NUCLEAR RECEPTORS AND COREGULATORS IN METABOLISM AND IMMUNITY

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EDITORIAL
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Editorial: Nuclear Receptors and Coregulators in Metabolism and Immunity

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Editorial on the Research Topic

Nuclear Receptors and Coregulators in Metabolism and Immunity

Dysregulated tissue metabolism and inflammation are associated with many human diseases such as metabolic disorders, autoimmune diseases and cancer (1, 2). The transcriptional alterations in both metabolic and immune cells in response to microenvironment-derived pathological stimulus are mostly linked with abnormalities of transcription factors (TFs). Nuclear receptors (NRs) are a family of ligand-dependent TFs. For most of them, their activities can be controlled by both endogenous and exogenous molecules such as metabolites, steroid hormones and synthesized chemicals (3). As a result, NRs have been appealing drug targets for many decades, with already approved compounds with promising therapeutic outcomes.

One of such NRs is the Peroxisome Proliferator-Activated Receptor γ (PPARγ). PPARγ is among the most extensively studied NRs. It is well-known as the master regulator of adipose tissue biology (4). Using an *in vitro* 3T3L1 adipocyte cell model, Dias et al found that PPARγ phosphorylation at serine 273 (S273) by cyclin-dependent kinase 5 (CDK5) caused a coactivator-to-corepressor switch and thereby decreased PPARγ activities and reduced mRNA expression of metabolically protective adipokines. In addition to adipocytes, many studies have discovered that PPARγ have pleiotropic functions in various other cell types and tissues, including colon, breast, prostate and bladder, as well as immune cells such as monocytes/macrophages, dendritic cells and lymphocytes. The multi-organ functions, dysregulations (mRNA expression changes, gain or loss of functional mutations, etc.) and molecular mechanisms underlying PPARγ activities are summarized by Hernandez-Quiles et al.

Another well-studied NR is glucocorticoid receptor (GR). GR is an important regulator of many physiological processes (5). Due to the strong anti-inflammatory function of GR, its agonists have been widely applied in the clinic for severe immune diseases. The usage of GR agonists is limited by the side effects including severe responses in key metabolic organs such as liver. Ongoing efforts aim to: 1) understand the regulatory mechanisms of GR, i.e. the coregulatory factors and complexes, functionality of different isoforms, in major metabolic organs such as liver, which is reviewed by Præstholm et al; and 2) identify so-called selective GR modulators (SGRM) with more tissue- or isoform-specificity in order to minimize the unwanted metabolic effects of GR, which has not been successful so far. Van Moortel et al. discussed the bottlenecks of pharmaceutical discovery of better GR ligands with emphasis on both ongoing research developments and potential solutions.

A classic paradigm of NR biology relies on lipid-sensing NRs as hubs to connect metabolism and inflammation. Lipids such as cholesterols play crucial roles in physiology and thus are tightly

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regulated by multiple NRs, including farnesoid X receptors (FXRs) and liver X receptors (LXRs). Johansson et al. investigated the hepatic FXR/fibroblast growth factor 19 (FGF19) axis in cholesterol excretion as bile acids (BAs). FGF19 is derived from both liver and intestine in response to BAs and is believed to be essential for the FXR to inhibit BA synthesis. The FXR/FGF19 connection was studied in primary human hepatocytes. Despite that FXR activation upregulated FGF19 secretion in the hepatocytes, FXR did not require FGF19 to inhibit BA synthetic genes. The authors therefore proposed independent regulatory roles of FXR and FGF19 in human liver BA production.

Overload of lipids (especially cholesterol) is related to many human diseases. In macrophages, accumulation of cholesterol causes inflammation and plaque development in atherosclerosis (6). Ramírez et al. discovered that ligand activation of LXR induced caveolin-1 expression. Because caveolin-1 is responsible for the formation of caveolae, multi-functional lipid raft microdomains of the membrane with high concentrations of cholesterol, LXR-induced caveolin-1 eliminated cholesterol in macrophages and alleviated atherosclerosis. Excessive cholesterol is also involved in the progression of multiple sclerosis (MS). MS is an autoimmune disease caused by constitutively activated immune cells in the brain. Systemic changes of cholesterol and oxysterol may contribute to the disease by modulating the activities of LXRs, and thereby causing immune cell dysregulation in the human MS development. The interplay between cholesterol, oxysterols and LXRs, as well as the potential therapeutic application of LXR agonists in human MS pathology have been reviewed by Pineda-Torra et al. in this Research Topic.

At the molecular level, genome-wide analysis of lipid-sensing NR binding with next generation sequencing (NGS) has revealed a major portion of the NR cistromes that are not responsive to ligand activation. In tissue macrophages, the lipid-sensing NRs work as lineage determining TFs (LDTFs) to define macrophage subsets. They can also recruit coregulators independent of ligand binding to regulate the epigenetic remodeling and 3D structure of chromatin. The new concept of the non-classic or 'unorthodox action' of lipid-sensing NRs are reviewed by Czimmerer et al.

The NRs also participate in innate immunity *via* a crosstalk with inflammasome pathways in the macrophages. Several NRs have been reported to work with inflammatory TFs such as NFκB to control inflammasome priming by regulating its component gene expression. NRs such as FXRs can physically interact with the NLRP3 and caspase1 to directly inhibit the complex assembly and the enzymatic activities. On the other hand, activated inflammasome also modulates NRs, i.e. by directly cleaving the NRs at conserved cleavage sites. The NR/inflammasome interaction is involved in multiple diseases, which is systemically reviewed by Duez and Pourcet. Beyond that, NRs are important regulators of adapted immune responses by regulating T cells. The NR4A family of orphan nuclear receptors (receptors with unrecognized ligands) not only controls T cell differentiation and development, but also defines the acute and chronic responses of CD4+ and CD8+ T cells. The underlying mechanisms of NR4A-mediated adaptive immune regulation were reviewed by Odagiu et al. in this Research Topic.

It has long been observed that metabolic and inflammatory responses differ between males and females. This is partially attributed to sex-specific steroid hormones that act as ligands of NRs such as estrogen receptors (ERs). Among the metabolic organs, liver shows the highest degree of sexual dimorphism. This aligns with the regulation of ER in both metabolic and inflammatory pathways in the liver, which is summarized by Della Torre in this Research Topic. Fluctuation of ER activities during physiological and pathological conditions leads to altered functions in both metabolic organs and immune cells and is linked with diseases such as breast cancer. Brundin et al. investigated the expression of ER subtypes in different cell subsets of human peripheral blood mononuclear cells (PBMCs), and their correlation with multiple inflammatory genes. The study confirmed the association of ER dysregulation with altered inflammation in PBMC cells during the menstrual cycle. Estradiol signaling through ER plays crucial roles in breast cancer cell development. Cervantes-Badillo et al. investigated the interaction between regulatory components of ER activities and identified the interferon alpha inducible protein 27 (IFI27/ISG12). IFI27/ISG12 could be induced by both interferon and estradiol in breast cancer cells. The protein then facilitated the interaction of ER with CRM1/XPO1 which retained ER in the cytoplasm and impaired its activities. As a result, IFI27/ISG12 elevation was associated with reduced overall survival of ER^+ breast cancer patients and resistance to tamoxifen treatment.

There are still many challenges remaining in the NR research field despite the huge efforts invested. Further studies are required for better understanding of the molecular events regulated by NRs in different tissues. Such information will be of great value to develop ligands or NR-targeted therapeutic strategies with more specificity.

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REVIEW
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Non-alcoholic Fatty Liver Disease as a Canonical Example of Metabolic Inflammatory-Based Liver Disease Showing a Sex-Specific Prevalence: Relevance of Estrogen Signaling

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There is extensive evidence supporting the interplay between metabolism and immune response, that have evolved in close relationship, sharing regulatory molecules and signaling systems, to support biological functions. Nowadays, the disruption of this interaction in the context of obesity and overnutrition underlies the increasing incidence of many inflammatory-based metabolic diseases, even in a sex-specific fashion. During evolution, the interplay between metabolism and reproduction has reached a degree of complexity particularly high in female mammals, likely to ensure reproduction only under favorable conditions. Several factors may account for differences in the incidence and progression of inflammatory-based metabolic diseases between females and males, thus contributing to age-related disease development and difference in life expectancy between the two sexes. Among these factors, estrogens, acting mainly through Estrogen Receptors (ERs), have been reported to regulate several metabolic pathways and inflammatory processes particularly in the liver, the metabolic organ showing the highest degree of sexual dimorphism. This review aims to investigate on the interaction between metabolism and inflammation in the liver, focusing on the relevance of estrogen signaling in counteracting the development and progression of non-alcoholic fatty liver disease (NAFLD), a canonical example of metabolic inflammatory-based liver disease showing a sex-specific prevalence. Understanding the role of estrogens/ERs in the regulation of hepatic metabolism and inflammation may provide the basis for the development of sex-specific therapeutic strategies for the management of such an inflammatory-based metabolic disease and its cardio-metabolic consequences.

Keywords: NAFLD (non-alcoholic fatty liver disease), estrogens, estrogen receptors, liver, sex differences

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INTRODUCTION

Liver Metabolism and Inflammation: Two Sides of the Same Coin

The liver is one of the most complex organs in the body, performing a multitude of functions, including the macronutrient metabolism, glucose, lipid and cholesterol homeostasis, protein and amino acid metabolism, detoxification and drug metabolism (1). The liver is also an immunological organ, being responsible for the production of acute phase proteins, complement components,

cytokines and chemokines, and contains large, diverse populations of resident immune cells (2). Under physiological conditions, the liver is constantly exposed to dietary and gut-derived bacterial products with inflammatory potential and is engaged in tissue remodeling, all process requiring a tight regulation of the inflammatory response to maintain tissue and organ homeostasis and to redistribute the energy resources during the rising of an inflammatory response (3–5).

A close and coordinated regulation of metabolic and immune responses has been conserved through evolution, with lower and higher organisms sharing common ancestral structures and common key regulatory molecules and signaling systems (6). However, the integration between metabolic and inflammatory pathways have been set up in the context of nutrient limitations and have not evolved and adapted to the current habits and lifestyles, where overnutrition and the reduced physical activity lead to chronic disturbance of metabolic homeostasis and to aberrant immune responses (4, 7). The metabolic overload and the lack of metabolic homeostasis typical of obesity and obesity-associated metabolic diseases trigger a sustained and chronic inflammatory response, that, by converse, can disrupt systemic metabolic functions, thus fostering a vicious cycle that favors the progression of metabolic diseases (4). In the liver, the inability to resolve inflammation may lead to chronic pathological inflammation and to a disrupted tissue homeostasis, which can promote hepatic steatosis, fibrosis, cirrhosis, and liver failure (3, 5, 8–10).

Although the higher prevalence of obesity among female population, women result to be somewhat protected from the obesity-associated cardio-metabolic consequences, such as non-alcoholic fatty liver disease (NAFLD), at least until menopause (11). The reason of that likely relies on the tight regulation of metabolic and inflammatory processes that may have reached its maximum degree of complexity in the liver of female mammals, where the regulation of hepatic metabolism is under the control of sexual hormones, estrogens in particular, and is subjugated to the reproductive needs (12–15). In view of the tight link between energy homeostasis and reproduction, liver diseases show a sex-specific prevalence (16, 17) and are associated with reproductive dysfunctions in women (14, 18). Nowadays, changes in dietary and lifestyle habits as well as the increased lifespan of women, that spend more than 1/3 of their lives in post-menopause, can explain the increased incidence in female population of cardio-metabolic diseases, which are previously considered male-prevalent (16, 19, 20). In this view, research programs aimed to unravel the role of estrogen signaling in the regulation of metabolic and inflammatory processes may have a significant impact on the design of new therapies that can counteract the development of NAFLD and the associated cardio-metabolic consequences in a sex-specific fashion.

NAFLD, a Canonical Example of Metabolic Inflammatory-Based Liver Disease Showing a Sex-Specific Prevalence

With respect to young, fertile women, men and post-menopausal women show an increased incidence of metabolic and inflammatory-based liver diseases (14, 18, 21). Among them, a canonical example is NAFLD, a syndrome characterized by excessive triglyceride (TG) accumulation within hepatocytes (22), that has reached epidemic proportions and represents an increasing public health issue due to its emerging association with several extra-hepatic diseases (23, 24), cardiovascular diseases (CVDs) in particular (25, 26). Indeed, cardiovascular mortality represents the commonest cause (45%) of death in NAFLD patients, followed by cancer (36%) and then liver-related mortality (7%) (27).

NAFLD is closely linked with peripheral insulin resistance and hepatic insulin resistance (28–30), a condition where insulin fails to suppress hepatic glucose production (HPG, which accounts for 90% of endogenous glucose production) but promotes lipid synthesis leading to hyperglycemia, hypertriglyceridemia and hepatic steatosis (31). Therefore, there is a significant correlation between HPG and the extent of liver fat in NAFLD patients (32) as well as between NAFLD and other metabolic insulin-resistant disorders such as type 2 diabetes mellitus (T2DM) (33, 34) and sarcopenia (35). Notably, women show an improved glycemic control, a greater peripheral and hepatic insulin sensitivity and a reduced HPG with respect to men (36–38), likely a consequence of a sex-dimorphic regulation of glucose homeostasis (39), to which the hepatic signaling of sexual hormones strongly contributes (40, 41), thus leading to a different susceptibility to NAFLD between the two sexes.

In the liver of NAFLD patients, TG accumulation it is due to increased *de novo* lipogenesis (DNL) (42, 43), increased delivery of fatty acids (FAs) to the liver (42, 44), and decreased lipid clearance consequent to impaired FA oxidation and lower lipid secretion (45, 46). Hepatocellular damage and fat-derived factors mediate the local activation of a pro-inflammatory response by hepatocytes and non-parenchymal cells, including Kupffer cells (KCs) and hepatic stellate cells (HSCs) (4, 47–49), that promote the recruitment of other immune cells, including neutrophils, T-lymphocytes and, mainly, macrophages (50).

The impaired mitochondrial oxidation (42, 51) and the up-regulation of both peroxisomal β-oxidation (52) and microsomal ω-oxidation (53) of FAs lead to chronic oxidative stress and result in the generation of reactive oxidative species (ROS) within the hepatocytes (42, 45). In addition to mitochondria—that are considered the most relevant source of ROS—and to peroxisomes and microsomes, the endoplasmic reticulum stress and enzymes as NADPH oxidase (NOX), cytochrome P450 2E1 (CYP2E1), cyclooxygenases, and lipoxygenases also produce ROS (54). According to the most valuable theories (22), the production of lipotoxic lipid intermediates and the excessive production of ROS further trigger a pro-inflammatory response that contributes to the progression of NAFLD to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (46, 55, 56). Pro-inflammatory cytokines released by immune cells intensify the inflammatory process, that hinders the liver to orchestrate a proper tissue regeneration by replacing the hepatocytes subjected to cell death or apoptosis, as occurs under physiological conditions (57). Possibly as an unsuccessful effort against liver injury and tissue regeneration, HSCs become activated and differentiate into myofibroblasts,

that, in turn, express actin and diverse types of collagen, leading to extracellular matrix deposition and fibrosis and liver degeneration (58–60).

