Target and Ligand Tested

Data from our siRNA experiments supported our original hypothesis that LXR biology in these transformed cells would be atypical. We found that the synthetic and natural LXR ligands T09, 25HC, and 24,25EC showed distinct LXR dependent and independent regulation of *ABCA1* mRNA in both LNCaP and PC-3 cells. The discovery that siRNA against LXRα or LXRß failed to inhibit T09- and 25HC-mediated *ABCA1* mRNA induction in LNCaP cells was compelling and contrasts with reports from numerous LXR functional studies demonstrating that T09 and oxysterols are potent LXR ligands and increase *ABCA1* mRNA expression through direct ligand activation of LXR (107). Although there are

reports in macrophages and skin fibroblasts that T09 can also activate the xenobiotic transcription factor PXR, we found that LNCaP and PC-3 cells treated for 24 h with multiple concentrations of T09 had no effect on expression of the PXR responsive genes *CYP3A4*, *CYP3A5* and *MDR-1*. We also did not observe any *ABCA1* and *ABCG1* mRNA changes in cells treated with multiple concentrations of the PXR agonist SR12813. These experiments support that T09 induction of *ABCA1* and *ABCG1* mRNA is not mediated through PXR in LNCaP or PC-3 cells.

These experiments revealed that, in comparison to other ligands tested, 24,25EC uniquely required LXRß in regulating both *ABCA1* and *ABCG1* mRNA expression in LNCaP cells. 24,25EC is synthesized exclusively in a shunt reaction from the mevalonate *de novo* cholesterol synthesis pathway and is believed to permit cellular sensing of excess cholesterol biosynthesis (108).

It was observed that *ABCG1* mRNA regulation by synthetic and natural LXR ligands in LNCaP cells strongly required the expression of LXRß. This was also observed for *ABCG1* and *ABCA1* mRNA expression in PC-3 cells transfected with siRNA against LXRß.   The lack of a consistent involvement of LXRß in ligand induction of *ABCA1* mRNA in LNCaP cells likely reflected the numerous molecular distinctions between these two cells lines (104, 110). The most prominent difference is the lack of functional AR and the need for androgen-driven growth in PC-3 cells, which is suggestive of a relevant cross-talk between androgen and LXR signaling in LNCaP cells (110). It has been reported in LNCaP cells that androgens strongly suppress the expression of ABCA1, and that

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T09 can act as an anti-androgen (102, 111). Conversely, the regulation of *ABCG1* mRNA by synthetic and natural LXR ligands in both LNCaP and PC-3 cells is consistent with reports that *ABCG1* mRNA transcriptional regulation comes exclusively under LXR (112). Given that our siRNA transfections against LXRα and LXRß achieved 70-80% silencing of these genes, our data strongly support a principal role of LXRß but not LXRα in the regulation of induction of *ABCA1* and *ABCG1* mRNA by T09 and oxysterols in these models.

The Stress kinase p38 is not involved in regulation of ABCA1 in LNCaP cells

Our pharmacological inhibitor studies initially suggest that T09 and 25HC regulation of *ABCA1* mRNA expression may involve the kinases-mediated pathway of p38, JNK and PKA. Both p38 and JNK mediate the cellular stress response and are naturally activated by a variety of stimuli including pro-inflammatory cytokines, osmotic shock and ultraviolet irradiation (113). There is a report of TNF-α induction of *ABCA1* mRNA expression in mouse peritoneal macrophages through the stress kinase p38 (114). Nevertheless, the p38 antagonist used in our experiments, SB203580, has been shown to also activate JNK and the ERK1/2 map kinase pathway (115, 116). The pleiotropic effect of SB203580 raised the possibility that its inhibitory effect on T09 and 25HC induction of *ABCA1* mRNA could occur through uncharacterized targets other than p38.

To add complexity to the regulation of *ABCA1* mRNA, we also observed that inhibition of JNK with SP600125 raised basal and LXR ligand inducible levels of *ABCA1* mRNA ~6 and 1.5-fold respectively in both LNCaP and PC-3

cells (Fig 7A and Fig 7B). Both p38 and JNK are typically redundant members of the stress-activated protein kinase pathway; however it has been shown that they can oppose each other depending on the downstream target (117). The observed induction of *ABCA1* mRNA by the JNK inhibitor could reflect a similar antagonism of JNK towards p38 signaling and could represent be a molecular "on" and "off" of *ABCA1* gene regulation. We observed a similar effect on *ABCA1* mRNA response in PC-3 cells pre-treated with p38 and JNK inhibitors supporting a common regulation of ABCA1 gene expression by a signal transduction pathway in both LNCaP and PC-3 cells.

siRNA experiments confirmed that p38 does not appear to be required in T09 regulation of *ABCA1* or *ABCG1* mRNA levels in LNCaP cells. To exclude the possibility that SB203580 was acting through a specific p38 isoform, LNCaP cells where transfected with a pool of siRNA oligonucleotides ensuring targeting of both p38α and p38β. Despite the observed inhibitory effect of use of this pharmacological inhibitor, data from these siRNA experiments contradict the pharmacological inhibition that was observed with SB203580 and illustrates the potential for pleiotropic effects of pharmacological inhibitors when targeting disruption of signal transduction pathways.

PKA is involved in regulation of ABCA1 in PC-3 cells.

Pharmacological inhibition of PKA with H89 strongly opposed T09 induction of *ABCA1* mRNA. However, unlike our experiments using p38 siRNA, transfection of PC-3 cells with siRNA oligonucleotides against PKA confirmed the involvement of PKA in this pathway. PC-3 cells treated with the cAMP

agonist and PKA activator forskolin resulted in significant upregulation of *ABCA1* mRNA levels but not *ABCG1*. This was reverse by siRNA against PKA confirming that PKA can upregulate ABCA1 in the absence of LXR ligand activation. This data is consistent with studies demonstrating that cAMP/PKA activation leads to upregulation of *ABCA1* mRNA, protein phosphorylation and cholesterol efflux in macrophages and lipid loaded fibroblasts (118). cAMP/PKA is activated by apo A-I in macrophages and leads to increased *ABCA1* mRNA expression and stabilization of protein levels via serine phosphorylation (119). PKA activation and stabilization of ABCA1 initiates and enhances cholesterol efflux even in the absence of LXR ligands (119). Our data are consistent with studies demonstrating a role for PKA in LXR mediated activation of ABCA1 and regulation of basal *ABCA1* mRNA levels (119). The observed differences in regulation of *ABCA1* mRNA between PC-3 and LNCaP are likely a consequence of the many molecular distinctions between these two cell types and warrants further testing.

LXR mediated cholesterol efflux decreases plasma membrane cholesterol raft domains.

Studies in lipid laden macrophage foam cells demonstrate that LXR activation shifts the dynamic balance of cholesterol metabolism towards efflux resulting in decreased cellular cholesterol levels and attenuation of foam cell development. Experimental data suggests that transformed prostate tumors have increased cholesterol levels compared to begin or normal prostatic tissue, demonstrating that the balance of cholesterol metabolism (synthesis/uptake and

degradation/efflux) shifts towards increases retention, but it is not clear which side of the dynamic regulation of intracellular cholesterol is altered in late stage PCa (61). There is some data demonstrating a discrete upregulation of *de novo* cholesterol synthesis in men with hormone refractory resistant PCa (103), but this may reflect a specific shift to prostatic cholesterol-steroid synthesis in response to castration. Prostatic tissue, like many peripheral tissue, preferentially derive cholesterol from circulating lipoproteins, this supports the population data demonstrating a positive correlation between risk of advanced PCa and serum cholesterol levels (61-64). However, all cells in peripheral tissue possess the machinery to remove excesses cellular cholesterol through reverse cholesterol transport mediated by LXR-ABCA1 (18). There is an information gap on whether the efflux side of the cholesterol metabolic equation is compromised in advanced PCa and equally important, whether increases cholesterol efflux can alter intracellular cholesterol levels. This is vital to understand the role of cholesterol in carcinogenesis and to aid in developing adjuvant therapies that can compliment the current belief that lifestyle and/or use of cholesterol synthesis inhibitors can protect against advanced PCa.

This project demonstrated that LXR mediated cholesterol efflux is active in PC-3 cells. PCR analysis demonstrated that the gene expression fingerprint during cholesterol efflux showed a strong increase in ABCA1 mRNA levels, but a dramatic induction of *ABCG1* mRNA (~130 fold), a level not previously observed in PC-3 or LNCaP cells. The hyper-augmented *ABCG1* mRNA levels might reflect higher ABCG1 protein as ABCA1 increased synthesis of immature HDL

particles from apo A-I. ABCA1 is considered the rate limiting step in HDL synthesis, allowing immature HDL particles to specifically interact with ABCG1 and continue cholesterol removal (18).

Examining cellular cholesterol levels during efflux stimulation revealed that cholesterol efflux was associated with a significant dose dependant apo A-I decrease in free cholesterol levels. The decrease in cellular cholesterol was observed after 4 hr of efflux but appears to be reversed by 12 h. This pattern of changes to cellular cholesterol levels corresponds to 4-6 h of linear cholesterol efflux typically observed under the current protocol. The changes in cellular cholesterol levels may not be a true reflection of the cellular response to LXR mediated efflux as serum free culture conditions deprive cells access to serum cholesterol to response to the shift towards cholesterol removal.

Analysis of genes related to *de novo* cholesterol synthesis and uptake revealed that under the efflux conditions, PC-3 cells did not appear to initiate a program of *de novo* cholesterol synthesis, but rather there was a small increase in expression of *LDLR* mRNA after 12 h of efflux conditions. This is in line with prostatic epithelial cells preferentially deriving cholesterol from lipoprotein endocytosis, rather than the mevalonate pathway (18). This change in gene expression does not likely reflect the increase in cholesterol levels that was observed by 12 hr because culture conditions where serum free, therefore the observed increase may have been from *de novo* synthesis or a reflection of basal differences in cholesterol between the 4 and 12 h groups. Also, mRNA levels of *SREBP2* and *HMGR*, both central actors in increasing cellular cholesterol

synthesis, are not always indicating of activity and therefore gene expression is a limited proxy to determine the response of PC-3 cells to cholesterol efflux.

Lipid raft domain cholesterol is a highly concentrated and metabolically active depot of cellular cholesterol (36). Diffusion of a number of membrane receptors and their adaptor proteins is dependent on the presence of cholesterol with these domains and experiments have demonstrated that agents that target raft cholesterol compromise membrane signal transduction because of disruption of raft integrity (36). Studies examining the molecular dynamics of LXR-ABCA1 mediated cholesterol efflux have demonstrated that raft domain cholesterol is a source of membrane cholesterol for efflux to lipid poor apo A-I (18). Therapies aimed at targeting raft associated signaling and adaptor protein assembly have demonstrated LXR mediated efflux can perturb raft integrity and assembly of raft adaptor receptors and protein complexes. Therefore a central question is whether LXR mediated cholesterol efflux can alter raft cholesterol in PCa cells and whether this might attenuate raft associated signaling.

Analysis of lipid raft images in PC-3 cells suggests that LXR-ABCA1 mediated cholesterol efflux does alter membrane raft domains in a pattern similar to those reported in macrophages. Live cell staining using the raft marker gm-1 is a reliable and sensitive tool used by others to demonstrate dynamic changes to raft architecture. Co-patching of gm-1 with other raft domains is highly dependant on the cholesterol content of these domains (100). Images taken of live cells cultured under efflux conditions demonstrated discrete changes to the number of lipid raft positive cells. What was interesting was the administration of the LXR ligand

alone, in the absence of apo A-I resulted in a slight but non-significant decrease in raft positive cells. This effect might have been as a result of a proposed step by ABCA1, which involves priming the membrane for efflux by re-location of cholesterol from raft to non-raft domains. This effect would appear as a decrease in raft domain cholesterol content in cells with high expression of ABCA1 without lipid acceptors. There was also a small but non-significant decrease in raft positive cells in apo A-I and vehicle treated cells. This effect was expected, as apo A-I will interact ABCA1 protein expressed in unstimulated cells and can initiate cholesterol removal (120). However, it was the combination of the LXR ligand T09 and apo A-I which demonstrated the strongest decrease in lipid raft positive cells. The decrease in free cellular cholesterol and raft domain positive cells under ABCA1, apo A-I dependent cholesterol efflux conditions strongly suggests that cholesterol efflux can lead to a decrease in raft domains in PC-3 cells. These data suggest that therapies aimed at increasing both LXR activation and circulating apo A-I synthesis, could increase the rate of raft domain cholesterol removal and negatively impact signal transduction dependant on raft cholesterol content. Further studies are needed to confirm or refute this.
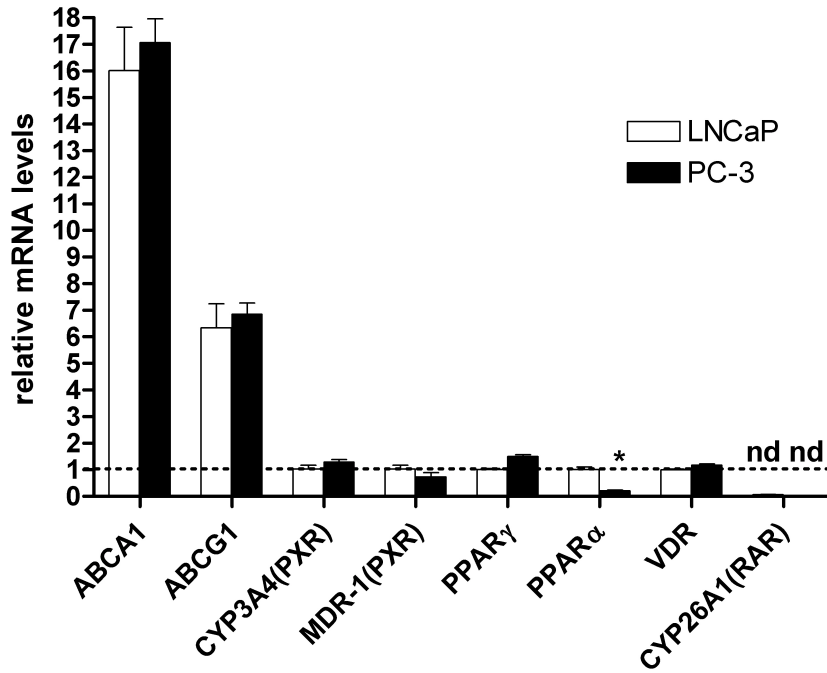
Figure 1. **Interactions of T0901317 (T09) with other type 2 Nuclear Receptors or Their Targets.** LNCaP and PC-3 cells were seeded at a density of 0.5×10^6 cells/well and grown for 24 h in RPMI 1640 10% FBS and then treated for 48 h with either vehicle or 10 μM T09. After treatments cells were subjected to RNA isolation and RT-PCR as described in Materials and Methods. mRNA expression is expressed as means ± SE of fold change relative to vehicle treated control (dotted line). Bar with an asterisk (*) denotes significant difference compared to vehicle treated controls at $p < 0.01$. nd= not detectable.
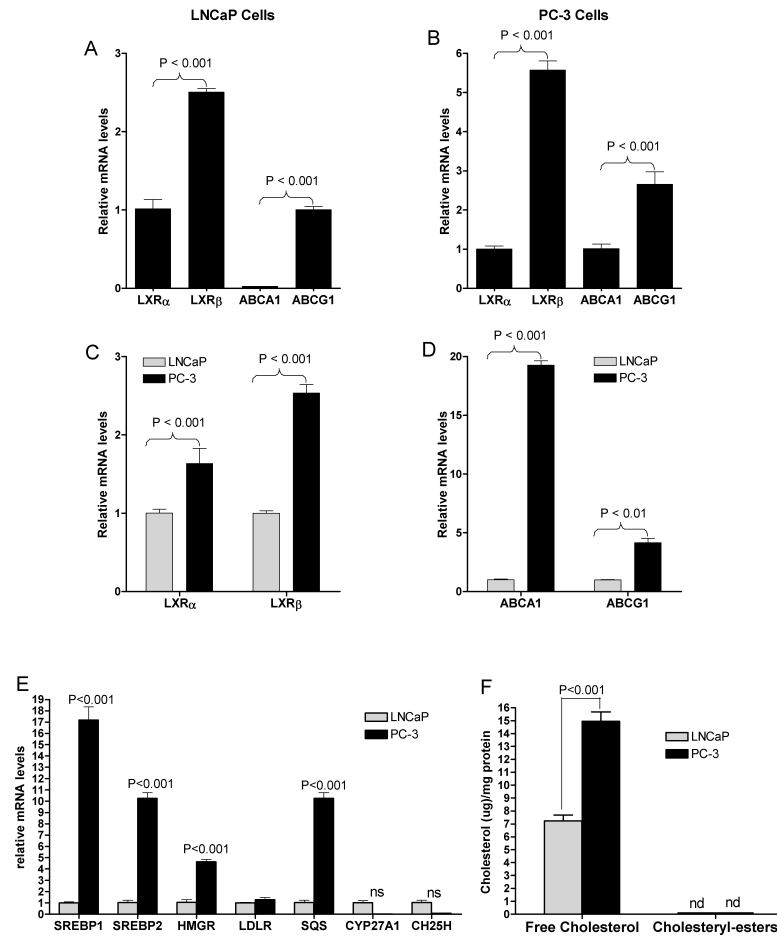
Figure 2. **Comparative expression of LXR, Cholesterol Metabolism Related Genes and Cholesterol Levels in LNCaP and PC-3 cells**. mRNA levels are expressed as means ± SE of comparative fold differences. Data are representative of three independent experiments. **A,** Relative mRNA levels of *LXRα, LXRβ, ABCA1,* and *ABCG1* in LNCaP cells. **B,** Relative mRNA levels of *LXRα, LXRβ, ABCA1,* and *ABCG1* in PC-3 cells. **C,** Comparative mRNA levels of *LXRα, LXRβ* between LNCaP and PC-3 cells. **D,** Comparative mRNA levels of *ABCA1* and *ABCG1* between LNCaP and PC-3 cells. PC-3 cell *ABCA1* and *ABCG1* mRNA expression levels were normalized to *ABCA1* and *ABCG1* mRNA expression in LNCaP cells. **E,** Relative mRNA levels of LXR and Cholesterol Metabolism related genes are expressed as fold change relative to LNCaP cells. ns=not significant. **F,** Cellular cholesterol profile of PC-3 and LNCaP cells. Cells were grown for 48 h in 10% FBS followed by lipid extraction and enzymatic cholesterol determination. nd=not detectable.
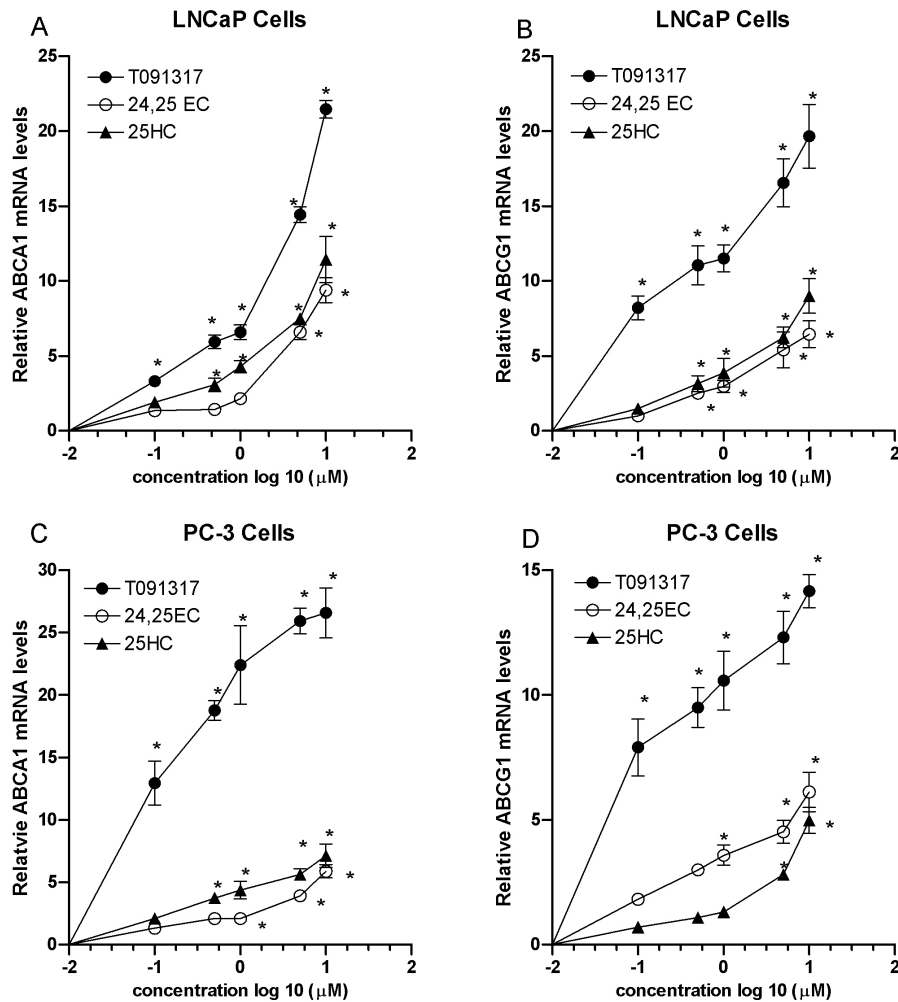
Figure 3. Concentration-dependent effects of T09, 24,25EC, and 25HC on
ABCA1 **and** *ABCG1* **mRNA expression.** LNCaP and PC-3 cells were seeded at a
density of 0.5 x 10^6 cells/well and grown for 24 h in RPMI 1640 10% FBS and
then treated for 24 h with or without 0.1 to 10 µM T09, 24,25EC, or 25HC. After
treatments cells were subjected to RNA isolation and RT-PCR as described in
Materials and Methods. Results are expressed as means ± SE of comparative fold
differences relative to control. Data are representative of three independent
experiments. Points with an asterisk (*) are significantly different than control
($p < 0.05$). **A and B,** Dose effects of T09, 24,25EC, and 25HC on *ABCA1* and
ABCG1 mRNA induction in LNCaP cells. **C and D,** Dose effects of T09,
24,25EC, and 25HC on *ABCA1* and *ABCG1* mRNA induction in PC-3 cells.
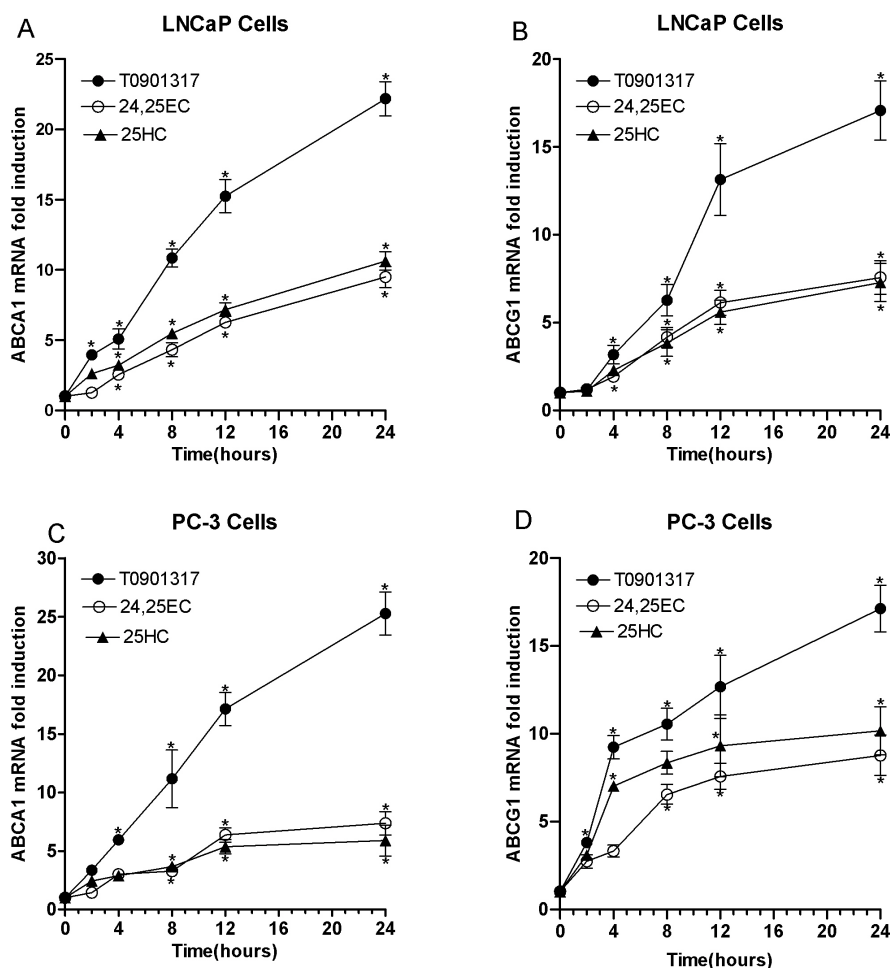
Figure 4. Temporal effects of T09, 24,25EC and 25HC on *ABCA1* and *ABCG1* mRNA expression. LNCaP and PC-3 cells were seeded at a density of 0.5 x 10^6 cells/well and grown for 24 h in RPMI 1640 10% FBS and then treated for 2 to 24 h with or without 5 μM of T09, 24,25EC, or 25HC. After treatments cells were subjected to RNA isolation and RT-PCR as described in Materials and Methods. *ABCA1, ABCG1* mRNA induction is expressed as means ± SE of fold change relative to control at each time point. Data are representative of three independent experiments. Points with an asterisk (*) are significantly different than control ($p < 0.05$) **A and B,** Temporal effects of T09, 24,25EC, and 25HC on *ABCA1* and *ABCG1* mRNA induction in LNCaP cells. **C and D,** Temporal effects of T09, 24,25EC, and 25HC on *ABCA1* and *ABCG1* mRNA induction in PC-3 cells
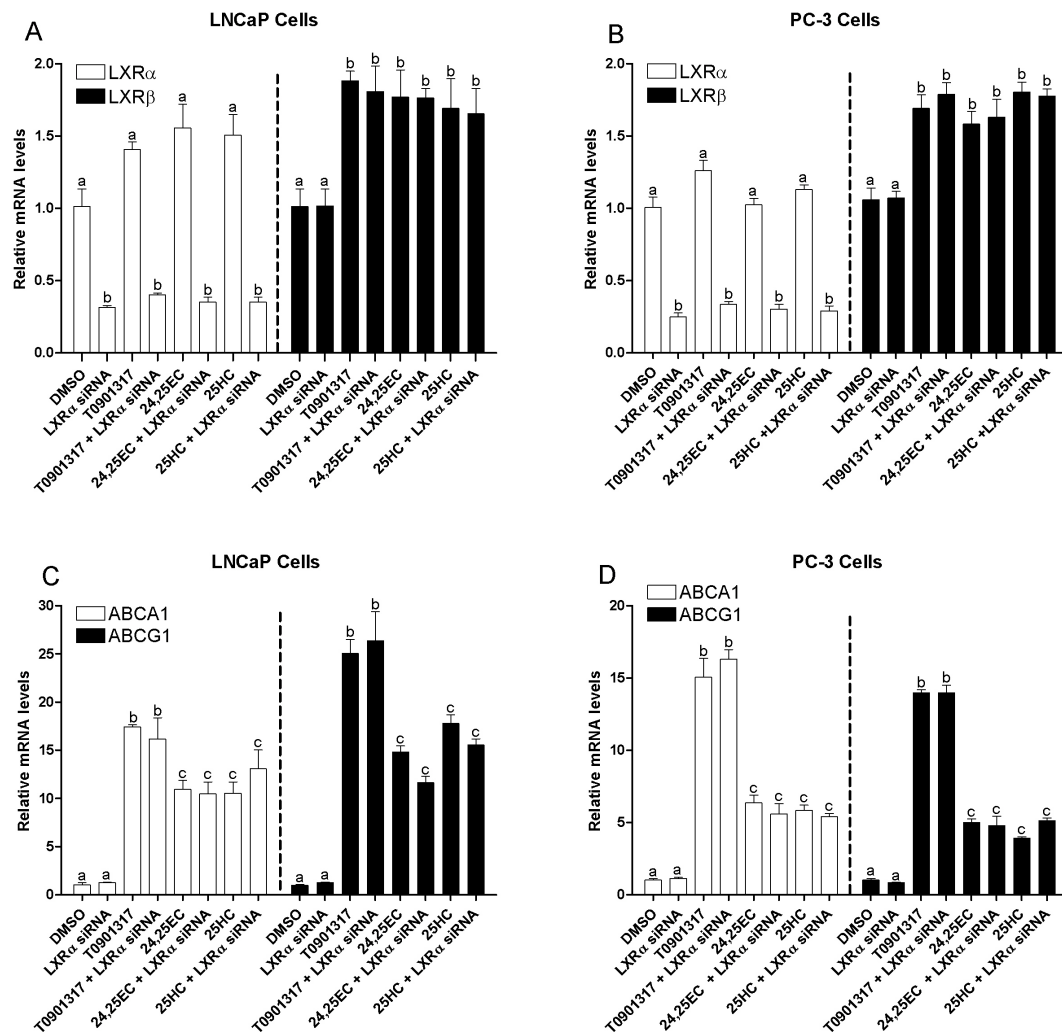
Figure 5. Effects of LXRα siRNA on ligand-mediated expression of *ABCA1* and *ABCG1* in LNCaP and PC-3 cells. LNCaP and PC-3 cells were transfected with or without LXRα siRNA oligonucleotides for 48 h and then treated with or without 5 μM of T09, 24,25EC, or 25HC for an additional 48 h. Relative mRNA levels are expressed as means ± SE of fold change relative to control. Data are representative of three independent experiments. Error bars with a different letter indicate significant differences between groups ($p < 0.05$). **A,** Effects of LXRα siRNA transfection on basal and ligand inducible *LXRα* and *LXRβ* mRNA levels in LNCaP cells. **B,** Effects of LXRα siRNA transfection on basal and ligand inducible *LXRα* and *LXRβ* mRNA levels in PC-3 cells. **C,** Effects of LXRα siRNA transfection on basal and ligand inducible *ABCA1* and *ABCG1* mRNA levels in LNCaP cells. **D,** Effects of LXRα siRNA transfection on basal and ligand inducible *ABCA1* and *ABCG1* mRNA levels in PC-3 cells.

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Figure 6. Effects of LXRβ siRNA interference on ligand-mediated expression of *ABCA1* and *ABCG1* in LNCaP and PC-3 cells. LNCaP and PC-3 cells were transfected with or without *LXRβ* siRNA oligonucleotides for 48h and then treated with or without 5 μM of T09, 24,25EC, or 25HC for an additional 48h. Relative mRNA levels are expressed as means ± SE of fold change relative to control. Data are representative of three independent experiments. Error bars with a different letter indicate significant differences between groups ($p < 0.05$). **A,** Effects of LXRβ siRNA transfection on basal and ligand inducible *LXRα* and *LXRβ* mRNA levels in LNCaP cells. **B,** Effects of LXRβ siRNA transfection on basal and ligand inducible *LXRα* and *LXRβ* mRNA levels in PC-3 cells. **C,** Effects of LXRβ siRNA transfection on basal and ligand inducible *ABCA1* and *ABCG1* mRNA levels in LNCaP cells. **D,** Effects of LXRβ siRNA transfection on basal and ligand inducible *ABCA1* and *ABCG1* mRNA levels in PC-3 cells.