In the presence of increased flux of free fatty acids (FFAs) and of chronic, low-grade inflammation, the liver acts as both a target of and a contributor to systemic chronic inflammation, triggering or boosting the progression of NAFLD and several extra-hepatic diseases (23), including atherosclerosis (61–63), cardiovascular diseases (62–64), chronic kidney disease (65), osteoporosis (66), and inflammatory bowel disease (67).

From Metabolism to Liver Injury: Role of Obesity and Nutrients in NAFLD Development

Although several factors might contribute to hepatic steatosis, including genetic and epigenetic factors (68, 69), obesity represents the main trigger of NAFLD development and progression. However, independently of energy intake, also the macronutrient composition of the diet can be associated with NAFLD/NASH development (70). Different epidemiological studies have, therefore, demonstrated that dietary habits may directly promote NAFLD/NASH, by modulating hepatic TG accumulation and antioxidant activity and, indirectly, by affecting insulin sensitivity and the postprandial TG metabolism (70). Several studies have identified the overconsumption of fats (saturated fats and *trans*-fats, in particular) and sugars (fructose, in particular) as the main nutritional mediators of NAFLD development (71–73), while the role of proteins and amino acids in NAFLD etiology has been less investigated and still raises controversies (70).

Obesity

The rising trends in obesity has been linked with the increase in the incidence and severity of NAFLD, with an estimated global prevalence of 25–30% worldwide, rising up to 90% in morbidly obese patients (26, 74). In obese NAFLD patients, ∼60% of hepatic FAs are derived from FFAs released by the adipose tissue as a consequence of an enhanced lipolysis and taken up by the liver *via* the increased uptake mediated by CD36 (cluster of differentiation 36) (75–77). To a less extent, hepatic lipid deposits derive from dietary FAs (∼15%) and from increased synthesis of new lipids (∼25%) from ingested carbohydrates that reach to a greater extent the liver due to the insulin resistance of the muscle (43, 75, 78). The exposure of hepatocytes to high lipid and carbohydrate levels promotes lipotoxicity and glucotoxicity, that, in turn, lead to mitochondrial defects, endoplasmic reticulum stress and oxidative stress (45, 79). The ectopic accumulation of lipid toxic intermediates triggers the activation of inflammatory pathways, cellular dysfunction, and lipoapoptosis, all features favoring NAFLD progression and liver injury (22, 80, 81).

Obesity also affects the liver through the unbalanced secretion of adipokines, exerting different effects on insulin resistance, hepatic steatosis, inflammation and fibrosis (82). For example, the obesity-associated reduction of adiponectin levels promotes insulin resistance and hepatic steatosis, while the increased levels of leptin foster hepatic inflammation (82). In the adipose tissue of obese people, the infiltration and activation of immune cells (macrophages, B-lymphocytes, T-lymphocytes and neutrophils) that produce pro-inflammatory cytokines (e.g., interleukin 1β, IL-1β; interleukin 6, IL-6; tumor necrosis factor-alpha, TNF-α) impair the dynamic antagonism between adipokines and cytokines and facilitate the progression of steatosis, inflammation and fibrosis (82).

Under obesogenic-like conditions, in addition to adipose tissue, the impaired regulation of metabolic process and signaling pathways in other tissues showing a strong interplay with the liver, including the skeletal muscle (83–85) and the gut-microbiota (86, 87), can further negatively affect the hepatic metabolic homeostasis and boost the progression of NAFLD.

In addition to genetic factors (88, 89), estrogen signaling strongly contributes to sex differences in obesity and associated cardio-metabolic consequences such as NAFLD (20, 21, 39, 90–93). With respect to pre-menopausal women, lean and obese men tend to accrue more visceral fat, that, having a greater lipolytic potential than subcutaneous adipose tissue, strongly contributes to increased FFA flux to the liver, where FFAs mediate insulin resistance and NAFLD pathogenesis (94, 95). After menopause, there is a redistribution of fat toward visceral depots and a lower inhibition of adipose lipolysis, all changes that fuel the FFA flux to the liver and increase the risk of developing NAFLD in post-menopausal women (92, 94).

Sex-specific and estrogen-mediated differences in obesity-induced NAFLD are ascribable also to impaired regulation of metabolic process in extrahepatic tissues showing a cross-talk with the liver, such as the adipose tissue and the skeletal muscle, that under obesogenic conditions display increased insulin resistance and increased inflammation that might further aggravate the hepatic dysmetabolism (96–106).

Dietary Sugars

Over the past century, the increased intake of added sugars, fructose in particular, is associated with increased incidence of hepatic steatosis and liver inflammation (107–109). Unlike glucose, ingested fructose by-passes the rate-limiting step of glycolysis and is preferentially metabolized by the liver, where it stimulates hepatic DNL acting mainly through SREBP1c (sterol regulatory element-binding protein 1c) and ChREBP (carbohydrate responsive element-binding protein), inhibits the mitochondrial β-oxidation of long-chain FAs, induces endoplasmic reticulum stress, and promotes TG formation and hepatic steatosis (73, 110, 111). Owing to the molecular instability of its five-membered furanose ring, fructose promotes protein fructosylation and formation of ROS, yielding to hepatocellular damage and to the development of a pro-inflammatory response (73).

Even after a single meal, fructose strongly up-regulates an inflammatory cascade through increased hepatic JNK (c-Jun N-terminal kinase) activity and induces hepatic insulin resistance, all effects occurring specifically in hepatocytes (112). Recent reports suggest that fructose can also induce liver inflammation by acting directly on inflammatory cells, where it drives the production of pro-inflammatory cytokines (IL-6 and IL-1β) that further promotes an aberrant lipid metabolism (107, 109, 112, 113). The high intake of fructose can also lead to gut microbiota

dysbiosis and contribute to inflammation, insulin resistance and NAFLD progression (114).

The consequences of extended fructose consumption on liver health are different between the two sexes, with males being more responsive to fructose and showing higher hepatic postprandial DNL and higher prevalence of NAFLD compared to females (115–117). These differences can likely be a direct consequence of the sex-specific modulation of glucose metabolism (39, 118) and of the specific relevance of estrogen signaling in regulating hepatic glucose metabolism and in promoting insulin sensitivity (119–121), acting also through FGF21 (fibroblast growth factor 21) signaling (122, 123). Accordingly, high fructose intake exacerbates the progression of NAFLD in ovariectomized (OVX) female mice by enhancing liver cell destruction, macrophage accumulation, and progression of fibrosis, all negative effects that can be reverted by 17β-estradiol supplementation (121).

Dietary Fatty Acids

The increased intake of dietary FAs is strongly associated with obesity and the development of obesity-associated metabolic diseases, such as NAFLD (124–127). Dietary regimens enriched in fats contribute to increase the hepatic pool of FAs, where they promote DNL and the generation of lipotoxicity through a sustained oxidation (128). Dietary FAs influence NAFLD pathogenesis also by modulating the gene transcription of specific enzymes and regulating various metabolic pathways involved in lipid metabolism (129, 130). Modern western diets are particularly enriched in saturated and *trans* FAs that are particularly detrimental for hepatic health, because they induce insulin resistance and fatty liver and promote liver injury by altering the composition of plasma cell membrane, thus impairing cellular homeostasis and amplifying the already sustained inflammatory signaling, that, in turn, boosts insulin resistance and apoptosis (127, 128, 131, 132). Conversely, diets enriched in ω3 polyunsaturated FAs (ω3 PUFAs), such as the Mediterranean diet (133), may be particularly effective in counteracting the early stages of NAFLD (134), limiting insulin resistance, oxidative stress, DNL and TG deposition in the liver (135, 136) and preventing the development of liver-associated cardio-metabolic diseases (137). ω3 PUFAs exert anti-inflammatory actions by preventing the alteration of cell membrane phospholipid composition and the disruption of lipid rafts, by inhibiting the activation of NF-κB (nuclear factor-kappa B), by reducing expression of inflammatory genes and by activating PPARγ (peroxisome proliferator-activated receptor γ) (138).

With respect to the female counterparts, men and male rodents show a higher propensity of developing hepatic steatosis/NAFLD that derives from the combination of increased FA import, DNL, and storage of lipids within the liver and lower dietary FA oxidation and secretion (91, 139). By comparing control and LERKO (liver-specific Estrogen Receptor alpha KO) mice, a recent study demonstrates that the liver ability of females to cope with the excess of dietary lipids strongly relies on the activity of hepatic ERα, that confers to females a higher metabolic flexibility (91).

Different dietary fatty acid regimens can also change the composition and the *ratio* of FAs in liver plasma cell membrane in gender-specific manner, another mechanism that can further explain the sex-specific incidence of NAFLD (140).

Furthermore, maternal high-fat diet can promote and even program hepatic steatosis/NAFLD and liver inflammation of offspring in a sexually dimorphic manner by altering gut microbiota (141) that has been shown relevant for the achievement of hepatic sexual dimorphism (142).

Dietary Amino Acids

While the hazardous effects of high-carbohydrate and high-fat diets upon hepatic structure/function are well-recognized, the potential effects of dietary regimens enriched in proteins and amino acids (AAs) on hepatic health are partly clarified and still raise controversies. Indeed, while several studies show a beneficial role exerted by high-protein diets in reducing body weight and in reverting hepatic steatosis, other studies suggest that high-protein diets can instead promote the development of NAFLD (143). The reasons of these contradictory effects on liver health can be ascribable to differences in dietary regimens (e.g., diet composition and protein source) and on the functional status of the liver (143).

Among AAs, branched chain amino acids (BCAAs: leucine, isoleucine, and valine), that account for 20% of total protein intake (144), exert beneficial effects on hepatic health as they alleviate hepatic steatosis and liver injury and prevent hepatic fibrosis and the development of HCC in NASH mouse models (145, 146). By contrast, elevated circulating BCAAs are strongly associated with several metabolic disorders, including obesity and insulin-resistant metabolic diseases (147, 148). NAFLD patients show a low hepatic content of BCAAs, that changes with the progression of the pathology, likely as a consequence of impaired expression of hepatic BCAA-degrading enzymes (149, 150). Furthermore, a recent study demonstrates that plasma BCAA levels display sex-dimorphic changes with increasing severity of NAFLD, independently of BMI, insulin resistance and age (151), suggesting a sex-specific regulation of BCAA metabolism and/or a sex-specific role of BCAAs in NAFLD development, as supported by pre-clinical studies (91). Indeed, although their causative or associative role has not yet clarified, among AAs and several other metabolites, BCAAs result the pathway most affected in the liver of a mouse model of diet-induced obesity (91). Notably, the decrease in AAs and, especially, in BCAAs correlates with increased lipid deposition in the liver of male, but not female mice; in fact, when exposed to an excess of dietary lipids, female mice, contrary to males, preserve the hepatic AA homeostasis, an effect associated with the ability to counteract liver lipid deposition (91), suggesting that the metabolism of BCAAs might have a key role in driving hepatic steatosis in a sex-specific fashion. The female-specific ability to preserve BCAA homeostasis and counteract liver lipid deposition is dependent on hepatic ERα, as it is lost in LERKO female mice (91), and it is likely a consequence of an higher metabolic flexibility conferred by hepatic ERα, that, in the female liver, adapts the hepatic metabolism to hormonal status and to nutrient availability, amino acids in particular (13, 15, 152, 153).

Sex Differences in NAFLD Onset, Development and Progression

NAFLD is more common in men, in whom it has a 2.0–3.5-fold higher prevalence than in fertile women; however, after menopause the incidence of NAFLD increases significantly to reach the levels seen in men, owing to the putative protective effect of estrogens (14, 17, 154). Indeed, gender-specific prevalence of NAFLD is related to age: while men commonly display an increasing prevalence of NAFLD during adulthood from young to middle-age, the prevalence of NAFLD in women occurs ∼10 years later than in men, rising after the age of 50 years, peaking at 60–69 years, and declining after 70 years (16). This last trend indicates that the increased incidence of NAFLD in aging women relies more on the lack of estrogens than on aging *per se*, even though aging may exacerbate the progression of NAFLD by negatively impacting on metabolic (155) and inflammatory processes (156). According to this view, young oophorectomized women (157) as well as young women suffering of other reproductive dysfunctions characterized by altered estrogen levels (such as Polycystic ovary syndrome, PCOS) (14, 158) show increased incidence of NAFLD with respect to young fertile women.

Even if the exact etiology of NAFLD in post-menopausal women is still unclear, the association of NAFLD with the cessation of ovarian activity and with other ovarian dysfunctions such as PCOS (159) suggests that estrogens protect against its development and progression. Notably, with respect to their control counterparts, pre-menopausal, post-menopausal, and PCOS women with NAFLD exhibit a significantly lower concentration of serum 17β-estradiol, which is the principal active estrogen (158). Accordingly, hormone replacement therapy (HRT) reduces the risk of developing NAFLD for post-menopausal women (16, 160).

In pediatric populations, NAFLD prevalence is higher in boys than in girls (161), even though sex differences are less relevant with respect to adult population, suggesting that the achievement of complete sexual differentiation is required to accomplish the sex-specific prevalence and features of such a pathology. Such a hypothesis is sustained by several studies showing a strict association between puberty and features of NAFLD (162) and between earlier age at menarche and the prevalence of NAFLD later in life (163–165).

Although the prevalence of NAFLD is undoubtfully higher in men than women, less clear is the sex-specific incidence of liver injury associated with NAFLD progression to NASH and fibrosis. In fact, some studies suggest that women have a lower risk of developing NASH and fibrosis (166–169), while others do not find differences between the two sexes (170, 171) or, even, indicate that women are more susceptible than men to an inflammatory-driven degeneration of NAFLD toward more harmful conditions (172–176). Most of these studies, however, has several limitations and important potential bias, as they do not differentiate between pre- and post-menopausal women or do not often consider the timing/duration of menopause, which may give confounding and contradictory results (12). By converse, consistent with the hypothesis that estrogens exert beneficial effects on liver health, menopause, premature menopause and prolonged estrogen deficiency have been independently associated with significant fibrosis in women with NAFLD (177, 178).

NAFLD incidence is increased in obese people suffering of other obesity-associated cardio-metabolic diseases; nevertheless, several mechanistic and longitudinal studies have indicated that NAFLD is an independent risk factor for atherogenesis (179–181) and CVDs (23, 63, 182–184) apart from other metabolic disorders. Although still debated, the causal relationship independent of other metabolic risk factors seems to rely on the systemic inflammatory milieu initiated in part by liver-secreted cytokines and molecules (23, 63). In addition to enhanced inflammation, a growing body of evidence indicates that, along with NAFLD progression, the alteration of cholesterol and lipoprotein metabolism (185–187) and the excessive generation of ROS may lead to the accumulation of oxidized low-density lipoprotein (ox-LDL) in the liver (188–190) and to macrophages transformation into foam cells, which is a hallmark of atherosclerosis.