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Figure 7. Effects of p38, JNK, MAPK and PKA inhibitors on T09 and 25HC mediated induction of *ABCA1* mRNA. LNCaP and PC-3 cells were pre-treated with or without 10 µM kinase inhibitors for p38 (SB203580), JNK (SP600125), ERK1/2 (PD98059), or PKA (H89) for 60 minutes followed by treatment with or without 5 µM of T09, 24,25EC, or 25HC for 24 h. Relative *ABCA1*, *ABCG1* mRNA levels are expressed as means ± SE of fold change relative to control. Data are representative of three independent experiments. Error bars with a different letter indicate significant differences between groups ($p < 0.05$). **A and B,** Effects of various kinase inhibitors on *ABCA1* and *ABCG1* mRNA expression in LNCaP cells. **C and D,** Effects of various kinase inhibitors on *ABCA1* and *ABCG1* mRNA expression in PC-3 cells.
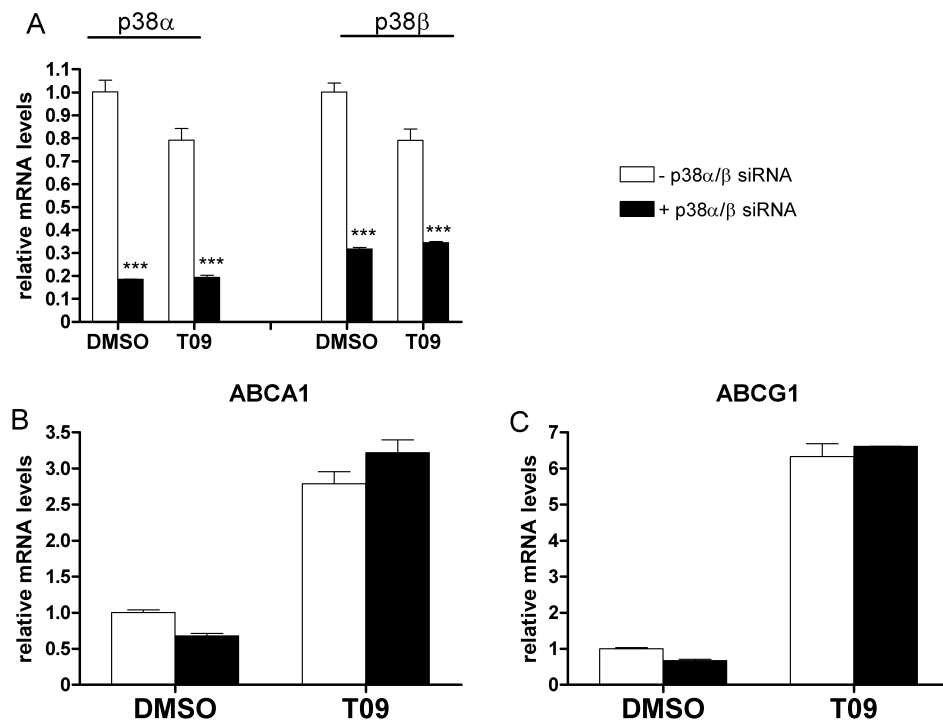
Figure 8. Effects of siRNA against p38α/β on T09 mediated induction of
ABCA1 **mRNA.** LNCaP cells transfected with either: negative control
oligonucleotides (-p38) or with 100 nM MAPK14 (+ p38α) and MAPK 11 (+
p38*β*) siRNA oligonucleotides for 48h followed treatment with either vehicle
(0.05% v/v DMSO) or 1 μM of T09 48 h. After treatments, cells were subjected to
RNA isolation and RT-PCR as described in Materials and Methods. Relative
mRNA levels are expressed as means ± SE of fold change relative to vehicle
treated control. Errors bars with an asterisk (***) indicates a significant difference
between negative control oligonucleotides (-p38α/*β*) and (+ p38α/*β)* siRNA
oligonucleotides. **A,** Effects of p38α/*β* siRNA on mRNA expression of *p38α* and
p38β in PC-3 cells. **B,** Effects of p38α/*β* siRNA on mRNA expression of *ABCA1*
in PC-3 cells. **C,** Effects of p38α/*β* siRNA on mRNA expression of *ABCG1* in
PC-3 cells.
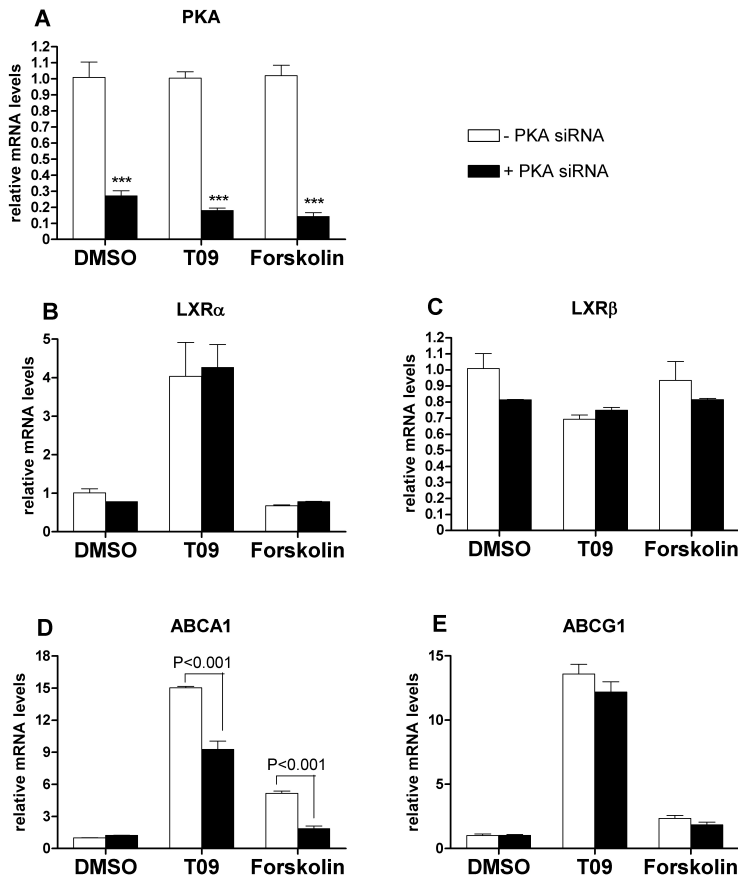
Figure 9. Effects of siRNA against PKA on T09 mediated induction of *ABCA1* **mRNA.** PC-3 cells were transfected with either: negative control oligonucleotides (-PKA siRNA) or with 100 nM PKA siRNA oligonucleotides (+ PKA siRNA) for 48 h followed treatment with either vehicle (0.05% v/v DMSO), 5 μM of T09, or 10 μM of the PKA activator forskolin for 48 h. Relative mRNA levels are expressed as means ± SE of fold change relative to vehicle treated control. Error bars with an asterisk (*) or a *p* value indicate significant differences between (-PKA siRNA) and or (+ PKA siRNA) where *** = $p < 0.001$. **A,** Effects of PKA siRNA on mRNA expression of *PKA* in PC-3 cells. **B,** Effects of PKA siRNA on mRNA expression of *LXRα* in PC-3 cells. **C,** Effects of PKA siRNA on mRNA expression of *LXRβ* in PC-3 cells. **D,** Effects of PKA siRNA on mRNA expression of *ABCA1* in PC-3 cells. **E,** Effects of PKA siRNA on mRNA expression of *ABCG1* in PC-3 cells.
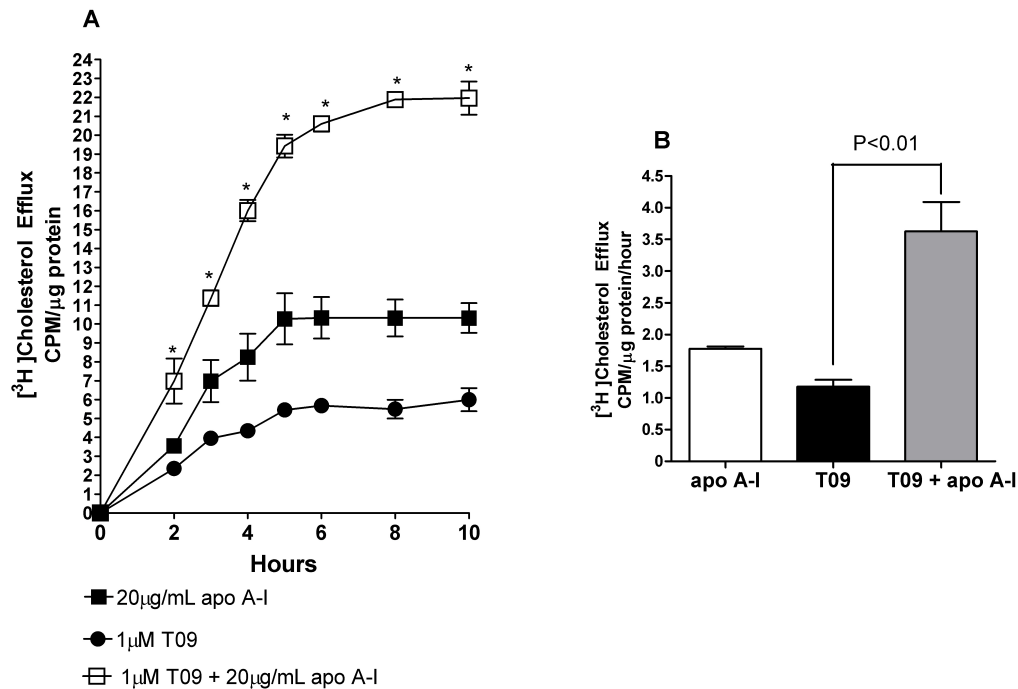
Figure 10. **LXR mediated Reverse Cholesterol Transport (RCT) in PC-3 cells.** PC-3 cells were loaded with 1μCi/mL of [^{3}H] labeled cholesterol. After 48 h PC-3 cells treated with either 1μM of the synthetic LXR ligand T09 or vehicle (DMSO 0.05% v/v) for 48 h in serum free, phenol free RPMI-1640 medium. After 48 h cells exposed to either vehicle (PBS) or 20 μg/mL of human apo A-I for 24 h in serum free medium. Aliquots of culture medium was collected every 2 h and radioactively determined using a scintillation counter. Data are representative of three independent experiments. Error bars with an asterisk (*) indicate significant differences between T09 and T09 + apo A-I ($p < 0.001$). **A,** Temporal changes in cholesterol efflux in PC-3 cells. **B,** Rate of cholesterol efflux in PC-3 cells.
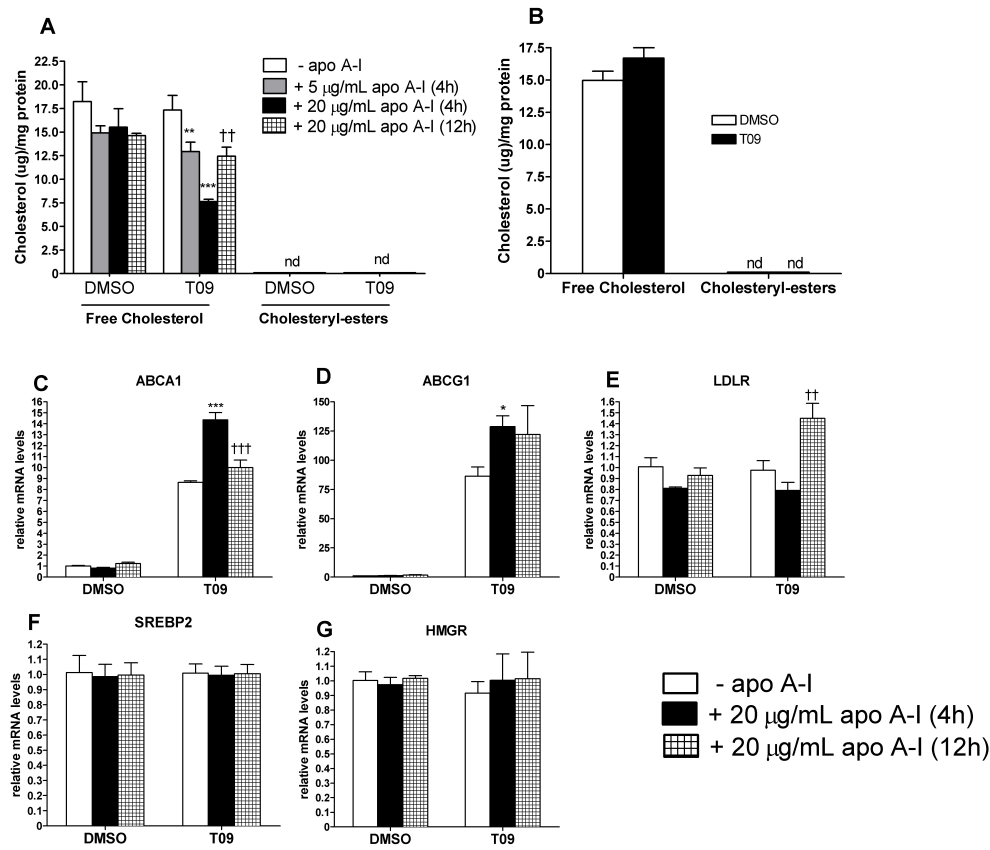
Figure 11. Cellular cholesterol levels and gene expression during RTC. PC-3 cells were seeded under efflux conditions as descried in materials and methods. RTC was initiated with 5 and 20 μg/mL of apo A-I in serum free conditions for 4 h and 12 h. At 4 and 12 h cellular lipids isolated as described in materials and methods. **A,** Cellular cholesterol levels after 4 and 12h of RTC in serum free conditions. Error bars with an asterisk (*) indicate a significant difference between T09 and T09 + apo A-I with * = $p < 0.05$ and *** = $p < 0.001$). Error bars with a cross (†) indicate a significant difference between T09 + apo A-I at 12 h and T09 + apo A-I at 4 h with ††= $p < 0.01$. nd= not detectable. **B,** PC-3 cells were treated for 48 h with 1uM T09 in 10% FBS followed by total cholesterol analysis. **C-G**, PC-3 cells were seeded under efflux conditions as descried in Materials and Methods. RTC was initiated with 20 μg/mL of apo A-I in serum free conditions for 4 h and 12 h. At 4 h and 12 h total RNA was isolated and used for analysis of gene expression. mRNA levels are expressed as means ± SE of fold change relative to vehicle treated control. Error bars with an asterisk (*) indicate a significant difference between T09 and T09 + apo A-I with ** = $p < 0.01$ and *** = $p < 0.001$. Error bars with a cross (†) indicate a significant difference between T09 + apo A-I at 12h and T09 + apo A-I at 4 h with ††= $p < 0.01$ and †††= $p < 0.001$.
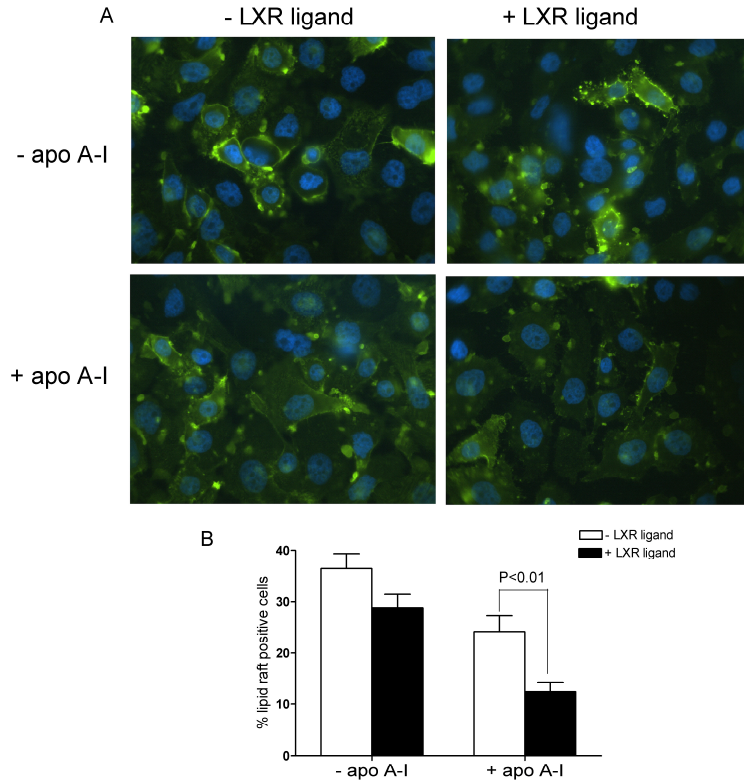
Figure 12. Effects of LXR mediated on Plasma Membrane Lipid Raft Domains. PC-3 cells were plated under conditions of cholesterol efflux as described in Materials and Methods and treated with 1 μM of the LXR ligand T09 for 48 h followed by 4 h of cholesterol efflux initiated with 20 μg/mL apo A-I. Following treatments lipid raft domains were labeled with Alexa Fluor conjugated cholera-toxin B-subunit (AF-CT-B) and visualized by immunofluorescence microscopy. Images captured and analyzed using Nikon NIS elements imaging software. Images (40X) **A,** Micrographs of lipid raft domains in PC-3 cells**. B,** Percent of lipid raft positive cells in PC-3 cells.

Chapter 3: Isoflavone regulation of ABCA1, ABCG1 mRNA levels in Prostate Cancer Cells

INTRODUCTION

Experimental Rationale for studies of Isoflavones and Cholesterol Metabolism in Prostate Cancer Cells

A number of population studies in East Asian males have found a correlation between consumption of soy derived foods and a reduced risk for developing prostate cancer (PCa) and other maladies (72-77, 79-81). Isoflavones are a class of polyphenols found at high concentrations in soy derived foods and are believed to be responsible for their anti-PCa properties (72-77). Studies have shown that habitual consumption of soy derived foods results in significantly higher concentrations of isoflavones in plasma, urine, and prostatic fluid (76). The most commonly found isoflavones found in soya are genistein, daidzein and glycitein. Equol, a metabolite of daidzein also found in biological specimens of individuals with high soy consumption (74).

Animal and *in vitro* studies have identified a number of anti-PCa properties of isoflavones including, tyrosine kinase inhibition, modulators of androgenic signaling, angiogenesis and apoptosis including inhibition of AR signaling (72, 77). Isoflavones can retard the growth of PCa tumors experimentally, however doses experimentally administered animals could be 10 times higher than the equivalent for human consumption (76). Epidemiological evidence is inconsistent as to whether these properties of soy or isoflavones result

in PCa prevention in individuals with high soy consumption or to healthy men without evidence of clinical PCa (76).

In addition to the anti-PCa properties of soy isoflavones, these compounds have also been reported to posses anti-atherogenic and cholesterol lowering abilities (79-81). This includes data demonstrating that consumption of soy and soy isoflavones can reduce risk factors for cardiovascular disease including lowering low density lipoprotein (LDL) cholesterol levels, increasing high density lipoprotein (HDL) cholesterol and increase HDL/LDL cholesterol ratios in animals and humans (79-81). It is still unclear how isoflavones discharge their lipid lowering properties. Nevertheless, the cholesterol lowering properties of soy isoflavones may provide beneficial to males at risk for PCa, as new data suggests that cholesterol is involved in prostate carcinogenesis (61-64). Therefore identifying pathways through which isoflavones might affect cholesterol metabolism in PCa cells is warranted.

In light of the apparent relationship between cholesterol and PCa, this study will examined whether isoflavones can modulate the mRNA expression of cholesterol reverse cholesterol transporters, ATP-binding cassette, sub-family A, member 1, (ABCA1) and sub-family member G 1, (ABCG1) in two cell models of PCa, LNCaP and PC-3 cells. ABCA1 and ABCG1 use energy from ATP hydrolysis to efflux excess free cellular cholesterol to circulating lipoprotein particles such as apo A-I and HDL. Both ABCA1 and ABCG1 come under the transcriptional regulation of the nuclear receptor transcription factors liver X receptors (LXRα, LXRβ), which act to prevent accumulation of excess cellular

cholesterol in peripheral tissue. Isoflavones will be tested for their ability to alter cholesterol efflux from PCa cells.

Specific Hypotheses Tested

Studies will be performed on LNCaP and PC-3 prostate cancer epithelial cells to examine the following hypotheses relating to isoflavone and LXR biology:

a) To test the hypothesis that soy isoflavones can activate mRNA expression of LXR responsive genes *ABCA1* and *ABCG1* in LNCaP and PC-3 cells.

b) To test the hypothesis that LXRβ and not LXRα will be involved in any isoflavone modulation of *ABCA1* and *ABCG1* mRNA levels.

c) To test the hypothesis that androgen receptor is involved in isoflavone regulation of *ABCA1* and *ABCG1* mRNA levels in LNCaP cells.

d) To test the hypothesis that isoflavones can increase the rate of cholesterol efflux from PCa cells.

ABSTRACT

Isoflavones are dietary compounds derived from soy foods studied extensively for their anti-prostate cancer (PCa) and cholesterol lowering properties. Although a strong link between cholesterol and PCa has been reported, there has been no examination of whether isoflavones can modulate mRNA expression of cholesterol metabolism related genes in prostate carcinoma. This study sought to examine whether five isoflavones, genistein, daidzein, equol, glycitein and glyceollin could modulate mRNA expression of two LXR responsive genes, ATP-binding cassette sub-family members A 1 (*ABCA1*) and G 1 (*ABCG1*) in LNCaP and PC-3 cells. Real time PCR data demonstrated that treatment of LNCaP and PC-3 cells with all isoflavones resulted in a one and ten fold increase in mRNA expression of *ABCA1*, *ABCG1*. In both cell lines, the strongest inducers of *ABCA1* and *ABCG1* mRNA were glyceollin and equol; with ~8-15 fold mRNA induction of *ABCA1*, *ABCG1* in LNCaP cells respectively and ~8 fold induction of both *ABCA1*, *ABCG1* mRNA in PC-3 cells. *ABCA1*, *ABCG1* mRNA induction in cells treated with genistein, daidzein and glycitein were weaker at ~1-3 fold. Using siRNA for LXR isoforms (LXRα and LXRβ), it was also observed that LXRβ is required for isoflavone induction of *ABCG1*, but not *ABCA1* in LNCaP cells. mRNA silencing of LXRβ did inhibit isoflavone mediated induction of both *ABCA1* and *ABCG1* mRNA in PC-3 cells. Tritium labeled cholesterol efflux assays revealed that a mixture of three glyceollins did not increase apo A-I induced cholesterol efflux from PC-3 cells.

MATERIALS AND METHODS

Chemicals and Reagents

Dharmacon ON-TARGET*plus SMART*pool siRNA reagents targeting LXRα (NM_005693) (Dharmacon Catalog # L-003413-00), LXRβ (NM_007121) (Dharmacon Catalog # L-003412-00) or androgen receptor (AR) (Dharmacon Catalog # L-003400-00-0005) were purchased from Thermo Fisher Scientific (Lafayette, CO). HiPerFect Transfection Reagent was purchased from Qiagen (Santa Clarita, CA). The synthetic LXR ligand T09 was purchased from Cayman Chemical Company, (Ann Arbor, MI). Genistein, daidzein, equol, glycitein and human purified apo A-I where purchased from Sigma Chemical Co. (St. Louis, MO). [1α,2α (n)-H^3] labeled cholesterol was purchased from Perkin Elmer (Waltham, MA). Lipoprotein free serum was purchased from Intracel (Rockville, MD). Amplex red cholesterol analysis kit was purchased from Invitrogen (Carlsbad, CA). Glyceollins mixture (glyceolins I, II and III) were a gift from Dr. Steve Boue (SRRC, ARS, USDA, New Orlean, LA)

Cell Culture

The human prostate cancer cell lines LNCaP and PC-3 were purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 1% glutamine and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA) (culture medium). Cells were incubated in the presence of 5% carbon dioxide and air at 37 °C.

RNA Isolation and Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT- PCR)

For gene expression experiments, LNCaP and PC-3 cells were seeded in 6 well plates at a density of 0.5×10^6 cells/well and grown for 24 h in culture medium, then treated with various concentrations (0.1-10 μM) of genistein, daidzein, equol, glycitein, glyceollin or vehicle (DMSO 0.05% v/v). After termination of experiments total RNA was isolated using the TRIzol® reagent (Invitrogen, Carlsbad, CA), and reverse transcribed to complementary DNA (cDNA) using StrataScript® First Strand cDNA Synthesis Kit (Stratagene**,** La Jolla, CA) (21). Real time PCR was carried out using a TaqMan Fast Universal PCR Master Mix on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The TaqMan probes and primers were purchased from Applied Biosystems using inventoried TaqMan gene expression assays: *LXRα*, (assay ID: Hs00172885), *LXRβ* (assay ID: Hs00173195), *ABCA1* (assay ID: Hs01059122), *ABCG1* (assay ID: Hs00245154), Human glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* (assay ID: Hs99999905) was used as an endogenous control for all gene expression. The following amplification parameters were used: 95°C for 20 sec, followed by 40 cycles of amplification at 95°C for 1 sec and 60°C for 20 sec. Quantitation of mRNA fold changes were derived using the Δ critical threshold (ΔCt) method (22).

Cell Transfection with Small Interfering RNA (siRNA)

LNCaP and PC-3 cells were seeded in 6-well plates at a density of 0.2 x 10^6 cells/well and grown for 24 h in RPMI-1640 10% fetal bovine serum (FBS)

RPMI-1640 containing 1% glutamine and 1% penicillin/streptomycin. After 24 h

cells were switched to 10% charcoal dextrin treated serum (CDS) media in RPMI-

1640 (without phenol red) containing 1% glutamine and 1%

penicillin/streptomycin and transfected with 5 nM of Dharmacon ON-

TARGETplus SMARTpool siRNA oligonucleotides targeting LXRα, LXRβ or

androgen receptor (AR) using the HiPerFect transfection reagent (Qiagen)

according to the manufactures protocol. Preliminary experiments determined that

5 nM siRNA oligonucleotides were optimal for 70-80% gene silencing without

any off target effects. After 48 h of siRNA transfection, cells were switched back

to 10% FBS and treated with various LXR ligands as described in Materials and

Methods for an additional 48 h. In experiments involving DHT, LNCaP cells after

transfection were kept in fresh 10% CDS and treated with 1 nM DHT for   48 h.

Cholesterol Efflux Assay

PC-3 cells were plated in 10% FBS in triplicate and loaded with 1μCi/mL

of [^{3}H] labeled cholesterol (Perkin Elmer) for 48 h. After 48 h PC-3 cells were

washed thrice with PBS and switched to serum free conditions (RPMI + 0.2%

fatty acid free bovine serum albumin (BSA) w/v). Cells were allowed 2 h to

equilibrate cellular cholesterol in serum free conditions, washed thrice with PBS

and treated for 48 h with either 1 μM of the LXR ligand T09 or 10 μM glyceollin.

After 48 h PC-3 cells were washed thrice with PBS + 0.2% BSA and incubated

with RPMI + 0.2% BSA plus 20 μg/mL of the cholesterol carrier apo A-I to

initiate cholesterol efflux. Aliquots of 100 μl were collected at multiple time

points for 24 h, mixed with scintillation liquid and measured for radioactivity on a

scintillation counter. After 24 h cells were lysed with RIPA buffer (Thermo) and protein measured using the bicinchoninic acid (BCA) assay (Thermo) according to the manufactures' protocol. Efflux of vehicle treated cells will be subtracted from efflux of all groups as background and efflux will be expressed as CPM of radioactivity in the cell culture medium per mg of cellular protein. Mean differences in efflux between isoflavone or T09 + apo A-I and apo A-I alone will be measured at time points within the linear range of efflux.

Statistics

Statistical analysis of data was carried out with the GraphPad PRISM4 program (GraphPad Software, Inc.). Contrasts of group means will be computed using one or two factor ANOVA followed by Bonferroni post hoc tests. The unpaired Student's t test will be used to compare experiments between two groups. Gene expression results will be expressed as means $\pm$ SE of comparative fold differences. Mean differences of post-hoc analyses are considered significant when p value is < 0.05.