Given such a correlation between NAFLD and CVDs, it is not surprising that, while in the general population women are less prone to CVDs under the age of 50 years, after menopause, women lose this protection and show a higher risk of developing NAFLD and cardio-metabolic associated consequences (191–193).

Sex Differences in the Regulation of Metabolism and Inflammation in the Liver

Sex-specific prevalence, progression and outcomes of hepatic diseases and their associated co-morbidities might be considered the resultant of sex differences typifying the male and female liver phenotype.

The liver is the major metabolic organ in mammals with the highest degree of sexual dimorphism (194, 195). Most of the sex differences in liver gene expression are dictated by the temporal pattern of circulating growth hormone (GH), which is sex dependent (highly pulsatile in males and more continuous in females) (196, 197) and under gonadal control (198–200). GH regulates the sexually dimorphic patterns of a large number of liver-expressed genes, including various plasma and urinary proteins, cytochromes P450 (CYPs, which contribute to sex differences in sex steroid hormone metabolism), enzymes devoted to steroid and foreign compound metabolism, and various receptors and signaling molecules involved in a broad range of physiological processes (194, 197, 201, 202). GH pattern carries out its sexual differentiating action of liver functions through multiple intracellular signaling pathways, including the transcription factor signal transducer and activator of transcription 5b (STAT5b) (203–205), hepatocyte nuclear factors 3β, 4α and 6 (HNF3β, HNF4α, HNF6) (206, 207) as well as their signaling cross-talk (208–210). GH dimorphic action on hepatic gene expression is also dependent on sex-specific regulation of DNA methylation and chromatin structure (197, 205, 211–215), resulting in major changes in sex-based liver functions. The hepatic responsiveness to GH dimorphic action

changes during development (216–218) and remains dynamic during adult life (205, 217), charging the liver of the possibility to adapt its functions to the needs of the organism throughout life.

GH and its signaling pathway, acting mainly through insulin-like growth factor-I (IGF-I), regulate lipid metabolism in the liver (219) and play an important role in antagonizing NAFLD, by directly reducing DNL in the hepatocytes and by inactivating HSCs, therefore limiting fibrosis (220). According to this, GH deficiency in adults and in obese children is associated with increased prevalence of NAFLD and NASH, while GH replacement therapy improves these conditions (220, 221). In mice, the liver-specific ablation of the GH receptor (GHR) increases lipid uptake and DNL, resulting in hepatic steatosis that cannot be reverted by IGF-1 treatment (219).

GH and its signaling may have a key role also in the liver disease progression, by regulating excessive inflammation and allowing liver regeneration (222). By converse, during inflammation, the liver can become resistant to GH actions, through mechanisms involving proinflammatory cytokines such as IL-6, TNF-α, and IL-1β (223–226), thus worsening metabolic alterations.

In addition to the well-known dimorphic activity of GH, hepatic sexual dimorphism depends on several other factors, including genetic (213, 215) and epigenetic (227, 228) factors, diet (141, 229, 230), circadian rhythm (231, 232), gut microbiota (142) and sexual hormones (12, 152, 233).

In spite of the fact that our knowledge of the entity of hepatic sexual dimorphism under physio-pathological conditions remains very limited (12), several evidences, including the sex-specific prevalence, incidence, progression and outcomes of hepatic diseases such as NAFLD (17, 234, 235), indicate that, among the factors contributing to hepatic sexual dimorphism, estrogens and their receptors recover a key role.

Estrogens can regulate sex differences in the liver through direct and indirect mechanisms, that are both affected by and able to prevail over sex-based genetic background and sexual hormone-dependent regulatory activities. Estrogen activity can contribute to the sexual dimorphism of the liver directly (21, 91, 152, 236, 237) and indirectly, by regulating GH action, both in the central nervous system and locally (198, 233, 238–240). Several experimental models with impaired/lost estrogen signaling support the involvement of estrogen dependent pathways in the regulation of hepatic metabolism, also in a sexually dimorphic fashion (14, 91, 152, 153, 241).

Estrogen-mediated contribution of hepatic sexual dimorphism likely arises from different metabolic costs of reproduction and from higher metabolic flexibility acquired and perfected through evolution by the female liver of mammals to adapt the hepatic metabolism to nutrient availability to sustain the energy needs of reproductive function (12, 13, 152, 242). In view of these evidences, although androgens and androgen receptor (AR) contribute to the sex-based hepatic phenotype in a direct or indirect fashion, by acting on GH dependent pathways (200, 215, 233, 243) and by regulating the accessibility of DNA to several transcription factors through chromatin remodeling (244, 245), this review will focus in particular on the role of estrogen signaling in the regulation of metabolic-driven inflammatory process at the basis of NAFLD development and progression.

NAFLD and Liver Inflammation

In NAFLD, the increased flux of FFAs, the generation of lipotoxicity and oxidative stress and insulin resistance concur in activating JNK and NF-κB signaling pathways, resulting in the increased production of pro-inflammatory cytokines, including IL-6 and TNF-α (4, 48, 49, 246). JNK is a member of mitogen activated protein kinases, which activation in fatty liver is associated with insulin resistance, activation of apoptosis and development of NASH (247–249). JNK pathway is differentially regulated between males and females during liver injury (250, 251), likely through an estrogen- and ERα-mediated inhibition of lipotoxicity-induced hepatic mitochondrial oxidative stress and, in turn, of JNK signaling pathway, thus avoiding the over-regulation of pro-inflammatory and pro-apoptotic process (252).

NF-κB is a transcription factor involved in innate and adaptive immune responses playing an essential role in the regulation of inflammatory signaling pathways in the liver. Under normal conditions, NF-κB is sequestered in the cytoplasm by the binding with IκB proteins; in response to stimulation by pathogenic stimuli, the degradation of the NF-κB inhibitor α (IκBα) allows the translocation of NF-κB to the nucleus, where it induces the expression of target genes encoding inflammatory mediators, such as TNF-α and IL-6 (4, 253). Persistent activation of the NF-κB pathway in the liver leads to a chronic inflammatory state and to insulin resistance, that further promote the development of NAFLD and NASH (81, 254). NF-κB and its downstream signaling pathway are under the inhibitory control of estrogen signaling (255–257), a regulation that accounts for sex- and menopause-associated over-regulation of hepatic inflammatory process and for the progression of NAFLD toward more harmful conditions such as NASH, fibrosis and HCC (16, 49, 230, 258, 259).

Homeostatic inflammation is tightly regulated by mechanisms acting to resolve inflammation in order to avoid excessive inflammation and pathological consequences. In the liver, the propagation or the resolution of inflammation mostly relies on the polarization abilities of KCs (the resident macrophages) and of the recruited macrophages (260, 261). Once activated by exogenous or endogenous danger signals, macrophages undergo pro-inflammatory or anti-inflammatory and reparative phenotype, respectively promoting or attenuating hepatic steatosis and liver injury in NAFLD (50, 258, 260, 261). As occurs in other physio-pathoplogical contexts (262, 263), estrogens might promote the skewing of pro-inflammatory macrophages toward anti-inflammatory macrophages and accelerate the resolution of inflammation and the tissue repair in the liver, thus contributing to limit NAFLD progression in pre-menopausal women with respect to men and post-menopausal women (258). Accordingly, a longer duration of estrogen deficiency increases the risk of developing fibrosis among post-menopausal women with NAFLD (177) as well as in OVX female mice fed with HFD (264).

Although the FA-induced activation of NOD-like receptor (NLR) NLRP3 inflammasome, which promotes IL-1β production, has been implicated in the progression of NAFLD to NASH (265–267), the potential role of estrogens in directly modulating NLRP3 inflammasome in the progression of NAFLD to NASH has been very poorly investigated (268) and remains unclear. By converse, estrogens suppress HCC through the ERβ-mediated upregulation of the NLRP3 inflammasome (269), likely contributing to the sex differences in HCC prevalence (270).

Liver Regeneration and Inflammation

Inflammation triggers many chronic and degenerative diseases, but it also aims to eliminate damaged cells and initiate tissue repair and regeneration, through highly conserved mechanisms (271). Tissue repair and regeneration is particularly important for the liver, especially in response to injury, an ability essential for the maintenance of the hepatic metabolic functions (57).

The process of liver repair and regeneration relies on the proliferative capacity of existing mature hepatocytes in response to environmental cues and can be divided in two phases: a "priming phase," where inflammatory mediators (e.g., IL-6, TNFα) trigger the inflammation-induced regeneration, and a "proliferation phase," where mitogens (including hepatocyte growth factor, HGF; transforming growth factor-α, TGF-α; epidermal growth factor, EGF) and auxiliary mitogens (including bile acids; endothelial growth factor, VEGF; insulin-like growth factor system, IGF system; estrogens) carry out the proliferation of hepatocytes, also through the interaction with the liver-resident immune cells (272–275).

Among inflammatory mediators, IL-6 plays a key role in the liver regeneration, as it is responsible for activating ∼40% of the genes that are immediately activated by transcription factors following partial hepatectomy (276, 277). According to that, mice lacking IL-6 show reduced hepatocyte proliferation, that can be restored with IL-6 administration (275). In addition to IL-6, also TNF-α is involved in the priming phase of liver regeneration, which requires the expression of inducible nitric oxide synthase (eNOS) to block the potential pro-apoptotic effect of TNFα signaling and trigger liver regeneration (5, 275). IL-6 and TNFα are released mainly by KCs, thus promoting hepatocyte proliferation. The KCs activation is mediated through the NF-κB signaling pathway triggered either by lipopolysaccharide (LPS)/Toll-like receptor4 (TLR4) signaling or by the components of the complement system like C3a and C5a (274, 275). While KC depletion is associated with impaired liver regeneration, the depletion of other liver-resident immune cells such as NK (natural killer) cells enhances liver regeneration due to reduced production of TNFα and IFNγ (interferon-γ), a negative feedback mechanism aimed at regulating the process of liver regeneration (5).

Males and females differ for their ability to regenerate the hepatic tissue in response to injury, with male animals showing a time-delay in the recovery process associated with a higher recruitment of monocytes (278), a difference that depends on both, estrogen and androgen signaling pathways (279–281). In regenerating livers, estrogens act mainly through ERα (281, 282), but also through ERβ (279), with ERα and ERβ orchestrating cell proliferation and differentiation, respectively. The relation between estrogens and IL-6 could be particularly complex, being IL-6 able to influence estrogen levels and, therefore, estrogen-dependent modulation of liver regeneration process (275).

A recent study demonstrated that estrogen and ERα might play an important role also in the accumulation of fats in the liver by modulating CD36 during the early phase of liver regeneration, when fatty acids, triglycerides and cholesterol are required for the proliferation of hepatocyte and for the formation of new cell membrane (283).

The Lack of Estrogen Signaling Impairs the Regulation of Hepatic Metabolism and Inflammation: Lessons From Estrogen Deficient and Knockout Mice

Estrogen Deficiency in Females

The relevance of estrogen signaling in the regulation of female hepatic metabolism and inflammation has been investigated in several pre-clinical studies recapitulating the effects of estrogen deficiency observed in post-menopausal women (14, 18, 169, 284, 285).

In the liver of ovariectomized (OVX) female mice, the lack of estrogens leads to hepatic insulin resistance, to enhanced DNL and FA import, and to reduced FA oxidation and secretion, resulting in increased body weight and fat mass and in fatty liver (14, 21, 153, 286, 287). In OVX females, the administration of estrogens improves insulin sensitivity and suppresses gluconeogenesis *via* the transcription factor FOXO1 (Forkhead Box O1) (288), prevents hepatic fat deposition by inhibiting DNL (153, 289), facilitates the VLDL (very low density lipoprotein)-mediated export of lipids from the liver by increasing hepatic VLDL-TG production and expression of microsomal triglyceride transfer protein (21, 290, 291) and sustains the β-oxidation of FAs by inducing expression of PPAR-α (peroxisome proliferator-activated receptor α) and FGF21 (123, 289).

Although estrogen replacement has been shown effective in reducing hepatic steatosis (123, 153, 287, 289, 291), however, the administration of constant amount of estrogens or SERMs (selective estrogen receptor modulators) partially restores a proper regulation of hepatic metabolism (123, 292). The reason for that likely resides on the fact that the administration of constant amount of estrogens does not reproduce the physiological oscillation of estrogen levels typical of the reproductive cycle and, therefore, fails to reproduce the cyclic activation of hepatic ERα, which is responsible for a tuned modulation of hepatic metabolism in females (15, 153, 292).

Moreover, estrogens may have a significant impact on hepatic metabolism depending on their route of delivery. For example, while transdermal estradiol reduces plasma TGs by increasing the rate of VLDL-TG clearance without affecting VLDL-TG production (293, 294), oral delivery of estradiol increases VLDL production and plasma TGs, indicating the liver the most responsible of estrogen's effects on increasing VLDL-TGs (21).

The lack of estrogens is associated with increases in lipotoxicity, pro-inflammatory cytokines (e.g., TNFα, IL-1β, and IL-6) and oxidative stress and with decreases in anti-inflammatory cytokines (e.g., IL-10, interleukin 10) and antioxidant defense, all changes that can be reverted or, at least, mitigated by HRT (295–297). When exposed to high intake of dietary lipids, the liver of OVX female mice displays increased expression of *Mcp-1* (monocyte chemoattractant protein-1) and *Ccr2* (monocyte chemokine receptor 2) that trigger the recuitment of macrophages and promote hepatic fibrosis, endoplasmic reticulum stress and apoptosis, all changes that are improved by estradiol treatment (264).

Estrogen Deficiency in Males

Even in the liver of males, estrogen action is relevant for the regulation of glucose homeostasis, insulin sensitivity, lipid metabolism, and in the prevention of hepatic steatosis (298, 299). Estrogen deficiency in men with mutations in the gene codifying for aromatase (CYP19A1, the enzyme converting testosterone in estrogen) show impaired glucose and lipid liver metabolism (300, 301). Aromatase KO (ArKO) mice display increased adiposity, glucose intolerance and insulin resistance in both sexes (302); in male ArKO mice, increased insulin resistance is primarily due to increased hepatic gluconeogenesis through the induction of *G6Pase* (glucose 6-phosphatase) and *Pepck* (phosphoenolpyruvate carboxykinase) expression (299). By contrast, only ArKO males, but not females, show impaired lipid and lipoprotein metabolism and develop hepatic steatosis (302, 303). The administration of estrogens reverses the hepatic steatosis, by reducing the expression of genes involved in DNL (e.g., *Fasn*, fatty acid synthase; *Acaca,* acetyl-CoA carboxylase α; *Scd-1*, stearoyl-CoA desaturase-1) and fatty acid uptake (e.g., *Adrp*, adipocyte differentiated regulatory protein) (302, 304) and by restoring the expression of enzymes involved in FA oxidation (e.g., *Cat*, catalase; *Mcad*, medium-chain acyl-CoA dehydrogenase) (305). Although the precise mechanism of estrogen action in the liver of males have not been fully elucidated, studies performed in KO mice suggest that estradiol mediats PPARα signaling in protecting against hepatic steatosis (306, 307).

Estrogen deficiency in ArKO males is also responsible for increased hepatic mitochondrial apoptosis and altered permeability of the mitochondrial membranes, that are restored by supplementation of 17β-estradiol (308).

ERα in Females

Estrogens can mediate their biologic effects in the female liver acting mainly through the estrogen receptor α (ERα, the isoform most expressed at the hepatic level) (15, 152) through a number of mechanisms, including the regulation of gene transcription by the direct binding to specific estrogen responsive elements (ERE) or by the tethering with other DNA-binding factors and by non-genomic action through membrane-associated ERα (15, 153, 309–313).

While the lack of ERβ does not affect hepatic phenotype (314), the role of ERα in the regulation of hepatic metabolism and inflammation has been highlighted by several studies performed with total body (ERαKO) and liver-specific (LERKO) ERα knockout mice (311). ERαKO mice mostly recapitulate the metabolic phenotype of OVX animals, with increased body weight, visceral adiposity, glucose production, insulin resistance, and hepatic steatosis associated with increased hepatic inflammatory signaling (21, 314–316). Differently from control mice, ERαKO mice are not able to antagonize the induction of cytokines in consequence to a pro-inflammatory stimulus, indicating that ERα protects the liver against liver inflammation (317).

The LERKO mouse represents a useful tool to elucidate the specific relevance of ERα in the liver, especially in the hepatocytes, as this mouse model has been obtained by crossing mice expressing *floxed* ERα with mice expressing *Cre*-recombinase under the control of albumin promoter, that it is specifically expressed in the hepatocyte cells (13). Although improperly, LERKO can be considered as liver-specific ERα KO mice, being hepatocytes the most abundant cell type in the liver (57) and being ERα the receptor for estrogens most expressed in the hepatocytes (152, 241).

Compared to control counterparts, LERKO females show an impaired regulation of genes relevant in the regulation of hepatic lipid and lipoprotein metabolism during estrous cycle progression (15), with aging and after ovariectomy (153). As a consequence, LERKO females show increased deposition of lipids in the liver and an impaired regulation of lipoprotein synthesis, leading to a reduced cholesterol efflux to the liver, impaired hepatic cholesterol clearance, high circulating cholesterol levels and increased susceptibility to atherosclerosis (15, 237).

Additional studies have confirmed the role of ERα in preventing hepatic steatosis by showing that liver-specific knockdown of ERα is sufficient to induce hepatic steatosis through a mechanism that seems to involve the regulation of small heterodimer partner (SHP), a transcription factor important in the regulation of hepatic metabolic processes and in the protection against hepatic inflammation (318, 319).

The livers of LERKO mice exhibit a greater expression of genes involved in the inflammatory process (e.g., *Tnfα; Il-1β;* interleukin-12 beta, *Il-12β; Ccr2)* and collagen deposition (sequestosome1, *Sqstm1*; vimentin, *Vim*; serpine1, *Serpine*); according to that, LERKO females display portal infiltration of mononuclear leukocytes and portal or centrilobular collagen deposition in the liver (15).

The action of the hepatic ERα is particularly relevant when mice are subjected to excess of dietary lipids: with the lack of hepatic ERα, LERKO females result no more protected against the excess of dietary lipids and accumulate lipids in the liver (91), a condition resembling what happens in OVX mice and post-menopausal women (14). However, differently from control OVX females, estrogen treatment fails to prevent lipid deposition in the liver of LERKO females, further stressing the specific relevance of hepatic ERα in the regulation of female hepatic metabolism (287, 320).

Also transgenic mice in which the expression of ERα is limited to the cytoplasm develop hepatic steatosis (312), suggesting that the protective effects of estrogens on liver health can be mediated by both, classical and non-nuclear mechanisms (321, 322).

ERα in Males

Similar to females, ERαKO male mice develop insulin resistance, impaired glucose tolerance, increased adiposity and marked hepatic steatosis (314, 315, 323). Despite its reduced expression compared to females (15, 152), also in males the liver-specific disruption of ERα signaling leads to altered expression of genes involved in carbohydrate and lipid metabolism (241, 324–326). Hepatic ERα plays a key role in the maintenance of hepatic metabolism, by suppressing hepatic gluconeogenesis and by decreasing DNL through its direct binding to the promoters of genes involved in gluconeogenesis (e.g., *Pepck*, *G6Pase*) and lipid metabolism (e.g., *Fasn*, *Acaca)* (241) and through the modulation of FOXO1 phosphorylation (326). As a consequence of the lack of ERα-dependent regulatory activity, LERKO males display elevated hepatic glucose production (HGP), liver insulin resistance, increased hepatic lipogenesis and liver lipid deposition (241, 326).

Recent studies suggest that hepatic ERα is required for the estrogen-mediated programming of the hepatic metabolism of males, contributing to hepatic sexual dimorphism (152) and accounting for the sex-specific metabolic response to diets enriched in lipids (91). In the liver of males, ERα is required also for optimal immune-metabolic function, as its lack causes increased expression of several inflammatory genes (327).

GPER

In addition to membrane localized ERs, estrogens can signal through G-protein coupled Estrogen Receptor (GPER, also called Gpr30), a cell surface receptor which role in the regulation of liver metabolism has recently emerged (328–330). After the binding with estrogens, GPER activate multiple non-genomic pathways, as well as the transcriptional programs through the regulation of target genes (331–334) in diverse cell types and tissues, including the liver (335).

GPER has been functionally implicated in several physiological and pathological process (335) and, in particular, in the regulation of metabolism (328, 332, 336–338) and immune response (339–341). In the liver, GPER plays a role in modulating lipid metabolism, in lowering circulating lipid levels and in reducing inflammation (20), as confirmed by several pre-clinical and clinical studies. Individuals carrying a hypofunctional genetic variant of GPER show increased plasma LDL cholesterol; according to that, the activation of GPER induces the expression of the LDL receptor (LDLR) in HepG2 liver cells (342). A recent study demonstrates that GPER mediates the estrogen-dependent reduction of LDLR degradation by preventing the internalization of PCSK9 (proprotein convertase subtilisin/kexin type 9), thus resulting in a higher LDL uptake by liver cells and, consequently, to lower circulating LDL cholesterol (343).

In OVX female mice, the activation of GPER lowers the levels of circulating lipids, reduces the expression of lipogenic and pro-inflammatory genes, and increases the expression of genes involved in lipid oxidation in the liver (329). In a KO mouse model, the lack of GPER leads to increased lipid accumulation in the liver and decreased circulating HDL levels in females, but not males (344), highlighting a sex-specific role of GPER in the metabolic homeostasis (329).

GPER signaling is associated with the immune and anti-inflammatory response, as revealed by its role in counteracting a variety of pathological conditions, including diabetes and obesity (330, 338, 345), atherosclerosis (346, 347), asthma (348), neuroinflammation (349, 350), and cancer (97, 351). In the liver, the lack of GPER enhances immune cell infiltration, fibrosis, and the production of inflammatory factors, such as IL-6, IL-1β, and TNFα in a mouse model of HCC (351). The activation of GPER signaling is effective in reducing the expression of IL-6, but not the viability and proliferation of hepatoma cells, suggesting that GPER action against hepatic tumorigenesis occurs through the regulation of inflammatory response rather than the direct modulation of tumor growth and invasion (351).

Although these studies suggest a direct involvement of GPER in the regulation of metabolism and inflammation in the liver, especially in females, it cannot be excluded that the hepatic effects due to the lack of its signaling are the results of a more complex interaction among metabolic tissues. Indeed, mice lacking GPER show increased adiposity, decreased insulin sensitivity, defective glucose/lipid homeostasis, and inflammation (337, 338, 352), all features that might indirectly affect the hepatic metabolism, pointing to the need of liver-specific GPER models to clarify the specific role of GPER in the hepatic tissue.

Estrogens and Key Cell Types in Liver Metabolism and Inflammation

The liver is composed of several cell types, each of them having unique functions in the regulation of metabolism and immune response and showing interactions with the other cell type, thus cooperating at multiple levels in the regulation of the hepatic function. The major cell types contributing to the main liver functions are hepatocyctes, Kupffer cells, hepatic stellate cells, liver sinusoidal endothelial cells, and cholangiocytes.

Hepatocyctes

Hepatocyctes represent the most abundant cell type in the liver (accounting for 80% of liver mass) and are involved in several functions, including lipid and carbohydrate metabolism (353), protein synthesis (354), detoxification and drug metabolism (355, 356), and the secretion of coagulation and complement factors (353, 354, 357). In the hepatocytes, estrogens, mainly acting through ERα, limit gluconeogenesis (241, 288) preventing increased HGP and insulin resistance (288), limit the uptake of FFAs, inhibit DNL (153) and promotes FA oxidation (289) and export (287), thus preventing lipid deposition in the liver and the generation of lipotoxicity and ROS (252) that trigger a pro-inflammatory response acting as the driver of NAFLD progression and liver degeneration (81, 247). Estrogen signaling facilitates the resolution of inflammation by inhibiting the production of pro-inflammatory cytokines (264), regulates apoptotic process (358, 359), and promotes liver cell regeneration (279–282), thus limiting or preventing liver injury. As recapitulated by studies performed in OVX and LERKO females, the lack of the regulatory activity of estrogens in the hepatocytes favors the development and progression of NAFLD and, likely, of the associated cardio-metabolic diseases (e.g., atherogenesis) (16).

Kupffer Cells (KCs)

Kupffer cells (KCs) represent one-third of the non-parenchymal cells in the liver and account for 80–90% of tissue macrophages present in the body, acting as immune sentinels (360). KCs are important members of the innate and adaptive immune systems, serving as a first line of defense against bacteria, microbial debris and endotoxins derived from the gastrointestinal tract. Once activated, KCs trigger an inflammatory response by producing a panel of pro-inflammatory cytokines, including TNF-α, IL-1β, and IFN-γ, and provide to the clearance of phagocytosable particles (361). Given their role in the regulation of inflammatory and innate responses, KCs are considered as potential targets for the treatment of liver diseases, including NAFLD (360, 362, 363). Male and female KCs are different from a morphological (364) and functional point of view (258), contributing to sex differences in liver inflammation and regeneration (280) and in the prevalence and progression of NAFLD (17, 258), ALD (alcoholic liver disease) (365) and HCC (259, 366). Estrogens result involved in the sensitization of KCs to toxic stimuli (367) and in driving the pro/anti-inflammatory polarization of KCs, that exerts a key role in the resolution or progression of inflammation, thus counteracting or promoting the development of liver diseases (50, 368, 369). The estrogen-dependent regulation of cytokine production by KCs is predominantly mediated *via* ERα (370, 371), resulting the isoform most expressed in these cell types (372).

Hepatic Stellate Cells (HSCs)

Although comprising only 5% of the liver cells, *hepatic stellate cells (HSCs)* play a central role in liver metabolism, especially in retinol metabolism and lipid storage (373). In healthy liver, HSCs are quiescent and store 80% of total liver retinol, that is released depending on its extracellular status. In injured liver, HSCs become activated and transform into myofibroblasts; activated HSCs lose their retinols and produce a considerable amount of extracellular matrix, thus leading to liver fibrosis (59). Although sex differences in the morphological expression of male/female HSCs has not been observed (364), several studies have demonstrated that estrogen inhibits the activation of HSCs and reduces liver fibrosis (374, 375), suggesting that estrogen signaling might account for the sex-specific prevalence of hepatic fibrosis. Although the molecular mechanism has not been fully clarified, estrogens seem to act through ERβ (376) and GPER (377), given that ERα is not expressed in these cells (378).

Liver Sinusoidal Endothelial Cells (LSECs)

Liver sinusoidal endothelial cells (LSECs), which comprise ∼50% of liver non-parenchymal cells, are highly specialized endothelial cells containing many small pores or fenestrations, which provide open channels that facilitate the transfer of substrates between the blood and the liver parenchyma and regulate lipoprotein traffic to and from the hepatocytes (379, 380). Their unique morphology gives to LSECs a high endocytic capacity, enabling them to act as effective scavengers and promote the clearance of lipids and macromolecules and small particulates from the blood. The impairment of their function is associated with the development of extra-hepatic pathologies, including atherosclerosis (380). *LSECs* exert a key role in the innate and adaptive immunity, promoting the presentation of antigens and favoring the removal and clearance of circulating antigens and viruses (381). In addition to their roles as pathogen recognition and antigen-presenting cells, LSECs also have a critical role in the recruitment of leukocytes into liver tissue, thus influencing the composition of hepatic immune population. The balance between tolerance and effector immune responses driven by LSECs might promote the resolution or the progression of the immune response, eventually leading to several chronic liver diseases, including NAFLD, cirrhosis, fibrosis, liver failure and HCC (381, 382).

In LSECs, estrogens, even by modulating the levels and the nuclear occupancy of ER (372), enhance the production of nitric oxide (NO) and regulate the hepatic sinusoidal microcirculation (383, 384), likely explaining the higher incidence of liver cirrhosis with portal hypertension in men and post-menopausal women than pre-menopausal women (385).

Cholangiocytes

Cholangiocytes are the epithelial cells lining the intrahepatic and extrahepatic bile ducts; these cells participate in bile production and secretion and, although to a less extent than hepatocytes, have a role in the liver development, regeneration and repair (386, 387). Cholangiocytes can be activated by a variety of insults, including infections, cholestasis, and xenobiotics (386), leading to increased proliferation and to pro-fibrotic and pro-inflammatory secreted factors (388), that can favor the development of cholangiopathies and cholangiocarcinoma (389–392).

Cholangiocytes are targets of estrogen action: by acting through both ERα and ERβ and by activating either genomic or non-genomic pathways, estrogens play a key role in the regulation of proliferative and secretory activities of cholangiocytes (393, 394). The lack of estrogens in OVX females decreases the expression of ERs (2.5-fold for ERα and 35-fold for ERβ), leading to reduced cholangiocyte proliferation and bile duct mass; conversely, the administration of 17β-estradiol during bile duct ligation in OVX rats induced a normalization of bile duct mass, cholangiocyte proliferation, and apoptosis (395). Also in males, estrogens exerts a major role in stimulating cholangiocyte proliferation by preventing the increase of cholangiocyte apoptosis and loss of cholangiocyte proliferation (396). Notably, the altered expression and/or activation of ERα and ERβ is often associated with a high risk of primary biliary diseases (397–399).