RESULTS

Concentration Effect of Soy Isoflavones on ABCG1 and ABCA1 mRNA Expression

To measure the concentration effects of soy isoflavones on mRNA expression of *ABCA1* and *ABCG1*, LNCaP and PC-3 cells were treated with increasing concentrations (0.1-10 µM) of genistein, daidzein, equol, glycitein or glyceollin for 48 h. In both LNCaP (Fig. 1A) and PC-3 cells (Fig. 1C), all

isoflavones resulted in a dose response in *ABCA1* mRNA expression. Except for glycitein, all isoflavones resulted in a dose dependant increase in *ABCG1* mRNA in both cell lines (Fig. 1B and 1D). LNCaP cells treated with various concentrations of glyceollin resulted in a ~2-15 fold induction of *ABCA1* mRNA compared to a ~2-8 fold induction equol treated cells (Fig. 1A). Whereas, the magnitude of genistein's, daidzein's and glycitein's effect on *ABCA1* mRNA expression in LNCaP cells was similar for each at ~0.5-3 fold increase (Fig. 1A). Glyceollin treatment resulted in a ~2-16 fold induction of *ABCG1* mRNA in LNCaP cells, compared to a ~2-4 fold induction by equol and a ~1-2 fold induction by genistein, daidzein and glycitein (Fig. 1B).

Glyceollin and equol resulted in ~2-8 and ~2-6 fold increase in *ABCA1* mRNA expression respectively in PC-3 cells (Fig. 1C), where the effect of genistein, daidzein and glycitein on *ABCA1* mRNA induction was ~1-2 fold (Fig. 1C).   In a similar pattern to LNCaP cells, glyceollin and equol treatments in PC-3 cells resulted in a ~2-7 and ~2-4 fold increase in *ABCG1* mRNA expression (Fig. 1D), however treatments with genistein, daidzein caused an ~1-2 fold change, while glycitein had no effect on *ABCG1* mRNA expression in PC-3 cells (Fig. 1D).

Temporal Effect of Soy Isoflavones on ABCG1 and ABCA1 mRNA Expression

Temporal changes in *ABCA1* and *ABCG1* mRNA expression in LNCaP and PC-3 cells treated with 5 µM of genistein, daidzein, equol, glycitein or glyceollin for 8-72 h was also determined. Fig. 2A and B shows that glyceollin

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treatment in LNCaP cells lead to a significant increase in *ABCA1* and *ABCG1*

mRNA at 8 h compared to cells treated with genistein, daidzein, equol, or

glycitein (~2-3 fold vs. ~0 fold). The same effect in glyceollin treated PC-3 cells

was also observed, with relative *ABCA1*, *ABCG1* mRNA levels increasing

approximately 2 fold at 8 h with little or no fold changes in cells treated with all

other isoflavones (Fig. 2C and D). In daidzein and equol treated LNCaP and PC-

3 cells, relative *ABCA1* mRNA levels significantly increased after 48 h (Fig. 2A

and C). LNCaP cells treated with genistein showed significant relative *ABCA1*

mRNA changes at 24 h, where genistein's effect on *ABCA1* mRNA in PC-3 cells

was apparent only at 72 h (Fig. 2A and C). Glycitein treatment in LNCaP and

PC-3 cells had no significant effect on *ABCA1* mRNA levels at any time point

(Fig. 2A and C). In contrast glyceollin treated LNCaP and PC-3 cells; a

significant increase in relative *ABCA1*, *ABCG1* mRNA levels at all time points

was observed (Fig. 2A-D). At 48 and 72 h equol treated LNCaP and PC-3 cells

showed ~3 and ~2 fold changes in relative *ABCG1* mRNA respectively (Fig. 2B

and D). Relative *ABCG1* mRNA levels in PC-3 cells treated with genistein,

daidzein and glycitein showed no increases at any time point, where LNCaP cells

treated with the same isoflavones showed significant increases in relative *ABCG1*

mRNA levels at 48 and 72 h (Fig. 2B and D).

**LXRβ Involvement in Isoflavones Regulation of ABCA1 and ABCG1 mRNA
Expression Varies between Cell Types**

Transfection of LNCaP cells with siRNA against LXRβ or LXRα could

not inhibit isoflavone mediated increase in *ABCA1* mRNA levels (Fig 3B and D).

A significant increase in *ABCA1* mRNA levels was observed in glyceollin treated cells with siRNA against *LXRβ* (Fig 3D). siRNA against *LXRβ* did significantly inhibit isoflavone mediated induction of *ABCG1* mRNA levels in LNCaP cells, while siRNA against LXRα had no effect (Fig 3B and D). In PC-3 cells, both *ABCA1 and ABCG1* mRNA induction by all isoflavones was inhibited in cells transfected with LXRβ siRNA (Fig. 4B and D); while LXRα siRNA had no effect on either *ABCA1* or *ABCG1* mRNA induction by any isoflavones (Fig. 4D).

Involvement of Androgen Receptor in Isoflavone Regulation of ABCA1 and ABCG1 mRNA Levels in LNCaP cells.

It has been reported and confirmed by this laboratory that androgens can oppose the mRNA expression of *ABCA1* in LNCaP cells (102). The antagonism of *ABCA1* mRNA by androgen in LNCaP cells may provide a new mechanism that can be tested in isoflavone regulation of *ABCA1*. The lack LXRα or LXRβ involvement in isoflavone regulation of *ABCA1* mRNA in LNCaP cells suggests an alternative pathway is involved. Work in this laboratory has demonstrated that some isoflavones such as genistein, equol and glyceollin can oppose the transcriptional program of AR (77, 121); therefore it was tested whether isoflavone regulation of *ABCA1* mRNA requires the presence of AR in LNCaP cells. To test this LNCaP cells were transfected with either 5 nM siRNA against AR or vehicle negative control for 48 h, followed by treatment with 5 μM isoflavones for an additional 48 h.

Transfection with AR siRNA lead to an increase in basal *ABCA1* mRNA levels, but this effect was not significant (Fig 5). AR siRNA significantly opposed

ABCA1 mRNA induction by glyceollin and glycitein ($p < 0.001$ and $p < 0.05$) respectively. There was no significant effect by AR siRNA on *ABCA1* mRNA levels in genistein, daidzein and equol treated LNCaP cells. AR siRNA significantly inhibited *ABCG1* mRNA induction by equol and glyceollin, ($p < 0.001$). AR siRNA lead to a significant increase in basal mRNA levels of *ABCG1* in control cells, ($p < 0.05$). These data suggest that the steroid hormone pathway, through AR, is involved in glyceollin and glycitein modulation of *ABCA1* mRNA and equol and glyceollin regulation of *ABCG1* mRNA levels in LNCaP cells, where the regulation of these targets in PC-3 cells appears to be exclusively through LXRβ.

Cholesterol Efflux from PC-3 cells

To evaluate whether isoflavone mediated increase in *ABCA1* mRNA levels can results in increased reverse cholesterol transport from transformed epithelial cells, PC-3 cells loaded with tritium labeled cholesterol were pre-treated with either vehicle, 1 μM T09 or 10 μM of glyceollin for 48 h and then stimulated to begin cholesterol efflux with lipid poor, human apo A-I. Cholesterol efflux was determined at multiple time points as described in Materials and Methods. Pre-treatment with the synthetic LXR ligand T09 resulted in significant increase apoA-I mediated cholesterol efflux compared to vehicle treated control (Fig 6) During initial experiments glyceollin significantly increased cholesterol efflux from PC-3 cells, however this was not specific as it occurred with and without the presence of the lipid carrier apo A-I. Further experiments showed no

effect of glyceollin on increased cholesterol efflux with the mean cholesterol rate of three independent experiments showing no effect in cholesterol efflux (Fig 6).

DISCUSSION

There is interest in identifying compounds from the diet which could protect against the onset of prostate and other cancers. These compounds, labeled as "bioactives" phytochemicals are believed to be responsible for health promoting associations, lower cancer risk, decreased heart disease associated with certain foods or dietary patterns. With the current interest in LXR as a putative anti-PCa target in models organism and cells, this arm of the study sought to examine whether a number of isoflavones studied for their anti-PCa and lipid lowering properties can active the LXR-ABCA1 pathway and cholesterol efflux in PCa cells.

Soy Isoflavones increase mRNA levels of ABCA1 and ABCG1 in LNCaP and PC-3 cells.

Isoflavones were first characterized for their potential estrogenic activity due to their structural similarity to estradiol (122). There is a body of data demonstrating that women have a lower risk of cardiovascular disease until they reach menopause, therefore research examining estrogen and estrogenic compounds as lipid lowering agents grew (122). Studies examining lipid lowering effects of soy, soy protein isolate or soy isoflavones has produced mixed results. The lack of consistent effect of soy or it's derivatives on lipid metabolism lead to the FDA in 2008 to retract a health claim suggesting that 25 g of soy intake per day can reduce the risk of cardiovascular disease (80). The new claim, suggests

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that soy or its derivatives, soy protein or isoflavones have only a modest effect at best on lowering serum cholesterol levels and attenuating risk for cardio vascular disease. However, it is possible that soy or soy isoflavones could have unmeasured effects on tissue lipid metabolism which may not be reflected by serum cholesterol or triglyceride profile. Therefore tissue or cell type examination of the effects of isoflavones may reveal biological properties on targets involved in lipid metabolism. The effects of isoflavones such as genistein and daidzein on activation of estrogen receptors have been well established (72), but less is known about promiscuous activation of other nuclear receptors such as PPARs, LXR and FXR in liver or other metabolically active tissue. Ricketts et al. demonstrated that the isoflavones genistein, daidzein and glycitein all activated LXR reporter vectors in HepG2 cells as strongly as the endogenous LXR ligand 22(R) hydroxycholesterol (82). In the current study we examined whether isoflavones could increase mRNA levels of LXR responsive genes *ABCA1* and *ABCG1*. Real time PCR data demonstrated that all the isoflavones tested in this study resulted in an increase in mRNA levels of *ABCA1* and *ABCG1* in both LNCaP and PC-3 cells. The effect of isoflavones on expression of ABCA1 and ABCG1 was dose dependant and, for glyceollin, the most robust inducer of these genes, lead to a significant increase in less than 24 h.

LXRβ is required for isoflavone regulation of ABCA1 and ABCG1.

In a pattern that mirrored those observed in the synthetic LXR ligand study, siRNA transfection studies revealed that isoflavones require LXRβ in mediating their induction of *ABCA1* and *ABCG1* mRNA, but there were some

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exceptions with neither LXRα nor LXRβ appearing to be involved in isoflavone regulation of *ABCA1* in LNCaP cells. The apparent lack of involvement of LXRs in isoflavone regulation of *ABCA1* mRNA levels suggests a either non-genomic regulation of *ABCA1* or activation of other nuclear receptors. Pharmacological inhibitor studies revealed the PKA inhibitor H89 might be involved in genistein regulation of *ABCA1*, however these findings studies were inconclusive and yielded data which was non-reproducible.

Androgen Receptor involvement in Isoflavone Regulation of ABCA1 and ABCG1.

This study also examined whether isoflavones may influence expression of ABCA1 and ABCG1 by blocking the AR antagonism on LXR responsive genes ABCA1 and ABCG1. Transfection of the hormone responsive LNCaP cells with siRNA against AR did significantly opposed glycitein and glyceollin induction of *ABCA1* mRNA levels (Fig 5B) and equol and glyceollin induction of *ABCG1* mRNA levels (Fig 5C). It was interesting to observe that silencing of AR lead to a decrease in isoflavone induction of *ABCA1* and *ABCG1*, because of the clear increase that occurred in basal levels of these genes when cells where transfected with AR siRNA. This data may suggest lack of AR also leads to changes to ill-defined pathways which isoflavone interact with to increase basal *ABCA1/G1* mRNA levels. Nevertheless, because of the interest in isoflavones as modulators of the sex steroid hormone axis, these data present a novel pathway to interrogate in understanding the biological interactions between isoflavones and PCa hormone signaling.

Glyceollin failed to increase LXR-ABCA1 mediated Cholesterol Efflux

As the most robust activator of *ABCA1* and *ABCG1* mRNA levels in both LNCaP and PC-3 cells, glyceollin was used to examine isoflavone effects on cholesterol efflux in PC-3 cells. This study was essential to examine whether the observed upregulation of *ABCA1*, *ABCG1* mRNA expression extended to functional changes to cellular cholesterol efflux. Despite some experimental evidence that treatment of PC-3 cells with glyceollin increased apo A-I mediated cholesterol efflux, there was not consistent evidence of this with the aggregate of data revealing no effect. This was not expected, as previous gene expression and cholesterol efflux with the synthetic LXR ligand showed parity between increases in *ABCA/ABCG1* mRNA levels and cholesterol efflux. However, the post-translational regulation and stability of ABCA1 protein levels involves a number of adaptor proteins including PKA phosphorylation of ABCA1 at a number of serine residues which help maintain ABCA1 levels during cholesterol efflux. In the current study neither ABCA1 stability nor protein levels where assessed and therefore cannot make judgment about the effects of isoflavones on protein levels of ABCA1, therefore it warranted to conduct further studies of ABCA1 protein stability and or phosphorylation status under the influence of isoflavones. Based on the structural requirements for ligands of LXRs, it is highly unlikely that isoflavones are direct activators of LXRs and therefore the increased expression of *ABCA1* and *ABCG1* mRNA might involve an indirect effect mechanism. However there is no evidence based on mRNA expression that isoflavones indirectly increase basal levels of either LXR isoform. Nevertheless, it does not

appear that isoflavones can act as modulators of LXR mediated cholesterol efflux in PC-3 cells. Despite the evidence that isoflavones might possess lipid lowering effect, the current study does not demonstrate this extends to functional changes to cholesterol efflux in PC-3 cells.

List of Figures with legends
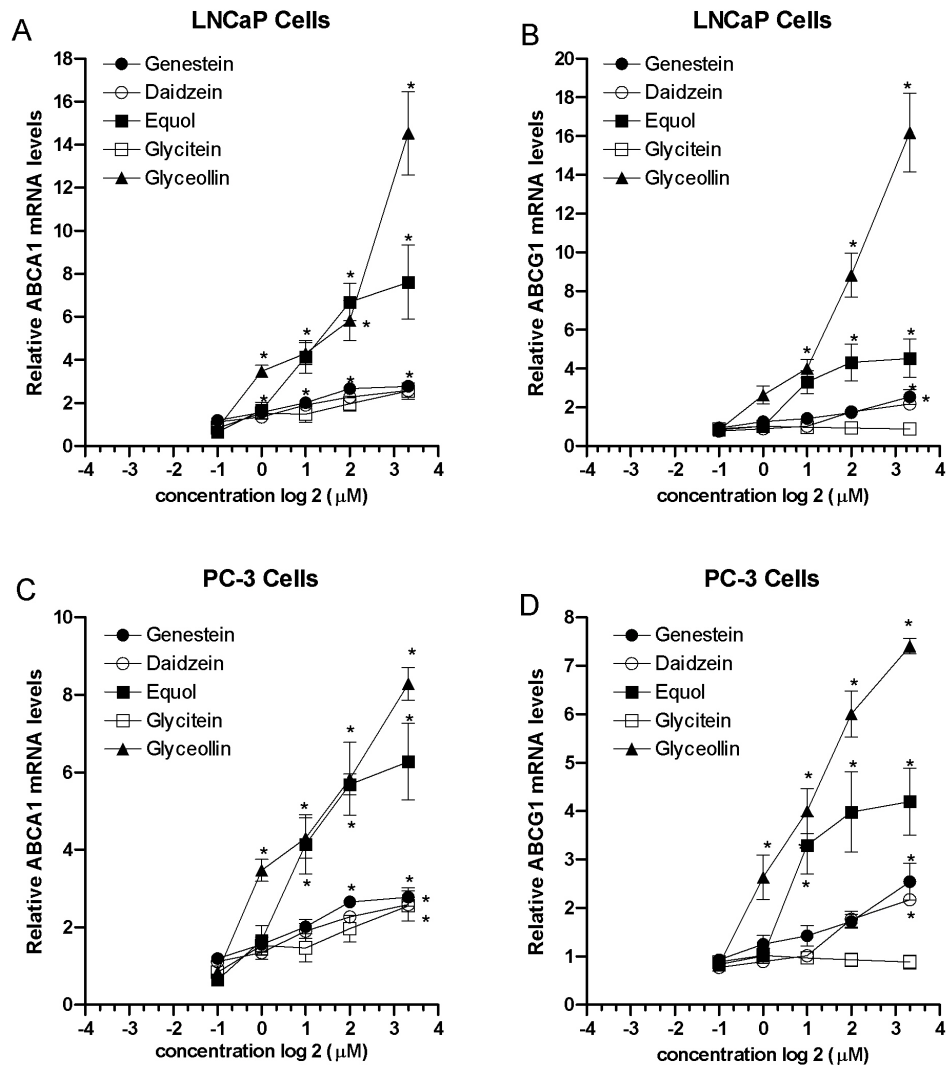
Figure 1. Concentration-dependent effects of Isoflavones *on ABCA1* and *ABCG1* mRNA expression. LNCaP and PC-3 cells were treated for 48 h with or without 0.1 to 10 μM genistein, daidzein, equol, glycitein or glyceollin. **A and B,** Dose effects of isoflavones *ABCA1* and *ABCG1* mRNA induction in LNCaP cells. *ABCA1*, *ABCG1* mRNA induction is expressed as means ± SE of fold change relative to control. Data are representative of three independent experiments. Points with an asterisk (*) are significantly different than control ($p < 0.05$). **C and D,** Dose effects of isoflavones on *ABCA1* and *ABCG1* mRNA induction in PC-3 cells. Points with an asterisk (*) are significantly different than control ($p < 0.05$).
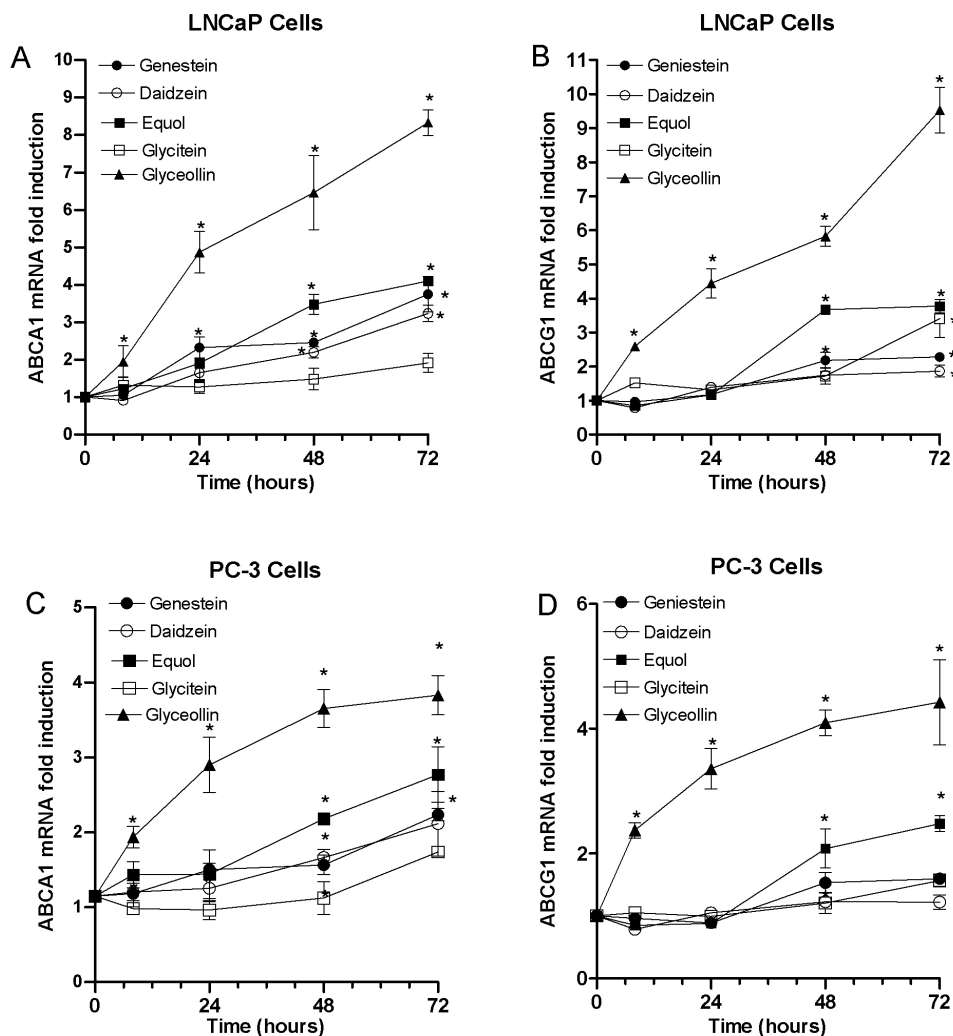
Figure 2. Temporal effects of Isoflavones on *ABCA1* and *ABCG1* mRNA expression. LNCaP and PC-3 cells treated for 2 to 24 h with or without 5 μM of genistein, daidzein, equol, glycitein or glyceollin. After treatments cells were subjected to RNA isolation and RT-PCR as described in materials and methods. **A and B,** Temporal effect of isoflavones on *ABCA1* and *ABCG1* mRNA induction in LNCaP cells. *ABCA1, ABCG1* mRNA induction is expressed as means ± SE of fold change relative to control at each time point. Points with an asterisk (*) are significantly different than control ($p < 0.05$). **C and D,** Temporal effect of isoflavones on *ABCA1* and *ABCG1* mRNA induction in PC-3 cells. *ABCA1, ABCG1* mRNA induction is expressed as means ± SE of fold change relative to control at each time point. Points with an asterisk (*) are significantly different than control ($p < 0.05$).