Impaired bile flow leads to cholestasis, a pathology characterized by elevated levels of bile acid in the liver and serum followed by hepatocyte and biliary injury, that shows an increased incidence in women receiving estrogen for contraception or hormone replacement therapy, or in susceptible women during pregnancy. Although the molecular mechanisms involved in cholestasis remain controversial, recent findings suggest that estrogens may influence its course by directly modulating the patho-physiology of cholangiocytes, which are the primary target of damage in this disease (393).

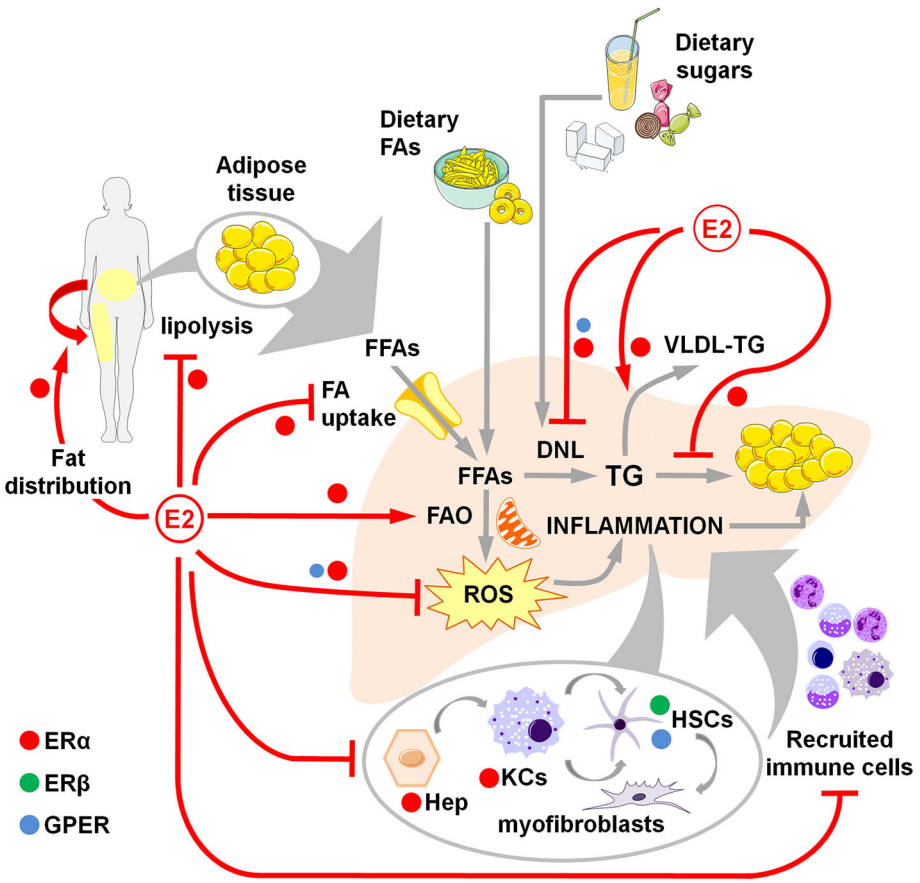

FIGURE 1 | Overview of estrogen action through ERα, ERβ and GPER in counteracting NAFLD development and progression in women. Estrogens favor fat distributionr to subcutaneous deposits, inhibit adipose tissue lipolysis and reduce the uptake of FFAs, thus limiting the flux of FFAs to the liver. Estrogens limit dietary-induced DNL and facilitate the export of lipids as VLDL-TG. Estrogens promote the FA β-oxidation and prevent the activation of a sustained alternative FA oxidation that triggers lipotoxicity and the generation of ROS that, in turn, activate a pro-inflammatory response. Hepatocellular damage and fat-derived factors mediate the local activation of a pro-inflammatory response by hepatocytes, KCs and HSCs, that promote the degeneration of hepatic tissue and the recruitment of extra-hepatic immune cells that boost the inflammatory response and worsen the metabolic alterations. DNL, *de novo* lipogenesis; E2, estrogens (mainly 17β-estradiol); FAs, fatty acids; FFAs, free fatty acids; FAO, fatty acid oxidation; Hep, hepatocytes; HSCs, hepatic stellate cells; KCs, Kupffer cells; ROS, reactive oxygen species; TG, triglycerides; VLDL, very-low density lipoprotein.

Although each cell type plays a specific role in the liver and expresses a unique gene (400) and proteomic profile (401), only the cooperation among different cell types enables the liver to achieve its functions (402), a consideration that should be taken into account when performing *in vitro* studies, in which the cross-talk among liver cell types is lost or partially reproduced (403, 404). In this view, although challenging, the recent advances in the generation of human liver organoids might represent a potential, more reliable tool for the *in vitro* analysis of liver-specific biological processes and for disease modeling and drug screening at near-physiological conditions (405).

CONCLUSION

The data summarized in this review outline the role of estrogens and their receptors in antagonizing the metabolic and inflammatory alterations that trigger and boost NAFLD development, thus determining its sex-dependent prevalence and its lower incidence in fertile females (**Figure 1**, **Table 1**).

Estrogen-mediated effects likely arise from higher metabolic flexibility gained and perfected through evolution by the female liver to adapt the hepatic metabolism to the reproductive function (12, 13, 152, 242). Playing the liver the most relevant role in the accomplishment of energy requirements, during evolution the hepatic metabolism has been sharpened, in a sex-specific fashion, to reach an accurate interconnection of regulatory mechanisms aimed to sustain the energy needs of reproductive functions that are greatly different between the two sexes. The dynamic regulation of hepatic metabolism should have acquired a maximum degree of complexity in the liver of females that, compared to males, have to be more flexible in adapting their hepatic metabolism to the different, more variable, reproductive stages (reproductive cycle progression, pregnancy, lactation) that entail different energy requirements.

TABLE 1 | Summarizing the relevance of estrogen signaling, ERα, ERβ, and GPER in the sex-specific regulation of metabolic and inflammatory pathways relevant in NAFLD development and progression.

Process/pathway	Regulation by estrogens	Mediators			Sex/Gender differences	References
		ERα	ERβ	GPER		
Hepatic glucose metabolism	●	●			●	(39–41)
Hepatic glucose production (HPG)	●	●			●	(36–38)
Hepatic insulin sensitivity	●	●			●	(20, 120–123)
Hepatic FFA uptake	●				●	(91, 303)
Hepatic *de novo* lipogenesis	●	●			●	(91, 116, 303)
Hepatic FA oxidation	●			●	●	(91, 139, 303)
VLDL-TG export	●				●	(91)
Hepatic lipid storage and deposition	●	●		●	●	(91, 115–117, 303, 304, 329, 344)
Hepatic AA metabolism	●	●			●	(91, 151, 152)
Hepatic JNK activation	●	●			●	(251, 252)
Hepatic NF-κB activation	●	●			●	(255–257)
Macrophage polarization (from pro- to anti- inflammatory phenotype)	●	●	●		●	(258)
Liver regeneration	●	●	●		●	(278, 279, 281, 282)
Subcutaneous fat distribution	●	●			●	(92, 94, 95, 406)
Adipose tissue lipolysis	●	●			●	(92, 94, 406)

The female liver had to develop and mold mechanisms able to sense and modulate efficiently the hepatic metabolism accordingly to the hormonal rhythm of estrogen fluctuations during the reproductive cycle and in other reproductive stages (pregnancy, lactation). In this view, in the liver of female mammals, estrogen signaling has therefore acquired a tight control on the hepatic metabolism through a sequence of well-tuned and intertwined events that have been perfectly tuned to secure reproduction only in favorable energy conditions and to support the energy needs of the different reproductive stages (14, 153, 235).

The high metabolic dynamicity conferred by estrogens to female liver contributes to prevent and limit the surge and progression of metabolic and inflammatory alterations in the liver, a mechanism underlying the increased incidence of NAFLD associated with the decline in liver metabolic flexibility after menopause.

The effects of estrogens and their receptors on the regulation of liver metabolism and inflammation may be direct or indirect, acting—for example—through other transcription factors and nuclear receptors (NRs) (407) with relevant and sex-specific activities in the liver (408) and in the NAFLD pathogenesis (409–411). Such an interplay might be particularly complex and regulated in the female liver: in view of its action in the regulation of reproductive process, ERα might have acquired in the female liver a regulatory role over these signaling pathways to adapt liver metabolism and inflammation to hormonal and nutritional status to accomplish the metabolic adaptations required to support the energy needs of reproduction. According with this idea, the lack of estrogens impairs the regulation of some NR signaling, including PPARα (289, 412) and glucocorticoid receptor (GR) (413), exerting pivotal roles in the regulation of hepatic metabolism and inflammation (414–416), thus favoring NAFLD development.

Despite the extensive, although probably underestimated, awareness on hepatic sex differences, the molecular mechanisms determining the sex-specific incidence of liver pathologies such as NAFLD are far to be unraveled. This knowledge has been prevented and affected by several limitations that stem from: (a) the paucity of available data on both sexes coming from pre- and clinical studies in which females are often underrepresented (12, 417); (b) the inability to enroll females in clinical studies (418–420); (c) the limited and, in some cases, misleading conclusions reached by experimental designs that did not take into account the relative contribution of genetic and hormonal backgrounds and exclude the sexual hormones as potential *confounding factors* (12, 417); (d) the lack of proper research tools helpful in investigating the genetic and/or hormonal factors relevant for the hepatic sexual dimorphism or the inability to use the available research tools in the best way (421, 422); (e) the fragmentary and still incomplete view coming from several studies that often do not share common protocols or lack of significance for the low number of the samples analyzed (422, 423); (f) the low, still insufficient commitment dedicated to dissemination of the results obtained from sex/gender research, such as educational programs addressed to health professionals (researchers, clinicians, scientific training programs, health institutions, etc.) and to society in general (419, 423–425); *g)* the still limited policies aimed at promoting sex/gender research programs (426–428). All these aspects contribute to our limited understanding of the nature and relevance of hepatic sexual dimorphism, thus preventing, so far, the development of more efficacious, sex-specific therapies against liver pathologies such as NAFLD, which incidence is greatly increasing in EU

and accounts for €35 billions only in Germany, France, Italy, and United Kingdom (234, 429). Furthermore, the partial unawareness of the relevance of hepatic sexual dimorphism in the liver physio-pathology is a contributing cause to the development of associated cardio-metabolic diseases, such as atherogenesis and CVDs (430, 431). Similarly, the lack of sex-specific pharmacological treatments (that should differ in terms of molecules, dose, timing and risk of adverse drug reaction between the two sexes) leads often to drug-induced hepatotoxicity, representing the main cause of withdrawal of drugs from the market and the main reason of liver transplants (17).

In this view, a deeper understanding of the mechanisms underlining the sex-specific incidence of NAFLD and the role of estrogen signaling pathways will likely yield the basis for the design of more personalized hepatic therapies that would significantly improve the quality of life of a large section of our society as well as of men and women which experience impaired/lost hormonal signaling (i.e., due to gonadal failure, aging, exposure to endocrine disrupting chemicals).

AUTHOR CONTRIBUTIONS

SDT: figure preparation, writing, review, and editing.

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PERSPECTIVE
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Improved Glucocorticoid Receptor Ligands: Fantastic Beasts, but How to Find Them?

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Exogenous glucocorticoids are widely used in the clinic for the treatment of inflammatory disorders and hematological cancers. Unfortunately, their use is associated with debilitating side effects, including hyperglycemia, osteoporosis, mood swings, and weight gain. Despite the continued efforts of pharma as well as academia, the search for so-called selective glucocorticoid receptor modulators (SEGRMs), compounds with strong anti-inflammatory or anti-cancer properties but a reduced number or level of side effects, has had limited success so far. Although monoclonal antibody therapies have been successfully introduced for the treatment of certain disorders (such as anti-TNF for rheumatoid arthritis), glucocorticoids remain the first-in-line option for many other chronic diseases including asthma, multiple sclerosis, and multiple myeloma. This perspective offers our opinion on why a continued search for SEGRMs remains highly relevant in an era where small molecules are sometimes unrightfully considered old-fashioned. Besides a discussion on which bottlenecks and pitfalls might have been overlooked in the past, we elaborate on potential solutions and recent developments that may push future research in the right direction.

Keywords: glucocorticoids, glucocorticoid receptor, selective GR modulators, drug discovery, inflammation, assay development, GR, SEGRM

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INTRODUCTION

Glucocorticoids (GCs) are endogenous steroidal hormones involved in metabolism, stress, development, and immunity (1). They exert their effects by binding the glucocorticoid receptor (GR), a nuclear receptor (NR) consisting of an intrinsically disordered N-terminal domain (NTD), a central DNA binding domain (DBD), a hinge region (HR), and a C-terminal ligand-binding domain (LBD) (2). Upon ligand binding, GR typically translocates from the cytoplasm to the nucleus where it acts as a genuine transcription factor to regulate target gene expression via multiple mechanisms (**Figure 1A**), which are discussed in detail in (3). The discovery of the anti-inflammatory effects of endogenous GCs preceded the development of synthetic GCs, which are used to treat, among others, inflammatory disorders, and hematological cancers (4). Unfortunately, the therapeutic efficacy of such exogenous GCs is, particularly for systemic use, overshadowed by an unacceptably high number of undesired side effects such as hyperglycemia, osteoporosis, mood swings, and weight gain (5).