Figure 3. Effects of LXRα siRNA on isoflavone mediated expression of *ABCA1* and *ABCG1* in LNCaP cells. LNCaP cells were transfected with or without LXRα siRNA oligonucleotides for 48 h and then treated with or without 5 μM genistein, daidzein, equol, glycitein or glyceollin for an additional 48 h. Relative mRNA levels are expressed as means ± SE of fold change relative to control. Error bars with a different letter indicate significant differences between groups ($p < 0.05$). **A,** Effects of LXRα siRNA transfection and isoflavones on *LXRα,* and *LXRβ* mRNA levels in LNCaP cells. **B,** Effects of LXRα siRNA transfection and isoflavones on *ABCA1* and *ABCG1* mRNA levels in LNCaP cells. **C,** Effects of LXRβ siRNA transfection and isoflavones on *ABCA1* and *ABCG1* mRNA levels in LNCaP cells. **D,** Effects of LXRβ siRNA transfection and isoflavones on *ABCA1* and *ABCG1* mRNA levels in LNCaP cells.

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Figure 4. Effects of LXRα siRNA on isoflavone mediated expression of *ABCA1* and *ABCG1* in PC-3 cells. PC-3 cells were transfected with or without LXRα siRNA oligonucleotides for 48 h and then treated with or without 5 μM genistein, daidzein, equol, glycitein or glyceollin for an additional 48 h. Relative mRNA levels are expressed as means ± SE of fold change relative to control. Error bars with a different letter indicate significant differences between groups (*p* < 0.05). **A,** Effects of LXRα siRNA transfection and isoflavones on *LXRα,* and *LXRβ* mRNA levels in PC-3 cells. **B,** Effects of LXRα siRNA transfection and isoflavones on *ABCA1* and *ABCG1* mRNA levels in PC-3 cells. **C,** Effects of LXRβ siRNA transfection and isoflavones on *ABCA1* and *ABCG1* mRNA levels in PC-3 cells. **D,** Effects of LXRβ siRNA transfection and isoflavones on *ABCA1* and *ABCG1* mRNA levels in PC-3 cells.
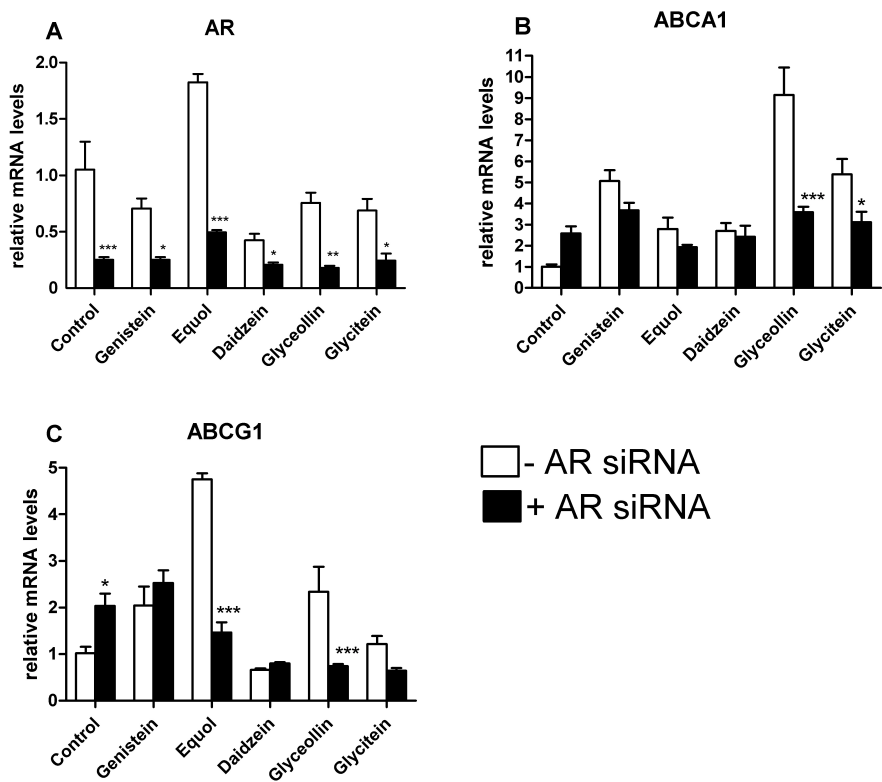
Figure 5. Effects of AR siRNA on isoflavone mediated expression of ABCA1 and ABCG1 in LNCaP cells. LNCaP cells were transfected with or without AR siRNA oligonucleotides for 48 h and then treated with or without 5 μM genistein, daidzein, equol, glycitein or glyceollin for an additional 48 h. Relative mRNA levels are expressed as means ± SE of fold change relative to control. Error bars with an asterisk indicate a significant difference between –AR transfected and +AR siRNA transfected cells with *=$p < 0.05$, **= $p < 0.01$ and ***= $p < 0.001$. **A,** Effects of AR siRNA on *AR* mRNA levels in LNCaP cells. **B,** Effects of AR siRNA transfection and isoflavones on *ABCA1* and *ABCG1* mRNA levels in LNCaP cells. **C,** Effects of AR siRNA transfection and isoflavones on *ABCG1* mRNA levels in LNCaP cells.
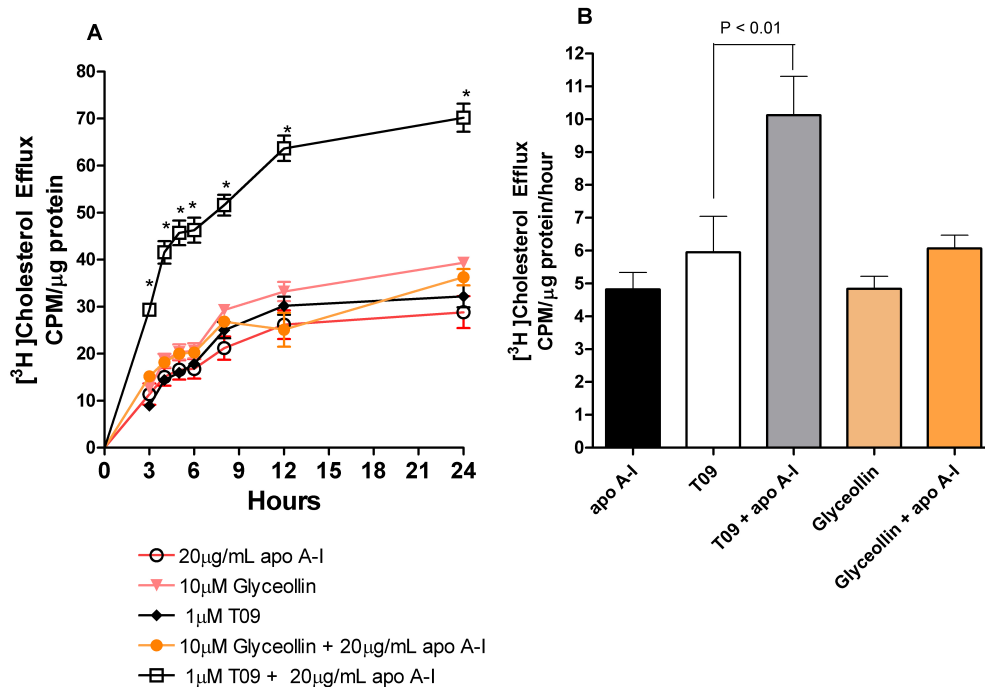
Figure 6. Effects of Glyceollin on apo A-I mediated Reverse Cholesterol Transport. PC-3 cells were loaded with 1μCi/mL of [³H] labeled cholesterol. After 48 h PC-3 treated with either 1μM of the synthetic LXR ligand T09, vehicle (DMSO 0.05% v/v), or 10 μM glyceollin for 48 h in serum free, phenol free RPMI-1640 medium. After 48 h cells were exposed to either vehicle or 20 μg/mL of human apo A-I for 24 h in serum free medium to initiate RCT. Aliquots of culture medium was collected every 2 h and radioactively determined using a scintillation. Data are representative of the mean ± SE of three independent experiments. Error bars with an asterisk (*) indicate significant differences between apo A-I and T09 + apo A-I ($p < 0.01$). **A,** Temporal changes in cholesterol efflux in PC-3 cells. **B,** Rate of cholesterol efflux in PC-3 cells.

Chapter 4: The Pro-Biotic, Lactobacillus casei, Prevents Cholesteryl-ester Accumulation and LPS modulation of the LXR and Inflammatory Axis in Alveolar Macrophages from Ossabaw pigs fed a High Fat Diet.

INTRODUCTION

Experimental Rationale

It is now well documented that obesity and hypercholesterolemia can promote a pro-inflammatory state and contribute to inflammation related morbidities such as atherosclerosis, stroke and cardiovascular disease (52). There is recent data demonstrating that obesity and hypercholesterolemia can also lead to altered pulmonary immunity (53-55). Alveolar macrophages have a fundamental role in pulmonary innate immunity through their ability to recognize and phagocytose pathogenic organisms and to orchestrate and expand the adaptive immune response through secretion of inflammatory mediators and antigen presentation (50). As coordinators of immune surveillance and pulmonary function, AM may contribute to altered pulmonary phenotype associated with obesity and hypercholesterolemia.

High fat, obesogenic diets increase adiposity and elevate serum cholesterol levels. Through a number of proposed mechanism discussed in more detail in citation (123, 124), it is has been demonstrated that serum low density lipoprotein cholesterol (LDL) can cross the sub-endothelial space of arterial sites where it is

prone to oxidation and is readily internalized by macrophages via a number of scavenger receptors (123,124). Macrophage endogenous cellular cholesterol metabolism is balanced between uptake (via LDL receptors and scavenger receptors) and efflux via LXR-ABCA1 (18). However, it has been demonstrated that feedback inhibition of scavenger receptors such as CD36 is impaired by phagocytosis of modified LDL cholesterol, potentially disrupting the balance of cholesterol removal and promoting cholesterol retention by macrophages (124). LXRs exist to protect macrophages and other cells from unchecked accumulation of cholesterol thought its ability to induce cholesterol efflux to lipid poor lipoproteins (18). However, if the micro-environmental conditions favor macrophage cholesterol retention due to either compromised efflux or increased uptake of modified cholesterol, accumulation of cholesteryl-ester rich cytoplasmic lipid droplets occurs and leads to onset on a macrophage foam cell development (123, 124). Continued accumulation of lipids will eventually lead to macrophage foam cell apoptosis and release of their internalized lipids and inflammatory mediators. There is a large body of data detailing an altered immunocompetency of peritoneal or atheroma derived foam cells macrophages against *Chlamydia pneumoniae* infection (125-127). However, in lungs, clinical detection of alveolar lipid laden macrophages is associated with poor pulmonary host defense and pulmonary function (128).

Experimental studies have led to the belief that hypercholesterolemia can overwhelm LXR regulation of cholesterol metabolism and the immune response and negatively impact both pathways (24). This may have overarching

consequences in tissue such as the lungs where lipid accumulation and inflammation can alter organ function and promote disease (51-54). Numerous studies link bacterial infection with the onset of foam cell macrophage phenotype (125-127). Pathogens can oppose LXR mediated cholesterol efflux in macrophages through activation of TLR4 and TLR2 downstream effectors MyD88 and IRF-3 and promote onset of a lipid laden foam cell phenotype (24, 128, and 130). Administration of LXR ligands can oppose this mechanism through direct antagonism of IRF-3 and by increases removal of excess free cholesterol (129, 130). This is the basis for intensive scrutiny of LXR as atheroprotective target in treatment of cardiovascular disease. In pulmonary macrophages it is less clear what role LXRs can have in attenuating either bacterial or diet induced onset of a foam cell phenotype. Studies have demonstrated that disruption of ABCA1 or ABCG1 mediated efflux from pulmonary AM leads impaired pulmonary function and leads to an augmented release of pro-inflammatory mediators such as IL-1β, TNF-α, IL-6 and IL-8 in response to bacterial endotoxin lipopolysacharide (LPS) (51-54).

Because AM macrophages contribute significantly to pulmonary inflammation, they represent an important target of therapies aimed at preventing or diminishing lipid and inflammation related pathologies. In recent years, a growing body of evidence has suggested that increased presence of some intestinal probiotic bacteria is associated with a decreased risk for inflammatory related conditions in the lungs such as asthma (131). Pro-biotics are host or commensal-derived bacteria such as *Lactobacillus reuteri, Lactobacillus casei*,

and *Bifidobacterium longum,* which can form bio-films in the lower intestinal tract of their host (132-134). Pro-biotic bacteria are defined by the World Health Organization as "any live microorganism which, when administered in adequate amounts, confer a health benefit on the host" (134). The basis for this definition comes from a body of data demonstrating that accumulation of these probiotic bacteria by diet can beneficially regulate host immunity in the gastrointestinal tract and in peripheral tissue (132-134). Clinical studies have demonstrated that daily supplementation with some probiotic species can increase gene expression of regulatory cytokines such as *IL-10* and *TGF-β* and oppose expression pro-inflammatory cytokines such as *TNF-α* and *IL-12* in the lamina propria of patients with irritable bowel syndrome, Crohn's' disease and ulcerative colitis as effectively as corticosteroids (132, 133). Supplementation with some strains of probiotic bacteria has also been shown to decreases the magnitude of pulmonary inflammation in patients with cystic fibrosis and lower serum cholesterol lipoprotein levels (132, 133). However, this effect is strain specific as some probiotic bacteria strains can actually increase systemic expression of proinflammatory mediators such as IL-6 and TNF-α (135). The observed disparities between some probiotic strains to benefits to host biology confounds broad advocacy for use of these bacteria to ameliorate inflammatory or lipid related maladies. Nevertheless, the evidence that some probiotic bacteria can positively affect inflammatory conditions outside of those observed in the gut and to lower serum cholesterol levels, warrants examination of each specifies to determine the presence of unique biological properties. Furthermore, the potential

for some probiotic bacteria to positively affect inflammation conditions and lower serum cholesterol levels make it an ideal therapy to test against conditions where both dyslipidemia and inflammation are involved.

This study will examine whether alveolar macrophages (AM) from juvenile Ossabaw pigs fed a HF diet undergo foam cell formation and exhibit and exaggerated immune response to LPS stimulation *ex vivo*. HF diets will also be examined for any interactions with the LXR mediated pathways in light of the central role that LXRs play in macrophage cholesterol metabolism (24). This study will also examine whether daily supplementation with the probiotic *Lactobacillus casei* can oppose any of the pro-inflammatory or lipid altering effects of a HF diet. It will also be examined whether LXR activation *ex-vivo* can oppose the inflammatory effect of LPS and whether this affect may interact with either PB or HF diets.

Specific Hypotheses Tested:

a) To test the hypothesis that an obesogenic, high fat (HF) diet or a HF diet supplemented with the probiotic *Lactobacillus casei* (HFPB) can modulate cholesteryl-ester accumulation and the LXR dependant pathway in alveolar macrophages (AM) isolated from juvenile Ossabaw pigs.

b) To test the hypothesis that HF feeding leads to an exaggerated immune response in AM stimulated by LPS *ex vivo*.

c) To test the hypothesis that LPS stimulation *ex vivo* can oppose LXR transcriptional activation in AM.

d) To test the hypothesis that AM from HFPB fed pigs will have decreased gene and protein expression of inflammatory mediators compared to HF fed pigs.

e) To test the hypothesis that LXR activation *ex-vivo* is anti-inflammatory.

ABSTRACT

Liver X Receptors (LXRs) are important regulators of cholesterol metabolism and the inflammatory response in macrophages. It is hypothesized that hypercholesterolemia can lead to impaired cholesterol metabolism by LXRs leading to macrophage cholesteryl-ester (CE) accumulation and an altered inflammatory response. High fat (HF) diets can lead to dyslipidemia and promote a pro-inflammatory phenotype marked by alterations of macrophage function and increased expression of pro-inflammatory mediators. As modulators of inflammation and cholesterol metabolism LXRs might prevent or diminish dyslipidemia and inflammation related pathologies caused by HF diets. Certain probiotic bacteria also have anti-inflammatory properties but have not been tested for any interactions with the LXR pathway in macrophages. The present study sought to determine whether dietary supplementation with the probiotics bacteria *Lactobacillus casei* (*L.casei*) to Ossabaw pigs would oppose the pro-inflammatory effects of a HF diet on expression of genes related to cholesterol metabolism and the pulmonary innate immune response in isolated alveolar macrophages (AM). AM isolated from pigs fed an HF diet had significantly higher concentrations of CE compared with AM from control (C) diet fed pigs suggesting the formation of a foam cell phenotype. AM from pigs fed HF diet supplemented with *L. casei* (HFPB) had no significant accumulation of CE. We observed that AM from pigs

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fed a control (C) diet supplemented with *L. casei* (CPB), had significantly higher mRNA levels of *IL-8* and *IL-6* in response to LPS. Protein analysis cell culture supernatants revealed that AM from HFPB fed pigs had significantly lower LPS mediated protein expression of IL-1β than AM from HF fed pigs. *Ex-vivo* activation of LXR with a specific LXR ligand T0901317 (T09) significantly opposed LPS mediated upregulation of *IL-1β*, *IL-6*, *IL-8* and *IL-10* mRNA levels in AM from HF, HFPB and CPB fed pigs. However, T09 only opposed LPS mediated protein expression of IL-1β in AM from HF fed pigs. Finally, it was observed that LPS stimulation lead to significant inhibition of LXR transcription of *LXRα, ABCA1*, *ABCG1*, cholesterol 25 hydroxylase (*CH25H*) and *PPARγ* in AM. This effect was abrogated by *L. casei* for *ABCA1*, *CH25H* and *PPARγ* mRNA expression. This study demonstrates that a HF, obesogenic diet supplemented with the probiotic *L. casei* can prevent CE accumulation in pulmonary AM and dampen LPS mediated stimulation of IL-1β and TNF-α. Moreover, dietary supplementation with *L. casei* opposed LPS antagonism of the LXR and cholesterol metabolism related genes: *ABCA1*, *CH25H* and *PPARγ*. Taken together these data demonstrate a new role for *L. casei* in modulating pulmonary inflammation and cholesterol metabolism.