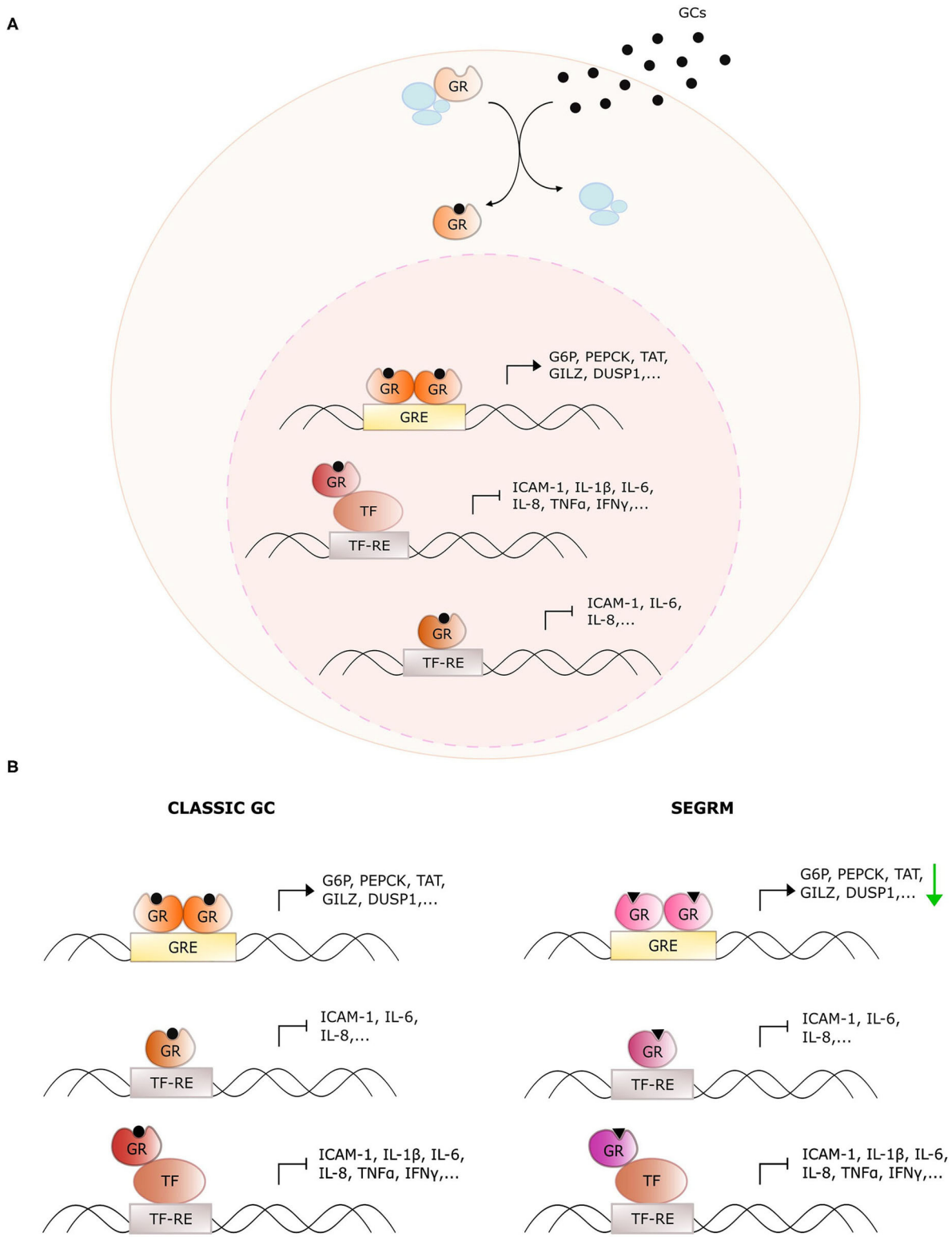

FIGURE 1 | Overview of glucocorticoid receptor activity with classic glucocorticoids and selective GR modulators. **(A)** General action mechanism of the glucocorticoid receptor (GR). Glucocorticoids (GCs) diffuse through the cellular membrane and bind GR. The latter dissociates from its chaperone complex and migrates to the nucleus. There, it dimerizes and binds glucocorticoid response elements (GREs) to upregulate downstream target genes. Monomeric GR also undergoes protein-protein interactions with DNA-bound pro-inflammatory transcription factors (TFs) to downregulate their activity, or it binds directly to the TF response elements (TF-RE). **(B)** Distinct actions of classic GCs and selective GR modulators (SEGRMs). In contrast to classic GCs, SEGRMs are hypothesized to reduce GR's capacity to dimerize and therefore reduce GRE-mediated transcription. Interference with TF activity is driven via monomeric GR and therefore maintained with SEGRMs.

Some of these side effects stem from direct binding of homodimeric GR to pseudopalindromic glucocorticoid reponse elements (GREs) in the promoter regions of genes controlling key metabolic pathways (**Figure 1**). The resulting GRE-driven upregulation of tyrosine aminotransferase (TAT), glucose 6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK) for instance leads to hyperglycemia (6). The suppression of nuclear factor (NF)-κB- and activator protein (AP)-1 activity on the other hand, is typically explained via protein-protein interactions with monomeric GR (called tethering) (7). Despite the controversies on the actual underlying mechanism (see further), the targeting of activities of pro-inflammatory transcription factors undoubtedly contributes substantially to the anti-inflammatory actions of GCs.

The discrepancy between monomer- and dimer-driven effects of GR was first suggested in 1994 with the demonstration that GR with a dimerization-disrupting mutation in the DBD (GRdim) is still able to repress AP-1-driven genes, while no longer able to induce GRE-mediated activation (8). Four years later, Reichardt et al. established that mice carrying this homozygous mutation were viable and healthy, in contrast to GR full knock-out mice (9), arguing for an equally viable mechanistic basis to separate beneficial from undesired effects. This was the starting point of the search for so-called dissociative or selective GR modulators (SEGRMs), GR ligands that can still repress inflammation via monomer-driven NF-κB, and AP-1 inhibition, while no longer inducing GRE-driven side effects (**Figure 1B**).

In the meantime, a few shades of gray have been added to the original black-and-white monomer-dimer paradigm. First of all, dimer-mediated gene activation also contributes to the anti-inflammatory effects of GCs via the upregulation of anti-inflammatory genes such as glucocorticoid-induced leucine zipper (GILZ) and dual specificity phosphatase (DUSP1) (10). This helps explaining why GRdim mice show increased sensitivity to acute inflammation such as septic shock (11). Secondly, it was shown *in cellulo* that Dex still promotes dimerization of the GRdim mutant (12). However, introducing an extra point mutation in the GR LBD almost completely disrupted dimerization and abrogated GRE-driven activity, but preserved the inhibition of NF-κB activity. Thirdly, monomeric GR was shown to bind directly to genomic NF-κB and AP-1 response elements, without the need for the transcription factor itself (13, 14). This finding challenges the original tethering hypothesis but still supports the notion that suppression of NF-κB and AP-1 activities does not require GR dimerization. Taken together, given the bodies of evidence on a large contribution of dimeric GR to particular side effects vs. the role of GR monomers to support anti-inflammatory actions in a chronic setting, the notion that compounds that favor signaling via monomeric GR can hold a therapeutic benefit against persistent inflammation, still stands.

The development of successful SEGRMs has proven to be a long and extremely bumpy road. Many compounds that showed promising initial results (listed in **Table 1**) never got past the pre-clinical stage or failed later on in clinical trials. It is well-known that the success rate for the development of any kind of small molecule drug from bench to clinic is very low, typically starting from 10,000 compounds to end up with one market-approved drug (44, 45). In addition, we believe that in the case of GR, multiple technical, and biological factors have been reducing the prospect to success even further. Fortunately, molecules are still being developed, trying to meet the hope of many patients who would benefit from GR modulators. For instance, AZD7495 (asthma, NCT03622112) and AZD9567 (rheumatoid arthritis, NCT03368235) are currently under evaluation in clinical trials.

This perspective offers our opinion as molecular biologists on the rationale why a continued search for SEGRMs still makes sense and bears significant relevance. We offer our view on a number of bottlenecks and pitfalls that might have hampered research progress in the past and elaborate on which new developments and insights could help overcome these issues.

SEGRMs: THE UNMET MEDICAL NEED

The need for more selective GR ligands remains highly relevant. Although more targeted therapies have successfully been introduced, such as anti-tumor necrosis factor (TNF) for arthritic disorders and inflammatory bowel diseases (IBD), these therapies are not without limitations. For one, anti-TNF therapy has been associated with a 250% increase in the occurrence of tuberculosis (46). Furthermore, these therapies have been reported to trigger multiple sclerosis (MS) and other demyelinating conditions (47–50). This is in line with the reported disease worsening in patients with pre-existing MS in clinical trials for Lenercept and cA2, two types of anti-TNF therapy (51, 52). Beside such side effects, monoclonal therapies are generally very expensive, laying a huge burden on health care systems, which will only increase with aging populations in western countries. Their price also makes them unaffordable for most people in low income countries, which is particularly a problem for asthma, for which 80% of disease-related deaths occur in low to low-medium income countries (53).

GCs on the other hand are generally much cheaper and are still the first-line treatment for asthma, multiple sclerosis, and multiple myeloma among others (54–56). However, their side effects are a well-known problem and not necessarily limited to patients receiving oral or intravenous GCs. While topical skin treatments, especially with the newest generation glucocorticoids, impose a very low risk for systemic side effects (57–59), topical eye treatments, and inhaled GCs (IGCs) have both been associated with adrenal suppression (60, 61). This can lead to growth retardation in infants and children, who form a large cohort of the asthma patient population. The long-term use of high doses IGCs has also been associated with decreased bone mineral density in both children and adults (62–64). Although the benefits of ocular and IGCs usually outweigh the risks, patients would still benefit from GCs with lower risks for systemic side effects.

Taken together, the need for more selective GCs reaches further than systemic treatments and is also high for ocular and inhalation therapies.

TABLE 1 | Available pre-clinical data of SEGRMs.

Compound	*In vitro* assays and *in cellulo* overexpression assays	*In cellulo* assays for endogenous anti-inflammatory and/or side effect targets	Inflammatory animal models	Status and latest progress	References
LGD-5552	Ligand-binding assays GR, AR, MR, PR MMTV-luciferase in CV-1 cells (overexpressed GR) E-selectin-luciferase in CV-1 and HepG2 cells (overexpressed GR) IL-6-luciferase in HepG2 cells (overexpressed GR) Cofactor binding assays	PEPCK and PDK4 mRNA in H4IIE cells COX2 and APOCIII mRNA in H4IIE cells POMC mRNA in ATT20 cells	Collagen-induced arthritis in mice Freund's complete adjuvant-induced arthritis in rats Experimental autoimmune encephalomyelitis in rats	Discontinued (preclinical)	(15, 16)
AL-438	Ligand-binding assays GR, PR RSV-LTR-GRE-luciferase in CV-1 cells (overexpressed GR) TAT-luciferase in HepG2 cells (overexpressed GR) E-selectin-luciferase in HepG2 cells (overexpressed GR) Cofactor binding assays	Eosinophil counts in BAL Human PBMC cell and rat splenocyte T-cell proliferation assays Osteocalcin protein in MG-63 cells Aromatase activity in hDSF cells IL-6 release in HSKF1501 cells	Carrageen-induced paw edema in rats Freund's complete adjuvant-induced arthritis in rats	Discontinued (preclinical)	(17–19)
MK-5932	MMTV-luciferase in A549 cells MMTV-luciferase in HeLa cells TNFα-β-lactamase in U937 cells	TNFα, IFNγ, IL-1β, IL-6 secretion in human whole blood TNFα, IL-6 secretion in rat whole blood	Oxazolone-induced contact dermatitis in rats	Discontinued (preclinical)	(20, 21)
GW870086	Functional selectivity MR, AR, PR, ER on MMTV-luciferase in CV-1 cells MMTV-LTR-luciferase in A549 and MG-63 cells E-selectin-κB-RE-alkaline phosphatase in A549 cells	Lymphotoxin-β, COX-2, Cyp24a1, MAP-7, GPR64, GILZ, DUSP1, MICAL2, FKBP5, CDKN1C, RGS2, SGK mRNA in A549 cells	Delayed-type oxazolone-induced contact hypersensitivity in mice Ovalbumin-induced airway inflammation in mice	Discontinued Phase II for asthma: no difference with placebo (NCT00945932) Phase II for atopic dermatitis: weaker effects than fluticasone propionate standard (NCT01299610)	(22–24)

(Continued)

TABLE 1 | Continued

Compound	*In vitro* assays and *in cellulo* overexpression assays	*In cellulo* assays for endogenous anti-inflammatory and/or side effect targets	Inflammatory animal models	Status and latest progress	References
BI653048	Ligand-binding assays GR, PR MMTV-luciferase in HeLa cells	IL-6 release in CCD-1112Sk cells Osteocalcin levels in MG-63 cells Human ERG potassium channel inhibition in Hek293T cells	Canine low dose endotoxemia model	Discontinued Phase I: no improvement on side effect profile compared to prednisolone (NCT02217631, NCT02224105, NCT02217644)	(25–27)
Mapracorat	Ligand-binding assays GR, PR, AR, MR MMTV-luciferase in HeLa cells Collagenase-luciferase in HeLa cells κB-RE-luciferase in SV-40 transformed hCEpiC cells TPA-RE-luciferase in SV-40 transformed hCEpiC cells	TAT activity in HepG2 cells IL-12p40, IFNγ secretion in PBMC cells Eotaxin-1 (+/– GR siRNA), –3, CCL5 (+/– GR siRNA), G-CSF, IL-6, IL-8, MCP-1 release in hConF cells Eotaxin-3, CCL5 (+/– GR siRNA), CCL27, ICAM-1 (+/– GR siRNA), IL-6, IL-8, MCP-1, TNFα release in hCEpiC cells IL-6, MCP-1 release and (p)p38, (p)JNK protein in SV-40 transformed hCEpiC cells IL-6, IL-8 release in hONA cells IL-1β, ICAM-1 release in hREC cells IL-6, IL-12p40, MCP-1 release in THP-1 cells (p)JNK, (p)p65, (p)p38, IκBα levels in hCEpiC cells MYOC levels in mkTM cells Migration, apoptosis, IL-8 release, annexin-1, and CXCR4 expression in human eosinophils IL-6, IL-8, CCL5, TNFα release in hMC-1 cells GM-CSF, TNFα, PGE2 production and COX-2, (p)p38, (p)MK2, DUSP1 protein in Raw 264.7 cells IL-6, IL-8, MCP-1, PGE2 release, COX-2, RelB, (p)IκBα protein, RelA and RelB DNA binding in human keratinocytes	Croton oil-induced irritant contact dermatitis in mice and rats Dinitrofluorobenzene (DNFB)-induced allergic contact dermatitis in mice and rats Dry eye model in rabbits Paracentesis model in rabbits Ovalbumin-induced allergic conjunctivitis in guinea pigs Compound 48/80-induced wheal and erythema skin inflammation in beagles	Discontinued Phase III for cataract surgery, no results reported (NCT01591655) Phase I for psoriasis, no results reported (NCT03399526) Phase I to assess corneal endothelial cell changes, no results reported (NCT01736462)	(28–36)

(Continued)

TABLE 1 | Continued

Compound	*In vitro* assays and *in cellulo* overexpression assays	*In cellulo* assays for endogenous anti-inflammatory and/or side effect targets	Inflammatory animal models	Status and latest progress	References
(Fos)dagrocorat	Gal4-RE-luciferase with Gal4-DBD-LBD in Huh7 cells Cofactor binding assays	IL-6 release in A549 cells IFNγ in human whole blood assays Human pre-adipocyte differentiation FABP4 mRNA in adipocytes TAT, PEPCK in human primary adipocytes Osteocalcin levels in human primary osteoblasts	Murine LPS-induced endotoxemia model	Discontinued Phase II for rheumatoid arthritis: no improved benefit-risk ratio compared to prednisone (NCT01393639)	(37, 38)
AZD5423	Ligand-binding assays GR, MR, PR, AR, ERα, ERβ TPA-RE-β-galactosidase stable in ChaGoK1 cells	TNFα-release in hPBMC cells	Sephadex-induced airway inflammation in rats	Discontinued Phase II for asthma (NCT01225549) Phase II for COPD (NCT01555099)	(39–41)
AZD7594	Ligand-binding assays GR, MR, PR, AR, ERα, ERβ TPA-RE-β-galactosidase stable in ChaGoK1 cells	TNFα-release in hPBMC cells	Sephadex-induced airway inflammation in rats	Ongoing Second phase II for asthma completed 11/2019 (NCT03622112) Phase I in adolescents ongoing (NCT03976869)	(39, 42)
AZD9567	Ligand-binding assays GR, MR, PR, AR, ERα, ERβ MMTV-β-galactosidase stable in ChaGoK1 cells TPA-RE-β-galactosidase stable in ChaGoK1 cells Cofactor binding assays	TAT in primary hepatocytes Osteoprotegerine in human fetal osteoblasts	Streptococcal cell wall reactivation arthritis model in rats	Ongoing Phase II for rheumatoid arthritis completed 11/2019 (NCT03368235)	(43)

Information on clinical trials was retrieved from clinicaltrials.gov. A549, human lung epithelial carcinoma cell line; APOCIII, apolipoprotein C18; AR, androgen receptor; ATT20, mouse pituitary tumor cell line; BAL, bronchoalveolar lavage; CCD-1112Sk, human foreskin fibroblast cell line; CCL (5), C-C motif chemokine (5); CDKN1C, cyclin-dependent kinase inhibitor 1C; hCEpiC, human corneal epithelial cells; ChaGoK1, human bronchogenic carcinoma cell line; hConF, human conjunctival fibroblasts; COX-2, cyclo-oxygenase 2; CV-1, African green monkey kidney cell line; Cyp24a1, vitamin D (3) 24-hydroxylase; CXCR4, C-X-C chemokine receptor type 4; hDSF, human dermal skin fibroblasts; ER, estrogen receptor; hERG, human ether-a-go-go potassium channel; FKBP5, 51 kDa FK506-binding protein; G(M)-CSF, granulocyte (macrophage) colony-stimulating factor; GPR-64, G-protein coupled receptor 64; H4IIE, rat hepatocellular carcinoma cell line; Hek293T, human embryonic kidney cell line; HeLa, human cervical adenocarcinoma cell line; HepG2, human hepatocellular carcinoma cell line; HSKF1501, human foreskin fibroblast cell line; Huh7, human hepatocellular carcinoma cell line; ICAM-1, intracellular adhesion molecule 1; IFNγ, interferon γ; IκBα, NF-kappa-B inhibitor α; IL(-12p40), interleukin (12 subunit p40); (p)JNK, (phospho-)c-Jun N-terminal kinase; κB-RE, NF-κB response element; LTR, long terminal repeat; MAP-7, microtubule-associated protein 7; hMC-1, human mast cell line; MCP-1, monocyte chemotactic protein 1; MG-63, human osteosarcoma cell line; MICAL2, molecule interacting with CasL protein 2; (p)MK-2, (phospho-)mitogen-activated protein kinase-activated protein kinase 2; MMTV, mouse mammary tumor virus; MR, mineralocorticoid receptor; MYOC, myocillin; hONA, human optic nerve astrocytes; (p)p38, (phospho-)mitogen activated protein kinase p38; (p)p65, (phospho-)nuclear factor kappa B subunit p65; PBMC, peripheral blood mononuclear cells; PDK4, pyruvate dehydrogenase kinase 4; PGE2, prostaglandin E2; POMC, pro-opiomelanocortin; PR, progesterone receptor; Raw264.7, mouse leukemia macrophage cell line; hREC, human retinal endothelial cells; RGS2, regulator of G-protein signaling 2; RSV, Rous sarcoma virus; SGK, serum/glucocorticoid regulated kinase; THP-1, human leukemic monocyte cell line; mkTM, monkey trabecular meshwork cells; TPA-RE, 12-O-Tetradecanoylphorbol-13-acetate response element; U937, human histiocytic lymphoma cell line.

BOTTLENECKS AND PITFALLS OBSERVED IN THE PAST

Current tools for screening potential SEGRMs suffer from shortcomings and do not always capture the complexity of GR signaling. First of all, the lack of three-dimensional structures of full-length GR highly restricts our knowledge of GR's structure-activity relationship and decreases the predictive power of molecular modeling and docking studies. Additionally, all existing crystal structures of ligands in complex with GR's LBD were obtained upon the introduction of one or more mutations in this LBD. Although these mutations were predicted not to influence the LBD structure, this can never be claimed with absolute certainty. F602S for instance, one of the most commonly used GR mutations allowing growth of LBD crystals, causes chemical shift perturbations in LBD nuclear magnetic resonance spectra compared to wild-type LBD (65). Furthermore, GR is allosterically regulated through interactions with its corresponding response elements and cofactors (66), and more general also for other NR members, conformational changes in one NR domain can allosterically alter the conformation of another domain within the same NR molecule (67). Thus, most probably the conformation of the LBD studied in isolation is an incorrect reflection of this domain's conformation in the full-length protein.

Further, while high affinity and selectivity for GR can be captured using *in vitro* ligand-binding assays, confirmations in a cellular or *in vivo* context are sometimes lacking. This harbors an inherent risk to miss out on off-target effects of the compound in question. Therefore, the confirmation of GR dependency in a cellular and an *in vivo* context is still an important validation to make, for instance by testing compounds in wild-type vs. GR knock-out models.

Table 1 provides an overview of the assays typically carried out to characterize GR-mediated actions of a set of well-known SEGRMs. To our opinion, a lack of predictive power is one of the problems most difficult to solve, especially when moving from simplified assays to more complex biology. Direct GRE-driven activity, potentially leading to side effects, is almost universally monitored via reporters driven by a mouse mammary tumor virus (MMTV) promoter. Although a fast and straightforward and thus defendable method for initial compound characterization, a GRE-driven reporter assay can be a poor predictor for regulation of endogenous GRE-driven genes, as was also observed for MMTV (15, 22). The effects of GCs are highly gene-specific and GRE-driven activity can differ depending on the sequence of the GRE and the surrounding chromatin environment (68–70). The use of overexpressed GR should also be avoided in such assays, as this may lead to compound potencies and efficacies that are not necessarily representative for an endogenous context. Additionally, not all side effects are dimer-driven and are therefore not predictable via GRE-driven reporters. Mimicking the right gene- and context-specificity of GR activity remains one of the greatest challenges. Making a switch from reporters driven by minimal recombinant promoters to more physiologically relevant promoters could already offer some benefit. These promoters would ideally belong to genes that are confirmed mediators of underlying therapeutic - or side effects. Validation on a well-representative set of relevant endogenous target genes is even more important (see below, section Potential Solutions: The Way Forward).

Cell- and tissue-specificity of GC actions is another variable parameter. The MMTV-driven reporter for instance showed stronger upregulation by GW870086 in bone osteosarcoma cells compared to lung epithelial carcinoma cells (22). It thus remains essential to screen compounds in cell types that are the best proxies for the underlying therapeutic and/or side effects *in vivo*, for instance the use of hepatocytes to study effects on glucose and lipid metabolism, or the use of osteoblast or osteoclast cell lines for drugs that would be used in arthritis patients. Further, although characterization of compound activity *in cellulo* is essential, this will always be an oversimplification of the situation in a living organism. Therefore, validation of an improved therapeutic benefit depends on representative animal models. While this is readily implemented for anti-inflammatory effect scoring, concomitant testing of side effect parameters (such as glucose tolerance, insulin tolerance, cortisol levels, bone mineral density) presents a bottleneck, because a longer treatment protocol may be needed to surpass the thresholds of measurable results for these parameters or because of species differences (see below) (22, 43).

Lack of translatability from animal models to human patients is yet another hurdle to overcome. Differences in ligand activity between species can be an underlying cause, as observed for AL-438 and MK-5932, which both had stronger anti-inflammatory effects in rat vs. human blood (17, 20). While it would be recommended to perform initial cellular tests in human cells as much as possible, *in vivo* interspecies differences remain a hurdle in the entire field of drug discovery and are currently difficult to overcome. Another concern is when animal models used to study a particular disease insufficiently mimic the pathology observed in humans. A careful design and set-up of animal models remains key to study anti-inflammatory as well as side effects. If a well-known side effect (marker) of a classic GC in man is not observed in the animal model used, this model will obviously have no predictive power on (markers of) this particular side effect in patients and will therefore be unsuited to evaluate the improved benefit-risk ratio of SEGRMs over classic GCs. For instance, in a canine model of low dose endotoxemia used to investigate the anti-inflammatory and bone-sparing effects of BI653048, neither BI653048 nor prednisolone treatment affected osteocalcin levels (25). However, prednisolone does reduce bone mineral density in dogs and decreases bone formation markers in humans after 1 day (71, 72). Indeed, in a phase I clinical trial, BI653048, and prednisolone both caused decreased serum osteocalcin levels (26). Studies with other SEGRMs also concluded that osteocalcin levels *in cellulo* do not always reflect *in vivo* decreases in bone density (18, 26, 27, 37, 38), casting doubts on the value of osteocalcin as proxy for the *in vivo* reduction of bone mineral density.

Lastly, notwithstanding the notion that dissociating GCs may improve the benefit-risk ratio in chronic inflammatory disorders,

a portion of the anti-inflammatory effects of GR does remain dimer-driven (73). Hence, the likelihood decreases for truly dissociating compounds to match the therapeutic efficacy of the strongest classic GCs. Taking into account that some side effects, such as osteoporosis, are at least partially mediated by monomeric GR (18), makes the quest to find a SEGRM that scores better on multiple side effects even more challenging.

POTENTIAL SOLUTIONS: THE WAY FORWARD

Although pre-clinical characterization of compounds will never suffice to accurately predict their effects in patients, particular improvements on current screenings could increase the predictive power. First of all, reporter genes driven by physiological promoters relevant for the clinical context of the tested SEGRM should be preferred over artificial promoters. An example could be the use of the G6P- or PEPCK-promoters in liver cells to monitor hyperglycemia (74, 75), or a Runt-related transcription factor (Runx)2-driven promoter in osteoblasts or Smad-driven promoters in osteoclasts as markers for GC-induced osteoporosis (76, 77). A consistent and thorough screening of endogenous targets in a relevant human cellular context adds to importance. While monitoring GR activity in every targeted pathway for every compound is impossible to achieve, identification of reliable *in cellulo* biomarkers with a higher predictive power for species-independent *in vivo* anti-inflammatory and/or side effects would be a tremendous help. This requires a full understanding of the molecular mechanisms driving both anti-inflammatory and side effects in human tissues as well as in animal models. This is, particularly for side effects, not always the case. Continued efforts to unravel the underlying molecular mechanisms driving particular GC side effects are therefore crucial. However, some important side effect mediators have already been identified and could be suitable markers. Examples are muscle ring finger (MuRF)1, atrogin-1, and Krüppel-like factor (KLF)15 in muscle atrophy (78), regulated in development and DNA damage response (REDD)1 in skin (and muscle) atrophy (79, 80), and G6P and PEPCK in liver (74, 75). In bone, the upregulation of cleaved caspase 3 and −9 or the reduction of bone morphogenetic protein (BMP)2 and Runx2 activity are important predictors for reduction in osteoblast numbers (81, 82), while upregulation of receptor activator of nuclear factor-κB ligand (RANKL)-RANK signaling and cathepsin K activity are important markers for increased osteoclast differentiation and activity (respectively) (76, 83).

Reduction of publication bias toward "negative results" and joining forces between pharmaceutical companies and academic groups should push the current boundaries and drive research forward. At times, underlying reasons for discontinuation of (pre-)clinical research remain enigmatic. As one concrete example of many other examples that can be brought forward, results from three completed phase III clinical trials on the use of Mapracorat for post-operative treatment of cataract surgery (NCT01230125, NCT01591161, and NCT01591655) await publication, leaving fundamental scientists on the sideline wondering why Mapracorat was never market approved. More insights on where exactly discontinued SEGRMs failed, if those reasons are on the scientific level, will encourage academic labs with the right expertise to dig deeper into the underlying causes, and create feedback-knowledge that may flow back to industrial programs.

Even though fully dissociating SEGRMs might never reach the therapeutic efficacy of the most potent classic GCs, they can still offer relevant therapeutic benefit. Many inflammatory disorders are characterized by a disease course that alternates between periods of remission and exacerbation or relapse. SEGRMs might not trigger the full-on anti-inflammatory cascade that is required to suppress an exacerbation, but might be ideal for maintenance therapy. To maintain disease control, lower GC doses often suffice. SEGRMs could match the anti-inflammatory efficacy of the lower dose classic GCs while still showing a reduced side effect burden. Combination of classic GCs with SEGRMs or other therapeutic agents is another strategy to increase benefit-risk ratios. Combination of Dex with CpdA was for instance shown to increase anti-inflammatory effects while reducing GRE-driven signaling *in cellulo* (84). Finally, the development of compounds that do not bind the classic ligand-binding pocket but instead target the dimerization interface might be an interesting alternative strategy to disrupt GRE-driven signaling.

The intrinsically disordered nature of the GR NTD (85), has so far prohibited resolving a crystal structure of full-length GR. However, some smaller (however technically still challenging) advances could already lead to important new insights. Crystal structures of wild-type LBD in absence of stabilizing mutations would already give more confidence in the reliability of current docking approaches. Secondly, crystal structures of the DBD-hinge-LBD portion would not only lead to a better understanding of the structure-activity relationship of GR, but might pose extra advantages for molecular modeling or docking studies. Since efficient GR dimerization seems to require both DBD and LBD (12), crystal structures of at least the DBD-hinge-LBD portion of GR should improve predictions on those molecular entities that are truly dimer-disrupting.

Another important emerging strategy to find more efficacious GCs is to minimize exposure to non-inflamed tissues. IGCs can for instance be optimized to undergo rapid elimination once they enter the systemic circulation, a strategy that was applied for the development of AZD7594 (39). For systemic GCs, the use of liposomal formulations is showing very promising results. While these not only improve distribution to tissues that are anatomically difficult to reach (86–88), they can lower the side effects of systemic GCs by maximizing concentrations at the inflamed tissues while minimizing distribution to other tissues (89, 90).

While it may be utopia to try and develop compounds that alleviate all side effects, improved profiles for particular side effects may be achievable. Skin thinning and ocular hypertension are for instance among the most problematic side effects for topical and ocular GC treatments, respectively (5). For systemic treatments, liposomal formulations in combination with selective improvement of particular side effects may be a viable way forward. Liposomal SEGRMs that do not affect bone metabolism

might for instance have an increased benefit-risk ratio over classic GCs for the treatment of arthritic disorders.