MATERIALS AND METHODS

Animals and Diets

Twenty, 7 week old female Ossabaw pigs were obtained from Indiana University Ossabaw production facility. Upon arrival pigs were housed in barn stalls with a nonabsorptive concrete floor surface, with one pig per pen until

adjustment to new surrounds. After adjustment, two pigs were placed in each pen and had access to water ad libitum and feed was measured daily. Animals (n=20) were randomly assigned to receive one of the following diets: i) control (n=10), (C) diet at 2900 cal/ kg body weight per day [13% kcal fat, 22% kcal protein and 64% kcal carbohydrate] or ii), a high fat (n=10), (HF) diet at 3500 cal/kg body weight per day [33% kcal fat, 17% kcal protein, 48% kcal carbohydrate]. Within the C and HF diet groups, animals (n=5) where further randomized to receive either a daily oral gavage of either 1×10^{10} colony forming units (cfu) of the probiotic *Lactobacillus casei* (admixed in maltrodextin) or vehicle placebo (maltrodextin alone) . These dietary groups were further categorized as either: iii) control diet + probiotic (CPB), or iv) a HF diet + probiotic (HFPB). Animals were maintained on each of the four diets for 28 weeks until study end.

Pulmonary Bronchial Alveolar Lavage

Alveolar macrophages (AM) were isolated from pigs at necropsy by bronchial alveolar lavage (BAL) as previously described (136). Briefly, the right lobe of a lung was gravity filled with 500 ml of PBS, followed by massaging for 30 s and draining of the cell suspension into 50 ml polypropylene tubes. The cells were washed in PBS and resuspended in RPMI 1640 medium and used for *ex-vivo* experiments the same day.

Unstimulated AM and *Ex-vivo* AM Experiments

Cell counts and viability of AM isolated from BAL were determined using trypan blue staining and hemocytometer counting. Determination of a diet effect on AM gene expression, 1×10^7 cells were counted from freshly isolated BAL

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fluid and immediately frozen at -80 °C without serum for future gene expression

analysis. For *ex vivo* experiments, AM from each animal were resuspended in

macrophage culture medium [RPMI 1640, 10% FBS, 1% non-essential amino

acids, 1% sodium pyruvate, 10 mM HEPES, 100 U/mL penicillin, 100 µg/mL

streptomycin, 1.5 g/L sodium bicarbonate] and seeded at a density of 2.5 x 10^6

cells/mL in 6 well plates. AM from each animal were treated with either: vehicle

(0.125% DMSO), 0.5 ng/mL lipopolysaccharide (LPS), 2.5 µM of the synthetic

LXR ligand T09 or LPS + T09 for 24 h. After 24 h, cell culture supernatants were

collected and frozen for subsequent protein analysis and cells where lysed with 2

mL of TRIzol® reagent (Invitrogen, Life Technologies, Carlsbad, CA), and frozen

for subsequent RNA isolation and gene expression analysis.

Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT- PCR)

Total RNA from unstimulated and *ex vivo* AM cell lysates was isolated

using the PureLink® RNA isolation kit (Invitrogen, Life Technologies, Carlsbad,

CA) according to the manufacture's protocol. Integrity and concentration of

purified RNA were determined using Experion™ RNA gel electrophoresis

analysis chips (Bio-Rad, Hercules, CA). Sample RNA integrity was compared to

an intact RNA standard and scored using a validated RNA quality indicator (RQI)

assigned by the Experion RNA analyzer software, version 3.2. All the samples

scored between 9.6 and 10, with a score of 10 indicating no RNA degradation.

First-strand cDNA synthesis was made from 1 μg of total RNA using

Superscript™ III reverse transcriptase according to the manufacturer's protocol

(Invitrogen, Life Technologies, Carlsbad, CA). Real-time RT-PCR was used for

amplification of panel of genes related to inflammation and lipid metabolism listed in Table 1. All probes and primers for real-time RT-PCR were previously designed using the Primer Express (Applied Biosystems, Foster City, CA, USA) software package and nucleotide sequences obtained from GenBank or the TIGR porcine EST database. A list of the sequences used for primer design are available online at the USDA Porcine Immunology and Nutrition (PIN) database: www.ars.usda.gov/Services/docs.htm?docid=6065. Real-time RT-PCR was performed using a 25 ng of cDNA template (cDNA concentrations are based on RNA concentrations determined from gel electrophoresis and assume 100% conversion of total RNA to first strand cDNA) and 25 μl of Absolute™ QPCR low ROX master mix (ABgene, Epsom, UK) per sample on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). Quantitation of mRNA fold changes was derived using the delta threshold cycle (Δ Ct) method. Data are presented as the mean fold change in gene expression relevant to either C fed animals or vehicle treated controls.

Macrophage Cholesterol Analysis

On the day of necropsy, 1 x 10^8 cells of AM were aliquoted and frozen at -80° C for future analysis of cellular cholesterol levels. On the day of analysis, cells were thawed on ice followed by lysis with cold RIPA buffer (Thermo) using 1 mL of RIPA per 10^7 cells. Cell homogenates were sonicated at 50% intensity for 30 s on ice followed by incubation for 30 min on ice. Total cellular cholesterol and neutral lipids were extracted from cell lysates using the Folch method as previously described (100). Briefly, total lipids were extracted from aliquots of

cell homogenates using chloroform: methanol (2:1) and partitioned using dH_2O. Organic phase solvents containing lipids were evaporated under nitrogen gas and resuspend in 0.5% (v/v) Triton X-100 solution in water. Total and free cholesterol were determined enzymatically using Amplex red cholesterol quantitation kit (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacture's protocol. Cellular cholesterol content was normalized to whole cell lysate protein concentrations. Protein was determined using the BCA assay (Thermo).

Cell Culture Supernatant Protein Analysis

Protein expression of five pro-inflammatory mediators (IL-1β, IL-6, IL-8, TNF-α and IL-10) in AM cell culture supernatants collected at termination of *ex-vivo* experiments were measured using a multiplex Procarta® Cytokine Profiling Kit (Affymetrix Santa Clara, CA) according to the manufacturer's protocol. Profiling kits were processed on a Bio-Plex 200 multiplex bead-based array system and analyte concentrations determined using Bio-Plex Manager software suite version 6.0 (Bio-Rad, Hercules CA).

Statistics

Statistical and power analysis of data was carried out with the GraphPad PRISM4 program and GraphPad Stat Mat 2 (GraphPad Software, Inc.). Contrasts of group means were computed using one or two factor ANOVA followed by Bonferroni post-hoc tests. Gene expression results will be expressed as means ± SE of comparative fold differences. One and two factor repeat measures ANOVA will be used to analyze BAL cell gene and protein expression from

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Ossabaw pigs. Diet, LPS and LXR were treated as independent variables. Mean

differences of post-hoc analyses are considered significant when p value is < 0.05.

RESULTS

Body Weight and Lipid Profile after 28 Weeks of HF diet

At the end of 34 weeks of HF feeding mean body weights were

significantly higher in pigs fed a HF and HFPB compared to pigs fed either a C or

CPB diet ($p < 0.01$, Table 2). Serum cholesterol levels in HF and HFPB fed pigs

were significantly elevated compared to C and CPB fed animals ($p < 0.001$, Table

2). Daily gavage with *L. casei* had no effect on serum cholesterol levels in either

C or HF fed pigs. There were no differences in plasma triglycerides among all the

diet groups (Table 2).

Cholesterol Analysis in Alveolar Macrophages

To determine whether an obesogenic, HF diet led to abnormal lipid

accumulation in pulmonary macrophages, cellular CE and free cholesterol (FC)

were analyzed in AM from pigs fed a HF obesogenic diet. Our data revealed that

AM from HF fed pigs had significantly higher concentrations of CE and FC

compared to AM from C and CPB fed pigs ($p < 0.001$, Fig 1). However, AM

from pigs fed a HFPB diet did not have any increase in CE or FC compared to

AM from C, CPB or HF fed pigs (Fig 1). There were no significant differences in

CE and FC concentrations between AM from C and CPB fed pigs. These data

suggest that daily gavage with the probiotic *L. casei* can protect against AM lipid

accumulation due to a HF, obesogenic diet, even in the face of higher serum

cholesterol levels.

Gene Expression in Unstimulated AM cells

In order to determine whether an obesogenic, HF diet or supplementation

with *L. casei*, was associated with changes to gene expression of the LXR axis,

markers of foam cell development, and immune related genes (Table 1), RT-PCR

analysis of unstimulated AM cells was undertaken. Figure 2A demonstrates that

animals fed a HF diet showed no significant changes to LXR or foam cell related

marker gene expression compared to C fed pigs. However two genes related to

cholesterol uptake, *LDLR* and esterification of free cholesterol, *ACAT* were

significantly decreased in AM from CPB fed pigs compared to AM from C fed

pigs ($p < 0.05$). Gene expression of *CYP27A1*, a gene involved in metabolism of

cholesterol to the oxysterol 27, hydroxycholesterol, was increased 5-6 fold (Fig 2)

in AM from HF and HFPB fed pigs, but this was not significant. Analysis of a

panel of genes (Table 1) associated with onset of foam cell phenotype did not

reveal any dietary effects on these markers in AM from any of the diet groups

(Fig 2B). Analysis of gene expression of inflammatory mediators also failed to

demonstrate any dietary effect either from HF or from *L. casei* supplementation

(Fig 3).

Elevated mRNA Levels of Pro-inflammatory Mediators in LPS-treated

Alveolar Macrophages from High Fat and Pro-biotic fed pigs is Opposed by

LXR Activation

Augmented responsive of LPS mediated IL-1β mRNA levels in AM from HF fed pigs.

Others have reported that genetic ablation of ABCA1 leads to macrophage foam cell formation and an increased sensitivity to LPS. We observed that AM isolated from HF and HFPB fed pigs had a 1.9 and 2.4 fold greater mRNA expression of *IL-1β* in response to LPS stimulation compared to AM from C fed pigs (Fig 4A) ($p < 0.05$). There was no evidence that the LPS mediated mRNA increase in *IL-6, IL-10* or *TNF-α* was augmented in AM from HF fed pigs compared to AM from C fed pigs (Fig 4B-E). The statistical difference between LPS mediated expression of *IL-1β* mRNA in AM from HF compared to C fed pigs does suggests an augmented response due to diet. Two-way ANOVA, however, did not reveal a significant interaction between HF diet and LPS mediated *IL-1β* mRNA response. ($p = 0.117$).

We also observed that LPS stimulation resulted in significantly stronger mRNA response of *IL-6*, *IL-8* in AM isolated from CPB fed pigs compared to C fed pigs, ($p < 0.001$ and $p < 0.05$ respectively (Fig 4B and C). This effect was most pronounced for LPS stimulation of *IL-6* (Fig 4B). *IL-10* mRNA levels also where elevated in AM from CPB fed pigs compared to C fed pigs (~9 fold vs. ~4 fold), however this difference was not significant. There was a strong and significant interaction between CPB diet and LPS in *IL-6* mRNA response ($p = 0.0003$). Except for AM from HFPB fed pigs, LPS did not significantly increase mRNA levels of *TNF-α* in any of the diet groups.

Anti-inflammatory Effect of LXR activation.

Activation of LXR opposed the effects of LPS on mRNA expression of *IL-1β* in AM from HF and HFPB pigs ($p < 0.001$), but not in AM from C or CPB fed pigs (Fig 4A). The hyper-responsive mRNA expression of *IL-6* and *IL-10* in AM from CPB fed pigs was significantly inhibited by LXR activation ($p < 0.001$, $p < 0.05$), respectively (Fig 4B and 4D). LXR activation significantly opposed LPS stimulation of *IL-8* in AM from HF and CPB fed pigs ($p < 0.01$, $p < 0.05$), respectively, (Fig 4C).

Protein Analysis of Cell Culture Supernatants

To examine whether AM from HF fed pigs also had heightened secretion of pro-inflammatory mediators, protein expression of cytokines IL-1β, TNF-α, IL-6, IL-8 and IL-10 were analyzed in cell culture supernatants after 24 h of LPS and LXR stimulation from AM in each dietary group. The analysis revealed that protein expression of IL-1β was significantly increased by LPS stimulation in AM from all dietary groups (Fig 5A). IL-1β protein level from HF fed pigs was ~1.6 fold higher than AM from C fed pigs, however this difference was not significant (Fig 5A, $p = 0.021$). There was no significant difference between LPS mediated increase in TNF-α protein between AM from C or HF fed pigs (Fig 5B). LPS mediated protein levels of TNF-α protein was ~2 fold lower in HFPB fed pigs compared to AM from HF fed pigs, this difference was marginally significant ($p = 0.09$). No significant differences were observed for protein levels of IL-6, IL-8 or IL-10 from any treatments or diet groups (Fig 5C-E).

L casei supplementation opposed LPS mediated IL-1β protein levels

Protein levels of LPS mediated IL-1β protein expression in AM from HFPB was 2.5 fold lower than LPS mediated protein levels from AM HF fed pigs, ($p < 0.05$, Fig 5A black bars). LPS mediated TNF-α protein levels where 2.1 fold lower in AM from HFPB fed pigs compared to AM from HF fed pigs, however this effect was not significant (Fig 5B, $p = 0.08$).

Anti-inflammatory Effect of LXR activation

The LXR ligand T09 significantly opposed LPS induced IL-1β protein expression in AM from HF fed pigs only ($p < 0.05$, Fig 5A). No significant anti-inflammatory effect of LXR was observed for LPS mediated stimulation of TNF-α, IL-6, IL-8 and IL-10 in AM from C, CPB, HF and HFPB fed pigs (Fig 5B-E).

Effect of *L casei* Supplementation on LPS Antagonism of LXR and Cholesterol Metabolism Related Genes

Data from RT-PCR revealed that T09 mediated transcription of *LXRα* mRNA was opposed by LPS in AM from HF and HFPB fed pigs (Fig 6A, $p < 0.001$ and $p < 0.05$ respectively). LXR activation of *ABCA1* mRNA was significantly inhibited by LPS in AM from C and HF fed pigs ($p < 0.01$ and $p < 0.05$) respectively, but not in AM from CPB or HFPB fed pigs (Fig 6C). LPS also significantly inhibited LXR activation of *ABCG1* mRNA in HF fed pigs ($p < 0.001$, Fig 6D). This was not observed in AM from any other diet group (Fig 6D). LPS treatment had no effect on *LXRβ* mRNA levels either alone or in T09 treated AM from any of the diet groups (Fig 6B).

L. casei supplementation prevents LPS suppression of LXR mediated transcription of PPARγ and CH25H in AM from HF fed pig

25-hydroxycholesterol (25HC) is an LXR ligand synthesized from cholesterol by the microsomal enzyme CH25H (9, 56). We observed that LPS opposed LXR activation of *CH25H* mRNA in AM from C and HF fed pigs, but not in AM from CPB or HFPB fed pigs ($p < 0.001$ vs. ns, Fig 6E). LPS also significantly oppose LXR activation of *PPARγ* mRNA in AM from C and HF fed pigs ($p < 0.05$), this was not observed in AM from either CPB or HFPB fed pigs (Fig 6E).

DISCUSSION

There is growing evidence that supplementation with some probiotic bacteria can positively modulate gut immunity and have a positive clinical effect on treatment of inflammatory bowel diseases such as Crohn's, infectious diarrhea, and *Clostridium difficile* infection (90-93). The beneficial effects of probiotic are strain specific as some have been shown to positively influence inflammation (135). Until recently it was believed that the mechanisms of action of probiotic bacteria were limited to the gastrointestinal tract, but recent findings suggest that probiotics may lead to systemic benefits on host immunity and inflammatory conditions outside of the gut (90-93). In the present study we report that dietary supplementation with the probiotic bacteria *L. casei* is significantly associated with decreased CE accumulation in AM. We also report that dietary supplementation with *L. casei* opposed LPS mediated gene suppression of *ABCA1*, an LXR responsive genes vital to maintaining cholesterol homeostasis in

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pulmonary AM and pneumocytes (30). *L. casei* also significantly decrease the protein expression of LPS mediated IL-1β protein levels. Collectively, these data demonstrate a role for *L. casei* in modulating AM cholesterol metabolism, LXR responsive genes and pro-inflammatory cytokine expression. This suggests that supplementation with *L. casei* may benefit pulmonary abnormalities where both hypercholesterolemia and chronic inflammation are involved.

HF feeding was associated with Cholesteryl-ester Accumulation in AM

The hallmark of foam cell formation is accumulation of CE in macrophages (137). Analysis of cholesterol extractions from AM from BAL fluid lysates revealed that AM from pigs fed a HF diet had significantly higher concentrations of CE compared to AM from control diet fed animals (Fig 1). This strongly suggests an increase in the presence of foam cell AM in bronchoalveolar passages of HF fed pigs for three reasons: i. It is well documented that the majority of cells isolated from BAL lavage are AM, ii. Contamination from either epithelial cells, or other immune cells in the lavage would have little contribution to the cholesterol analysis because these cell types typically do not accumulate CE, and iii: The majority of cholesterol found in type 2 pneumocytes is unesterified and associated with lamellar body surfactant proteins (138-141).

The presence of foamy AM in BAL fluid of HF fed animals is consistent with the significant increase in serum levels of cholesterol observed in these animals (Table 1) and consistent with data demonstrating that dietary lipids can positively increase lipids levels in the alveolar compartment (138-141). Moreover, the CE concentration in AM from HF fed animals (~10 µg/mg protein)

is consistent with the CE concentrations observed in foam cell macrophages isolated from high fat fed mice (18). Future studies will be conducted to determine if the AM cholesterol profile is also mirrored in the AM within the lung parenchyma.

However an unanswered question is whether the source of excess cholesterol in AM was derived directly from the diet (i.e. LDL or scavenger receptors) or from a source of pulmonary cholesterol associated with surfactant protein (SAP). SAP is synthesized by pulmonary type 2 pneumocytes (type 2 cells), and is composed of 90% lipids, the majority of which is phospholipids and 10-15% neutral lipids, almost all of which are in the form of FC (138). Pulmonary surface SAP can be recycled and reused by type 2 cells or catabolized by AM. Internalization of adsorbed surfactant proteins and lipids is a vital function of AM and the cycle of maintaining pulmonary surface tension (138). Although both type 2 cells and AM can internalize SAP and lipids, *ex vivo* studies have demonstrated that AM typically internalize three to four times more surfactant protein and lipid than type 2 cells (138-141). SAP bound cholesterol represents approximately 80% of the cholesterol found in the lungs and the majority of this cholesterol is derived from circulating lipoprotein particles (138-141). Type 2 cells express LDL and VLDL receptor in their basolateral membrane where they can rapidly incorporate serum cholesterol and triglyceride from circulating lipoprotein particles (138-141). Therefore the largest pool of cholesterol that AM in the alveolar space would be exposed to is potentially from internalization of lipids associated with lamellar body surfactant proteins. Studies of rat lung perfusions with radio labeled

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lipoproteins demonstrated that surfactant cholesterol concentrations are increased by exogenous cholesterol incubations and type 2 cells preferentially incorporate cholesterol from serum lipoproteins, with less than 1% being derived from de novo cholesterol synthesis (138-141). Animal models have also demonstrated that obesogenic diet and hyperlipidemia can increase lipid uptake by type 2 cells and increased the deposition of SAP lipids on the surface of alveoli (138-141). Collectively, these data suggest that the source of the increased CE in AM from HF fed animals could be derived from type 2 cell surfactant lipids. This may have overarching consequences not only to normal lung physiology, but to the innate immune response of AM as it has been demonstrated that increased surfactant lipids can negatively impact AM innate immunity and that foam cell macrophages are, immuno-compromised, highly unstable, atherogenic and prone to apoptosis (18).

Gene expression profiling of AM from BAL fluid did not reveal any significant differences in relative mRNA levels of scavenger receptors such as *CD36*, *CD209*, *MSR-1* and *SCARB2* in AM of HF fed pigs (Fig 2B), but there was a significant decrease in *LDLR* and *ACAT* mRNA in AM from CPB fed pigs compared to C fed pigs (Fig 2A). The decreased expression of *LDLR* and *ACAT* mRNA was not reflected in the cholesterol profile of these cells (Fig 1), and also does not correlate with what would be expected in cells with lower cellular cholesterol levels, because decreased levels of cholesterol in the endoplasmic reticulum (ER) leads to an increase in LDLR receptor expression (9). *ACAT* mRNA levels might not necessary be reflected in the cholesterol profile of these

cells as it has been demonstrated in other cell types (142). However, if mechanistically *L. casei* supplementation resulted in a net decrease in cholesterol uptake by AM, this may not have been reflected in the gene expression changes in AM from HFPB fed pigs because of the dynamics nature of cholesterol metabolism under a HF diet. It would also be important to examine the effects of *L. casei* supplementation on hepatic expression of cytochrome P-450 cholesterol 7-alpha-hydroxylase (CYP7A1). CYP7A1 is responsible for the rate limiting conversion of hepatic cholesterol to bile acids and is also under the transcriptional regulation of LXRα (9). There is experimental evidence demonstrating that the probiotic bacteria *Lactobacillus plantarum* can modulate cholesterol levels by increasing hepatic conversion of cholesterol to bile salts through upregulation of CYP7A1 (146).

Two studies have demonstrated that mice lacking expression of either ABCA1 or ABCG1, present with gross pulmonary lipidosis and the presence of lipid-laden foamy AM (51-54). This demonstrates a role for LXR and the ABCA1/G1 efflux pathway for maintain normal lung cholesterol homeostasis. Gene expression analysis did not reveal any significant differences in *LXRα, LXRβ*, *ABCA1* and *ABCG1* mRNA expression between AM from HF fed pigs and C fed pigs demonstrating that the observed accumulation of CE in AM is not reflected by mRNA changes to expression of LXR or ABCA1/G1 transporters. This does not rule out the involvement of the LXR-ABCA1 pathway in development of a lipid laden AM phenotype because mRNA and protein levels of ABCA1 and LXR expression in human atheromas do always not correlate (146).

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L. casei Supplementation Prevents Cholesteryl-ester Accumulation in AM from HF fed pigs.

Analysis of lipid extracts from BAL fluid in HF fed pigs supplemented with _L.casei_ revealed a significantly lower concentration of CE compared to AM from HF fed pig. There was no effect of _L casei_ alone on cholesterol accumulation in AM from control fed pigs, which may suggest that any hypo-cholesterolemic effect of this strain of _L. casei_ occurs only in the context of gross lipid burden. It is also important to note that the serum cholesterol profile of HFPB fed pigs significantly elevated (~100 ng/dl higher) than HF fed pigs (Table 1, $p < 0.05$), and despite this difference, the AM of HFPB clearly showed less evidence of CE accumulation (Fig 1). This suggests that the cholesterol lowering effect of this strain of _L. casei_ may be cell specific and is not reflected by the serum cholesterol profile. Two primary mechanisms for the lipid lowering effect of probiotics have been proposed. The first involves probiotic assimilation of intestinal lipids, thus decreasing the availability pool of post prandial cholesterol lipids for absorption (90). The second involves an increase in probiotic bile salt hydrolase (BSH) activity (90-93). Bacterial BSH catalyzed the deconjugation of the two major bile salts; cholic and chenodeoxycholic acid, which decreases their reabsorption across the entire length of small intestine and increases their removal in feces (93). It is estimated that approximately 5% of all bile acids and free cholesterol secreted in the small intestine are removed in the feces by this mechanism, which has been shown to have modest effects on serum cholesterol levels (93). However these mechanisms do not appear to be involved in the

cholesterol lowering effect observed in this study because *L. casei*

supplementation had no effect on serum lipids in either PB or HFPB fed pigs.

The findings in the current study may reflect a unique lipid modulating property

of this strain of *L. casei* and sugges t that studies reporting strain specific effects

on serum cholesterol in human and animals may overlook tissue or cell specific

lipid modulating properties.

Increased LPS mediated IL-1β mRNA Expression in AM from HF Fed Pigs

Gene expression and protein analysis revealed that a HF diet was

associated with a significantly higher mRNA expression of *IL-1β* in response to

LPS in AM from HF fed pigs compared to C fed pigs; however this effect did not

carry over to the protein levels (Fig 4A and 5A). AM from HF had approximately

a 2 fold increase in LPS mediated *IL-1β* mRNA levels compared to AM from C

fed pigs (Fig 4A). This is consistent with the increased levels observed in studies

of lipid laden-AM from ABCG1 -/- null mice which display an augmented

response to LPS (53). Still, in this study we did not observe the broad

augmentation of LPS mediated induction of inflammatory mediators as reported

in studies of lipid laden LPS or bacterial challenged macrophages (53-55). A post-

hoc power analysis suggests that the current study design with a 4 diet groups and

a sample of 5 animals, is approximately 30% underpowered to detect small

differences between LPS mediated responsive gene expression across four diet

groups and might explain the absence of a strong interaction between a HF diet

and AM inflammatory response in the current study. This information is

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important in for design of future studies designed to examine the same questions in the current pig model.

Despite the limitations of the current study design on these parameters, the significant increase in *IL-1β* mRNA levels in HF fed pigs suggests that animals on this diet would experience an augmented pulmonary immune response if challenged with an airborne pathogen. It is tempting to hypothesize that this would enhance host pulmonary immunity as it has been demonstrated in mice models of pulmonary lipidosis; however these studies also demonstrated that increased presence of AM foam cells is positively associated with increased pulmonary defects and host mortality. Future studies should be undertaken to determine whether HF feeding adversely affects pigs challenged with air borne pathogens. However deeper examination of the interactions between diet, pulmonary lipid accumulation and pulmonary inflammatory response, would require a larger cohort of animals in order to detect any small but significant differences which may occur due to these experimental parameters.

Effects of *L. casei* Supplementation on Expression of Inflammatory Mediators

Post-hoc analysis revealed that LPS mediated IL-1β protein expression in AM from HFPB fed pigs was significantly lower than that in AM from HF fed pigs ($p < 0.05$, Fig 5A). LPS mediated TNF-α protein expression was also decreased two fold in HFPB compared to HF fed pigs, but this effect was nearly significant at $p < 0.08$, Fig 5B). Protein expression of other cytokines did not reveal further evidence that *L. casei* had any anti-inflammatory effect in AM.

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However, taken together the decreased IL-1β and TNF-α protein levels do suggest that *L. casei* discharged an anti-inflammatory effect in AM from HFPB fed pigs. Both IL-1β and TNF-α are integral mediators of the innate immunity and remediation of pathogenic infection (51-54). However some conditions such as obesity, pulmonary cystic fibrosis or chronic obstructive pulmonary disorder (COPD) are associated with elevated levels of systemic and pulmonary cytokine levels and this is believed to have adverse effects on host immunity and normal tissue function (51-54). The observed anti-inflammatory effect of *L. casei* adds to a growing body of data demonstrating that probiotic bacteria can modulate systemic ant-inflammatory mediators outside of gut mediated mucosal immunity (90-93). *L. casei* may prove to be beneficial in ameliorating inflammation in pathologies where chronic inflammation has been observed. A fundamental question is whether the dampened immune response in AM from HFPB fed pigs would translate to an impaired immune response.

AM from PB fed pigs showed augmented mRNA expression of *IL-6*, *IL-8* and *IL-10,* however, these mRNA changes where not reflected in supernatant protein levels. Future studies examining this effect should examine earlier time points as other have demonstrated that *L. casei* inoculated mice have augmented release of IL-6 and TNF-α up to 8 h post-infection compared to control mice but have better clearance of air borne pathogens than non inoculated litter mates (143, 144).

L. casei Supplementation Prevents LPS Antagonism of ABCA1 and ABCG1 Expression

It is recognized that cholesterol plays an integral role in the pathogenesis of atherosclerosis and replication of human immunodeficiency virus -1 (HIV-1) in host cells (45, 46). Foam cells are highly apoptotic and undergo necrosis and calcification leading to release of their cholesterol and cytokine rich vacuoles (18). This cascade is central to the pathogenesis of atheroma formation, but may also negative impact host immunity as foam cell macrophage may be immuno-compromised (126, 127). Both viral and bacterial pathogenic products lead to impaired cholesterol metabolism in macrophages and lead to foam cell formation suggesting a potential mechanism for bacterial suppression of innate immunity (126).

In the current study we observed that the TLR4 ligand LPS opposed LXR transcription of *ABCA1*, *ABCG1* in AM. This is consistent with a study by Tontonoz et. al. that demonstrated TLR3 and TLR4 activation strongly oppose transcriptional activation of LXR responsive genes ABCA1 and ABCG1 in macrophages through a mechanism involving IRF3 trans-repression of LXR transcriptional activation (130). Others have demonstrated associations between viral and bacterial products and foam cell formation in macrophages, but the study by Tontonoz et. al. was the first to characterize specific cross talk between TRL4 and LXR in antagonism of macrophage cholesterol metabolism and cholesterol efflux (130). Other studies have subsequently shown that both IRF3 and MyD88 are involved in antagonism of LXR signaling (129, 130). Our data is

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the first to demonstrate that LPS/TLR4 can oppose LXR transcription in AM, but it is unclear what affect this may have on AM cholesterol metabolism. Loss of ABCA1 negatively impacts cholesterol efflux and can lead to macrophage foam cell transformation when accompanied by increased serum cholesterol levels. Therefore, this data suggests that if exposed to chronic TLR4 challenge, that pigs fed a HF fed diet may be at increased risk for AM foam cell development. Further experiments should be conducted to examine the effect that LPS antagonism may have on AM cholesterol metabolism and any potential involvement of LXR-ABCA1 *in vivo*.

Gene expression analysis also revealed that AM from both CPB and HFPB fed pigs were resistant to LPS antagonism of LXR mediated transcription of *ABCA1* mRNA revealing a potential novel mechanism behind the cholesterol lowering effect of *L. casei.* This finding was interesting because TLR4 antagonism of LXR responsive genes in peritoneal macrophages is unopposed by LXR ligand activation. Our data is the first to demonstrate probiotic opposition of TLR4 mediated suppression of the LXR axis. This effect by *L.casei* might specifically involve antagonism of MyD88 or IRF3 suppression of LXR since it has been demonstrated that both pathways can oppose LXR and MyD88 is involved in some of the immune-modulating properties of some lactobacillus bacteria (131). The diminished LPS mediated cytokine secretion of IL-1β and TNF-α in AM from *L casei* supplemented animals (Fig 6), does suggest an interaction between *L. casei* and TLR4 signaling and therefore provides the rationale to examine interactions between *L. casei* on some of the downstream

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effectors of TLR4. However gene expression in unstimulated AM did not demonstrate any antagonism of the TLR4 mediated expression of inflammatory mediators by *L. casei*, therefore a MyD88 independent pathway involving IRF3 might be equally involved. The apparent lack of LPS antagonism of LXR mediated transcription of *ABCA1* and *ABCG1* in AM from PB and HFPB fed supports a specific involvement of *L. casei* in antagonizing LPS suppression of the LXR axis.

Expression of *PPARγ*, and *CH25H,* two genes involved in cholesterol metabolism, was antagonized by LPS in T09 stimulated AM, however this was not observed in AM from PB and HFPB fed pigs. PPARγ, can indirectly promote cholesterol efflux in macrophages through increased regulation of LXRα, whereas CH25H is a key enzyme in metabolism of cholesterol to the LXR ligand 25HC (18). This data supports a broader mechanism through which *L. casei* can exert its modulation of lipid and cholesterol axis in AM.

It is feasible that hypothesize that *L. casei* antagonism of LPS/TLR4 suppression of the LXR axis is a mechanism supporting the decreased accumulation of CE in HFPB fed pigs. However future studies should examine whether LXR mediated cholesterol efflux is antagonized by LPS treatment and whether *L. casei* supplementation can abrogate that. Nevertheless, the data in the current study collectively these data demonstrate a cell specific lipid lowering effect of *L. casei* and a potential mechanism involving the LXR axis.

List of Figures with legends

LXR/Lipid Related Genes	Nuclear receptor subfamily 1, group H, member 3	NR1H3
	Nuclear receptor subfamily 1, group H, member 2	NR1H2
	ATP-binding cassette, sub-family A member 1	ABCA1
	ATP-binding cassette, sub-family G member 1	ABCG1
	Apolipoprotein E	APOE
	Fatty Acid Synthase	FAS
	Sterol-regulatory element binding protein, type 1 (SREBP-1)	SREBF1
	Sterol-regulatory element binding protein, type 2 (SREBP-2)	SREBF2
	Low density lipoprotein receptor	LDLR
	Scavenger receptor, type I (SR-BI)	SCARB1
	Scavenger receptor class B, member 2	SCARB2
	Monocyte/macrophage serine esterase 1A	CES1A
	Monocyte/macrophage serine esterase 1B	CES1B
	Acyl-Coenzyme A: cholesterol acyltransferase) 1	ACAT1
	Cytochrome P450-sterol 27-hydroxylase	CYP27A1
	25 cholesterol hydroxylase	CH25H
	Sulfotransferase family cytosolic 2B member 1	SULT2B1
	Cluster determinant 36	CD36
	Peroxisome proliferator-activated receptor, (PPAR) gamma	PPARG
Innate Immunity Related Genes	Tumor necrosis factor-α	TNFA
	Interleukin 1, β	IL1B
	Interleukin 6	IL6
	Interleukin 8	IL8
	Interleukin 10	IL10
	Interferon-gamma	IFNG
	Inducible NOS	iNOS
	Cyclooxygenase 2	COX-2
	Dendritic Cell-Specific Intercellular adhesion molecule	CD209
	Macrophage scavenger receptor 1	MSR1
	Macrophage receptor with collagenous structure	MARCO
	CD5 antigen-like (scavenger receptor cysteine rich family))	CD5L
Housekeeping genes	Cyclophilin A	PPIA
	60S ribosomal protein L32	RPL32
	Ubiquitin C	UBC

Table 1: List of Genes Measured in AM Isolated from Ossabaw Pigs

	C diet (n=5)	CPB diet (n=5)	HF diet (n=5)	HFPB diet (n=5)
Protein (% kcal)	22.1	22.1	17.7	17.7
Fat (% kcal)	13.3	13.3	33.4	33.4
Carbohydrates (% kcal)	64.6	64.6	48.9	48.9
Lactobacillus casei (cfu/day)	*placebo*	1×10^{10}	*placebo*	1×10^{10}
Kcalories/day	2810.0	2810.0	3500.0	3500.0
Body Weight (kg)	35.6 ± 0.6 [a]	36.4 ± 1.3 [a]	42.2 ± 1.1 [b]	43.0 ± 1.6 [b]
Plasma Cholesterol (mg/dL)	77.6 ± 5.2 [a]	87.6 ± 2.7 [a]	215.8 ± 19.1 [a,b]	307.4 ± 51.46 [a,b,c]
Plasma Triglycerides (mg/dL)	22.4 ± 3.2	30.4 ± 2.1	25.4 ± 2.6	20.4 ± 2.6

Table 2. Body weight, plasma cholesterol and triglycerides are expressed as mean ± SE.

Means with a different letter indicate a significant difference between groups when $p < 0.05$.
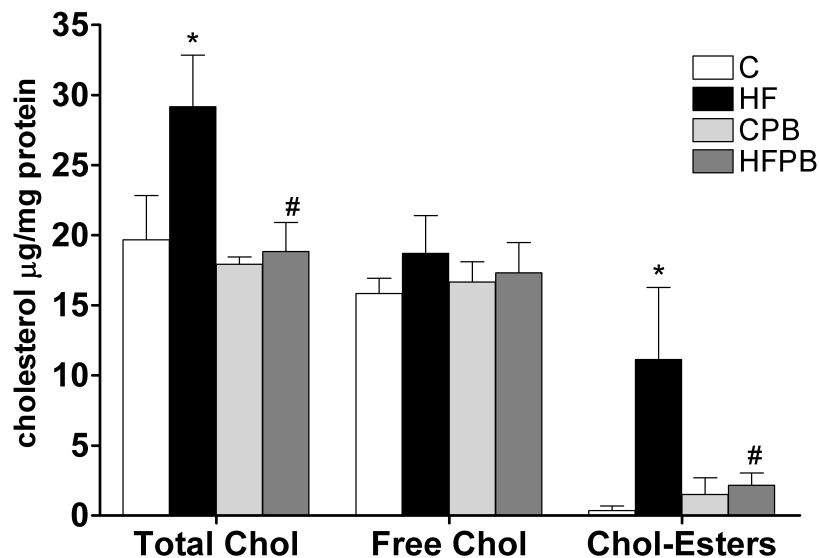
Figure 1. AM Cholesterol Profile. Total lipids were extracted from 1×10^8 AM isolated from Ossabaw pigs fed either a control diet (C), control + probiotic (CPB), high fat (HF) or high fat + probiotic(HFPB) and used to determine total cellular cholesterol-esters and free cholesterol. Cholesteryl-esters were calculated by subtracting the concentration of free cholesterol (µg/mg cellular protein) from total cholesterol concentration (µg/mg cellular protein). Data are representative of the mean ± SE of (n=5) animals per dietary group. Error bars with an asterisk (*) indicate significant difference between HF and C where * =$p < 0.05$. Error bars with a number sign (#) indicate a significant difference between HFPB and HF where # = $p < 0.05$.
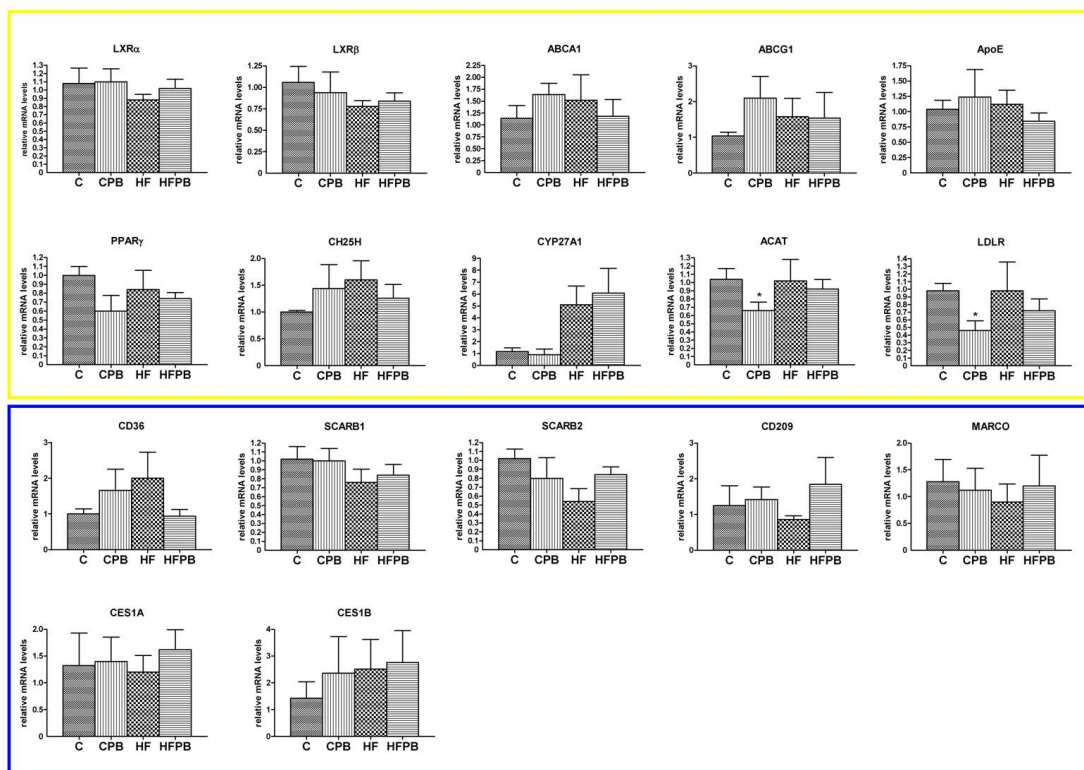
Figure 2. Gene Expression of LXR and Lipid Metabolism Gene in unstimulated AM from C, CPB, HF and HFPB fed pigs. Total RNA was extracted from freshly isolated BAL cells, reversed transcribed to cDNA and analyzed for genes expression using RTPCR as described above. Grouped genes surround by yellow box are related to LXR and cholesterol metabolism. Grouped genes surrounded by a blue box are markers of foam cells development. Gene expression is reported as means ± SE of fold change relative to C fed animals. Error bars with an asterisk (*) indicate a significant difference between C and CPB fed pigs with $p < 0.05$.
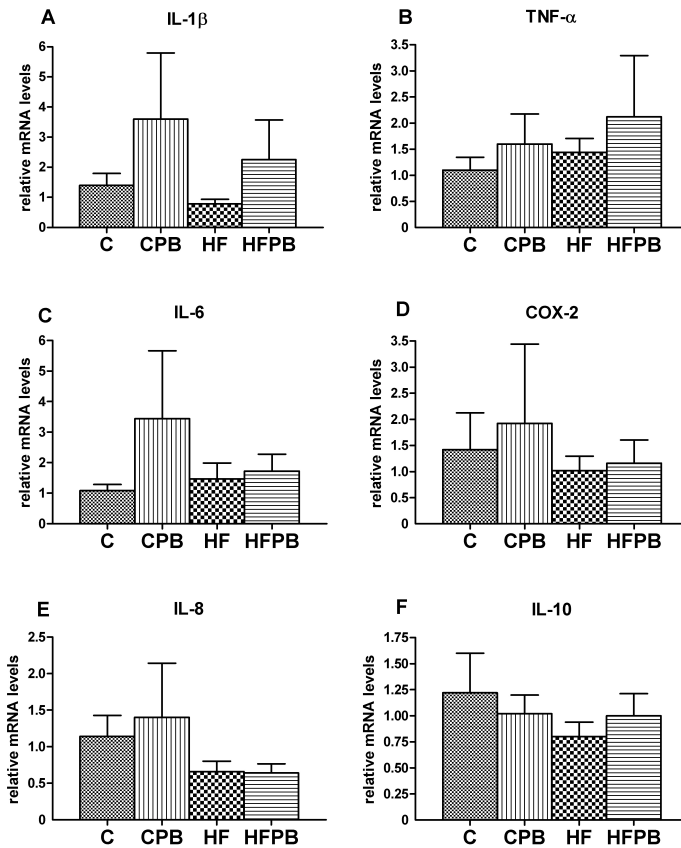
Figure 3. **Gene Expression of Inflammatory Mediators in unstimulated AM from C, CPB, HF and HFPB fed pigs.** Total RNA was extracted from freshly isolated AM, reversed transcribed to cDNA and analyzed for gene expression using RTPCR as described in Materials and Methods. Gene expression is reported as means ± SE of fold change relative to C fed animals. **A,** mRNA expression of *IL-β* in unstimulated AM. **B,** mRNA expression of *TNF-α-* in unstimulated AM. **C,** mRNA expression of *IL-6* in unstimulated AM. **D,** mRNA expression of *COX-2* in unstimulated AM. **E,** mRNA expression of *IL-8* in unstimulated AM. **F,** mRNA expression of *IL-10* in unstimulated AM.
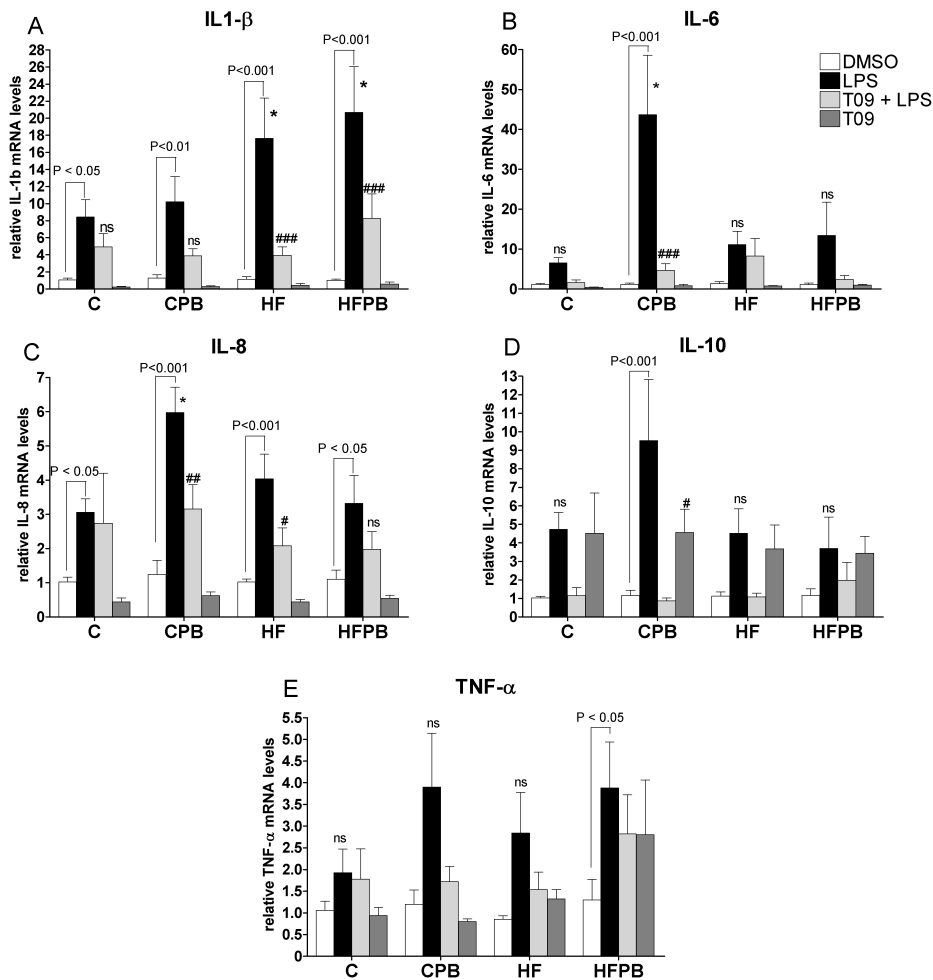
Figure 4. **Gene Expression of Inflammatory Mediators in AM treated with LPS and a LXR ligand from C, CPB, HF and HFPB fed pigs**. AM were seeded at a density of 2.5×10^6 cells/mL and treated with either: vehicle (0.125% DMSO), 0.5 ng/mL LPS, 2.5 μM of the synthetic LXR ligand T09 or LPS + T09 for 24 h. Gene expression is reported as means ± SE of fold change relative to vehicle treated control. Error bars with *p* values are contrasts between LPS and vehicle treated AM. Errors bars with an asterisk (*) represents a significant difference between LPS from either: HF, HFPB or CPB and LPS from C fed pigs with *p* < 0.05. Error bars with a number sign (#) indicate a significant difference between LPS and T09 + LPS treated AM with # *p* < 0.05, ## *p* < 0.01, and ### *p* < 0.001 or not significant, ns. **A,** mRNA expression of *IL-β* in AM. **B,** mRNA expression of *IL-6* in AM. **C,** mRNA expression of *IL-8* in AM. **D,** mRNA expression of *IL-10* in AM. **E,** mRNA expression of *TNF-α-* in AM.
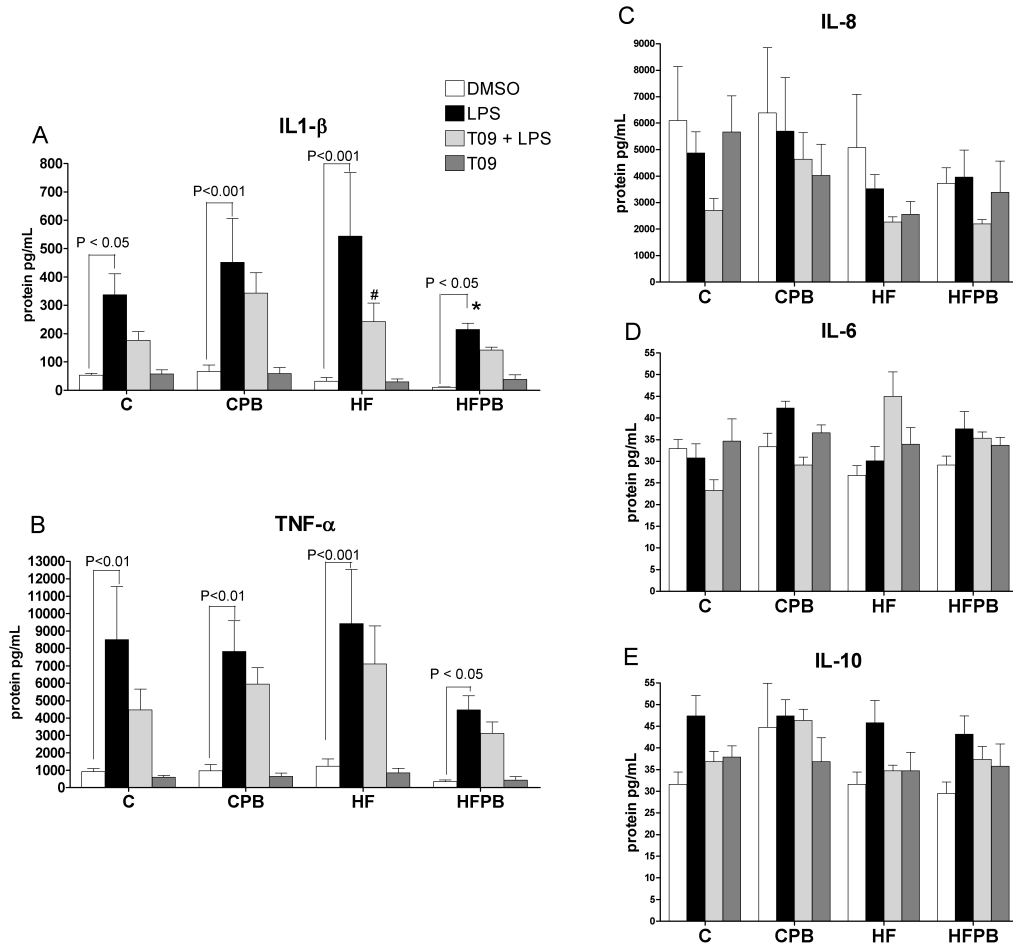
Figure 5. **Cell Culture Supernatant Protein Expression of Inflammatory Mediators in AM from C, CPB, HF and HFPB fed pigs.** AM were seeded at a density of 2.5 x 10^6 cells/mL and treated with either: vehicle (0.125% DMSO), 0.5 ng/mL LPS, 2.5 μM of the synthetic LXR ligand T09 or LPS + T09 for 24 h. Cell culture supernatant were collected and analyzed for protein expression on a Bio-Rad Protein Bio-plex system. Protein is expressed as pg/mL and reported as means ± SE. Error bars with *p* values are contrasts between LPS and vehicle treated AM. Errors bars with an asterisk (*) represents a significant difference between LPS from HFPB and LPS from HF fed pigs with *p* < 0.05. Error bars with a number sign (#) indicate a significant difference between LPS and T09 + LPS treated AM with # = *p* < 0.05. **A,** Supernatant protein expression of IL-β in AM from. **B,** Supernatant protein expression of TNF-α in AM. **C,** Supernatant protein expression of IL-8 in AM. **D,** Supernatant protein expression of IL-6 in AM. **E,** Supernatant protein expression of IL-10 in AM.

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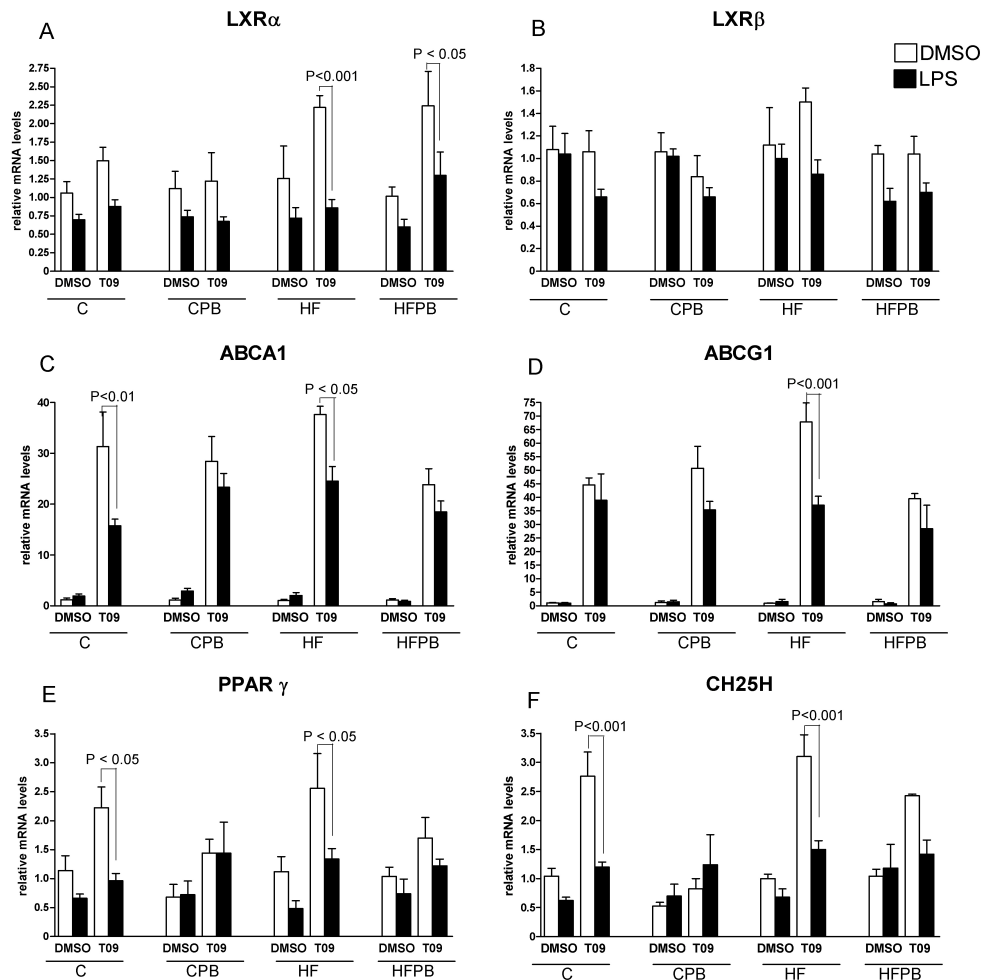
Figure 6. *L. casei* **prevents LPS antagonism of LXR and Lipid Related Gene Expression.** AM from C, CPB, HF and HFPB fed pigs were seeded at a density of 2.5 x 10^6 cells/mL and treated with either: vehicle (0.125% DMSO), 0.5 μg/mL LPS, 2.5μM of the synthetic LXR ligand T09, or LPS + T09 for 24 h. Gene expression is reported as means ± SE of fold change relative to vehicle treated control. Error bars with *p* values indicate significant differences between T09 and T09 + LPS. **A,** LPS modulation of *LXRα* mRNA in T09 stimulated AM. **B,** LPS modulation of *LXRβ* mRNA in T09 stimulated AM. **C,** LPS modulation of *ABCA1* mRNA in T09 stimulated AM. **D,** LPS modulation of *ABCG1* mRNA in T09 stimulated AM. **E,** LPS modulation of *PPARγ* mRNA in T09 stimulated AM. **F,** LPS modulation of *CH25H* mRNA in T09 stimulated AM.

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Chapter 5: Perspectives

Studies of LXRs as Modulators of Lipid Raft Domains in PCa Cells

The current study clearly demonstrated that under conditions of LXR-
ABCA1 mediated cholesterol efflux, that lipid raft domains are negatively
affected in PC-3 cells. There are still a number of questions that need to be
examined if LXR ligands are to be categorized as modulators of lipid raft
signaling in PCa. The first and the most important is disruption of raft domain
patching under LXR-ABCA1 or ABCG1 mediated cholesterol efflux results in a
decrease in raft associated receptor signal transduction. More specifically,
whether there is a decrease in the presence of receptors such as CXCR4 and IL-
6R in raft domains during ligand activation and whether this is associated with
attenuation of downstream signaling. Freeman et al. demonstrated that modulation
of de novo cholesterol synthesis by statins specially reduced assembly of IL-6R
into raft domains and AKT mediated signaling in LNCaP cells (97). Pommier AJ
et al. took this further and demonstrated that mice injected with LNCaP cell
xenografts and treated with the LXR ligand T09 showed a significant reduction in
raft domain integrity and AKT mediated survival. Unlike previous studies of the
anti-PCa effect of LXR ligands, the current study demonstrates for the first time
that raft domains in PC-3 cells are specifically impacted by cholesterol efflux
conditions involved apo A-I.

An inverse association between serum HDL cholesterol and risk for
prostate and other cancers has been reported (64). These data would suggest

therapies aimed at increasing either circulating apo A-I or LXR mediated RCT would also be protective because ABCA1 mediated cholesterol efflux is rate limiting in HDL genesis. The current project potentially ascribes a mechanism to the anti-PCa effect of increased HDL cholesterol, since raft domains where only negatively impacted when both apo A-I and T09 where present. Future studies examining the specific effects of LXR mediated cholesterol efflux on raft domain dependant signal transduction are warranted. Moreover, animal studies examining the effects of LXR mediated HDL biosynthesis on PCa progression would also increase understanding of the potential impacts that LXRs can have in not only protectively against atherosclerosis but also PCa and other cancers inversely affected by serum HDL levels.

Studies of Isoflavones as Modulators of the LXR axis in PCa Cells

Despite the significant increase in ABCA1 and ABCG1 mRNA levels in both PCa cells line, a significant increase in cholesterol efflux was not observed. One reason this might have occurred is because that protein levels of ABCA1 and ABCG1 were not significantly increased by isoflavones. This would need to be confirmed in future studies examining the effects of these compounds on the LXR axis. Secondly, ABCA1 stability is positively regulated by phosphorylation by a PKA during cholesterol efflux (118). The current study did not examine whether ABCA1 protein stability is varied compared to those observed when induced by endogenous or synthetic LXR ligands. Nevertheless if ABCA1 or ABCG1 protein levels are significantly increased by isoflavones, further studies examining other biological effects of these transporters in PCa cells are warranted. This

comes at a time when there is interest in developing novel ligands where are capable of activation LXR responsive genes specifically through LXRβ to prevent LXRα driven hepatic lipidosis. The current study did demonstrate a role for only LXRβ in isoflavone regulation of ABCA1 and ABCG1. Future studies examining specific interactions between isoflavones and the ligand binding domain of LXRβ could also determine whether there is a direct interaction between isoflavones and LXRβ or whether the observed effects are indirect.

Studies of HF diet and Probiotics on LXR and Inflammatory Mediators in AM

The current study examined a number of important questions involving both HF diets and dietary supplementation with probiotic bacteria. One important observation was the increased accumulation of CE in AM from HF fed pigs. This may have implications to human health for a number of reasons. If left unresolved, AM foam cells (defined by accumulation of CE) clearly have an altered immune response. This has been demonstrated in studies of animals with genetically impaired LXR mediated cholesterol efflux and shows that AM foam cells are hyper-responsive to the presence of pathogens. Although in the short term these animals appear to have better clearance of intra-tracheal derived bacteria, long term survival of animals with AM foam cells is significantly decreased post-infection. This is intuitive and may provide new insight to the deleterious effects of obesogenic diets and hyper-cholesterolemia on human health. The strength of the current project lies within the use of wild type animals

to demonstrate this effect of HF feeding on AM cholesterol profile, while other studies have used genetically altered mice to achieve similar effects.

Another important finding in this study was the apparent lack of antagonism of LPS on LXR mediated transcription of ABCA1 and ABCG1 in AM from CPB and HFPB fed pigs. This observation is relevant to human health because of the body of data demonstrating that bacteria and viral antagonism of LXR mediated transcription impairs macrophage cholesterol efflux and increases formation of foam cell an immunocompromised phenotype. The observations from this study demonstrate that *L. casei* feeding abrogated LPS mediated suppression of the LXR axis. This would be a novel finding as other studies have failed to demonstrate that synthetic LXR ligands can oppose LPS mediated suppression of ABCA1 and ABCG1. Moreover, these observations may also provide a mechanism for the lack of CE accumulation in AM from HFPB fed pigs. The current data suggests an enhanced effect of LXR ligand against the response of LPS. Future studies specifically examining whether *L. casei* feeding can inhibit LPS mediated opposition to LXR mediated cholesterol efflux should be considered.

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