CONCLUSION

While there still seems a long road ahead toward SEGRMs with a real improved benefit-risk ratio, there is light at the end of the tunnel. The pipeline of SEGRM compounds under clinical evaluation is not empty and new insights from ongoing (or future) research is expected to lead to optimized screening tools with maximized predictive power. Additionally, strategies to limit exposure to off-targets tissues, such as liposomal formulations for systemic treatments, show promising results (86–90). Combination of these approaches with the identification of reliable markers to predict on-target side effects, (e.g., ocular hypertension in ocular treatment, osteoporosis in rheumatoid arthritis, skin thinning in topic applications) may be an effective and achievable leap forward.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

LVM wrote the manuscript with contributions from KG and KDB. LVM made the artwork with contributions from KG and KDB. All authors approved the final version.

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IFI27/ISG12 Downregulates Estrogen Receptor α Transactivation by Facilitating Its Interaction With CRM1/XPO1 in Breast Cancer Cells

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The estrogen receptor alpha (ERα) is a ligand-activated transcription factor whose activity is modulated by its interaction with multiple protein complexes. In this work, we have identified the protein interferon alpha inducible protein 27 (IFI27/ISG12) as a novel ERα-associated protein. IFI27/ISG12 transcription is regulated by interferon and estradiol and its overexpression is associated to reduced overall survival in ER+ breast cancer patients but its function in mammary gland tissue remains elusive. In this study we showed that overexpression of IFI27/ISG12 in breast cancer cells attenuates ERα transactivation activity and the expression of ERα-dependent genes. Our results demonstrated that IFI27/ISG12 overexpression in MCF-7 cells reduced their proliferation rate in 2-D and 3-D cell culture assays and impaired their ability to migrate in a wound-healing assay. We show that IFI27/ISG12 downregulation of ERα transactivation activity is mediated by its ability to facilitate the interaction between ERα and CRM1/XPO1 that mediates the nuclear export of large macromolecules to the cytoplasm. IFI27/ISG12 overexpression was shown to impair the estradiol-dependent proliferation and tamoxifen-induced apoptosis in breast cancer cells. Our results suggest that IFI27/ISG12 may be an important factor in regulating ERα activity in breast cancer cells by modifying its nuclear versus cytoplasmic protein levels. We propose that IFI27/ISG12 may be a potential target of future strategies to control the growth and proliferation of ERα–positive breast cancer tumors.

Keywords: ISG12, IFI27, estrogen receptor, CRM1, nuclear export, breast cancer

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INTRODUCTION

The estrogen receptor α (ERα) is a ligand-activated transcription factor that mediates the effects of the hormone estrogen (17β-estradiol; E2) on cell proliferation and differentiation in mammary gland and participates in maintenance of skeletal system, metabolic homeostasis and in development of central nervous system (1). In humans, ERα activity is also associated to the development, progression and metastasis of 70–80% of all breast cancer tumors (2). ERα has also been shown to play a key role in mediating resistance to apoptosis, immunosurveillance and hormone treatment in breast cancer (3–5). ERα belongs to the family of transcription factors known as nuclear hormone receptors and its structure is characterized for possessing functionally independent domains that include a DNA-binding domain, ligand-binding domain and two transactivation domains designated AF1 and AF2 that are located at the N-terminal and C-terminal regions, respectively (6–8). The functional synergistic interaction between the unique transcriptional properties of AF1 and AF2 domains is responsible for the full ligand-dependent ERα transactivation.

Mechanistically, the binding of E2 to ERα produces a major structural rearrangement on its ligand binding domain that allows AF2 to interact with a large array of coactivator proteins that include SRC-1, SRC-2/GRIP1/TIF2/NCoA2, SRC3/RAC3/p/CIP/ACTR/AIB1, CREB-binding protein (CBP)/p300, and CBP-associated factor (P/CAF) that increase ERα transcriptional activity by relaxing the chromatin structure through their histone acetyl-transferase activity (9–11). In the absence of E2 or in the presence of ERa antagonists, such as tamoxifen (TOT), the AF2 domain recruits corepressor proteins including NCoR, SMRT, and histone deacetylases (HDACs) that increase the condensation status of chromatin (10–12). Functional and molecular studies have also identified a large number of AF1 specific coregulators including BTF3 (basal transcription factor 3), SRA1 (steroid receptor RNA activator), CoCoA (coiled-coil coactivator), hMMS19 (human homolog of the yeast nucleotide excision repair gene MMS19), SPBP (stromelysin-1 platelet-derived growth factor-responsive element-binding protein), Smad4, NHERF2, and Tristetraprolin (TTP) (13–23). The exchange of coactivators and corepressors is a mechanism that finetunes the ERα transactivation activity in hormone responsive tissues and allows this transcription factor to oscillate between its functions as activator and repressor of gene expression (24, 25).

The transactivation activity of ERα can also be modulated through its interaction with proteins that affect its cellular localization. In its unliganded form ERα binds to an Hsp90 chaperone protein complex, which keeps ERα in a ligand-binding competent but inactive state that prevents it from binding to estrogen-response elements in the DNA (26–28). Some cell factors affect ERα transcriptional activity by regulating its mRNA and protein expression levels or its translocation in and out of the cell nucleus (3, 29–33).

In recent years the study and characterization of nuclear receptor associated proteins have been an important target for molecular oncology because dysregulation of their cellular expression levels has been associated with different forms of cancer. For example, changes in protein levels of coregulators SRC-1; NHERF2, TTP have been shown to correlate with tumor proliferation, disease recurrence or poor disease-free survival in breast cancer (19, 20, 34). Similarly, changes in the expression levels of proteins that affect the nucleocytoplasmic translocation of ERα such as CRM1/XPO1 or prosaposin have also been linked to breast cancer development (3, 31, 35).

In this work, we screened a yeast two-hybrid library to identify new ERα-associated proteins. Our studies identified a 122-amino acid protein, previously identified as interferon/estradiol induced p27/IFI27/ISG12 (hereafter ISG12) and which is overexpressed in breast cancer cells. Expression of ISG12 in MCF-7 cells down-regulates ERα transactivation activity and transcription of its target genes suggesting it functions as a nuclear receptor corepressor. However, immunohistochemistry and Western blot analysis of MCF-7 cells showed that ISG12 expression, unlike bona fide corepressors, such as NCoR and TTP, does not co-localize with nuclear DNA but it is confined to the nuclear envelope and cytoplasm. We demonstrate that the effect of ISG12 on ERα transactivation is mediated by enhancing the interaction between ERα and nuclear exportin CRM1/XPO1. We show further that ISG12 overexpression reduces proliferation and migration of MCF-7 cells and their ability to form spheroids in 3-D culture assays. We propose that ISG12 plays a role in the control of ERα transactivation by participating in the regulation of its protein levels in the cell nucleus of breast cancer cells.

MATERIALS AND METHODS

Reagents and Antibodies

Estradiol (17 β-estradiol) and geneticin (G418) were from Sigma-Aldrich. Lipofectamine 2000 was purchased from Invitrogen. Human ERα and CRM1 antibodies were purchased from Santa Cruz Biotechnology, anti-FLAG antibody was from Sigma-Aldrich, and IFI27/ISG12 polyclonal antibody was purchased from Abcam.

Plasmids

pcDNA3.1- ERα and 2XERE-Tk-LUC vectors have been previously described (19, 20). Human full-length IFI27/ISG12 mRNA (GenBank TM accession no. NM_001130080) was amplified by RT-PCR and cloned into the mammalian expression vector pCMV-3Tag-1A (Agilent Technologies, Santa Clara, CA). The resulting vector is referred as pCMV-3Tag-ISG12.

Yeast Two-Hybrid Assay

The yeast two-hybrid screen was performed using matchmaker two-hybrid system kit (CLONTECH). Briefly, a cDNA fragment encoding the AF-1 domain (amino acids 1–180) of ERα was cloned into pAS2.1 vector to be used as a bait. A human mammary gland cDNA library in pACT2 plasmid was cotransformed with construct pAS2.1/AF1 into Y190 yeast cells. Yeast cells were plated on medium lacking tryptophan,

leucine and histidine (SD/-Leu -Trp -His) containing 25 mM 3-amino-1,2,4-triazole(3-AT) and incubated for 2 to 4 days at 30°C. Resulting colonies were assayed for β-galactosidase activity. The positive AD plasmids were transformed into Escherichia coliDH5α cells for DNA sequencing and identification using Basic Local Alignment Search Tool (BLAST) analysis.

Cell Culture and Transfection Assays

The luminal A breast cancer cell lines MCF7, T47D, and ZR-75-1 were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) inactivated fetal bovine serum (FBS) (GIBCO, Rockville MD, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Rockville MD, USA) in a humidified atmosphere containing 5% CO2 at 37°C. Cells were grown in tissue culture dishes containing phenol red-free DMEM supplemented with 5% charcoal/dextran-treated FBS and cultured for 24 h before all experimental treatments with 100 nM E2. For transient transfection assays cells were grown to 80% confluence in 96 well plates and then they were transfected using Lipofectamine 2000 (Invitrogen), with 50 ng of ERE-TK-Luc, and 100 to 300 ng pCMV-3Tag-ISG12 vector or empty vector. Luciferase activity was determined using Dual-Glo Luciferase Assay System Protocol (Promega) according to the manufacturer's instructions. For stable cell line transfection the ISG12 cDNA was subcloned into the pCMV-3Tag vector and the resulting pCMV-3Tag-ISG12 construct was transfected into MCF-7 cells using Lipofectamine 2000. The MCF7-ISG12 cells were plated in p150 plates containing G418 (500 μg/mL). G418-resistant cells were transferred to 96 well plates to select individual clones in the presence of G418. The MCF7-ISG12 clone used in this study was selected after confirming ISG12 over-expression by Western blot analysis. The effect of TOT and ISG12 on cell viability was determined using PrestoBlue (Thermo Fisher Scientific) following the manufacturer recommendations.

Proximity Ligation Assays

To analyze the interaction between endogenous ERα and ISG12 proteins *in situ*, we used the Duolink Proximity Ligation Assay (PLA) (Sigma-Aldrich) in MCF-7, T47-D, and ZR-75-1 cells following the manufacturer's instructions. Briefly, cells were grown on eight-well chamber slides (Lab-Tek) and stimulated with E2 for 1 h. The cells were fixed in 4% PFA in PBS, permeabilized in PBS-Triton X-100 0.05%, incubated in blocking solution for 1 h at 37°C and then in a solution containing mouse monoclonal anti-ERα antibody (D12, Santa Cruz Biotechnology) and rabbit anti-ISG12 antibody (Abcam) for 1 h at 37°C. The PLA probes consisting of secondary antibodies conjugated with complementary oligonucleotides were incubated for 1 h at 37°C. The ligation of the oligonucleotides was performed for 100 min at 37°C followed by an amplification step. Samples were analyzed under fluorescence microscopy using a Zeiss LSM710 Duo confocal microscope. Image acquisition was performed by imaging DAPI staining at a fixed Z Position while a Z stack of ± 5 μm at 1 μm intervals was carried out. The final image was stacked to a single level before further quantification. On each sample, at least three different fields were analyzed and fifty cells were counted in each. Results were represented as mean ± S.E. Significance (p-value) between cell lines was determined using the Student t-test. * $p < 0.05$; ** $p < 0.01$.

3-D Cell Cultures

For 3-D cultures 5000 control MCF-7 cells or MCF-7 cells overexpressing ISG12 were plated atop reconstituted basement membrane (Matrigel, Corning) in eight-well chamber slides as previously described (36). Cells were treated with vehicle (control), 100 nM 17 β-estradiol, or 1 μM Tamoxifen on day 14 and fixed on day 15. The 3-D cell cultures were then stained with Oregon Green Phalloidin (Thermo Fisher Scientific); 4',6 diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) and anti–Ki-67, or anti-cleaved PARP1 (asp214) antibodies (Cell Signaling Technology). Samples were analyzed under fluorescence microscopy using a Zeiss LSM710 Duo confocal microscope. Percentage of Ki-67–positive, and anti-cleaved PARP1–positive cells were scored on the basis of assessment of 30 spheroids per well. Bar, 50 mm. Results were represented as mean ± S.E. Significance (p-value) between cell lines was determined using the Student t-test. * $p < 0.05$; ** $p < 0.01$.

Wound Healing Assay

MCF-7 and MCF7-ISG12 cells were cultured to confluence in 6-well plates. The cell cultures were wounded with a sterile 10ul pipette tip, and then washed with PBS to remove floating cells. The cells were incubated with serum-free medium supplemented with 100 nM E2 for 24 or 48 h. Micrographs were taken and used to measure the migration of the cells. Cell migration into the wound surface was considered as the process of *in vitro* healing. Cell migration was calculated with the formula: $(A_0 - A_t)/A_0 \times 100\%$, where A_0 represents the area of the wound at 0 h, and A_t represents the area of the wound at 24 or 48 h.

Immunoprecipitation and Western Blot

MCF-7 and MCF7-ISG12 cells were lysed with TNTE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA containing 0.5% Triton X-100 plus a mixture of protease inhibitors). Proteins were immunoprecipitated with rabbit polyclonal anti-ERα (HC-20) or mouse monoclonal anti-CRM1 (C-1). Immunoprecipitated proteins were separated by PAGE and detected by WB with mouse monoclonal anti-ER (D-12) or anti-CRM1 antibodies. Proteins were visualized by incubation with anti-rabbit or anti-mouse secondary horseradish-peroxidase-conjugated antibodies (Pierce, Thermo Fisher Scientific Inc.) and using an enhanced chemiluminescence assay (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific).

Immunofluorescence and Confocal Microscopy Studies

The cellular localization of ERα and ISG12 was determined by indirect immunofluorescence microscopy. Briefly, MCF-7 cells were grown on glass coverslips and fixed with freshly prepared 2% paraformaldehyde solution. The cells were incubated first

with primary antibodies and then with secondary antibodies conjugated with Alexa-546 (red) and Alexa-488 (green; both from Molecular Probes, Eugene, OR). Prolong-Gold Antifade reagent with DAPI (blue; Invitrogen) was used to counterstain the DNA. Confocal analyses were performed using the Leica TCS SP8 confocal microscopy system and MRC600 laser-scanning confocal microscope (Bio-Rad, Hercules, CA). Each slide was examined at three excitation wavelengths (488, 546 and 633 nm). Quantification of nuclear ERα immunofluorescent signal (ERα signal/area) in control MCF-7 and MCF7-ISG12 cells is represented as mean ± SE. of three independent experiments (25–120 nuclei, each). Statistical significance (p value) for differences between MCF-7 and MCF7-ISG12 cells is shown as $p < 0.05$.

RNA Isolation and RT-PCR Analysis

Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality was assessed using spectrophotometric methods and formaldehyde-agarose gel electrophoresis, considering the 28S/18S rRNA ratio. Two micrograms of total RNA were DNase I (RNase-free) treated (Ambion, Austin, TX, USA). cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen), following the manufacturer's protocol. Quantitative PCR amplification was carried out using Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific) and the following primers: GREB1 Fw 5'-CAAAGAATAACCTGTTGGCCCTGC-3', GREB1 Rv 5'-GACATGCCTGCGCTCTCATACTTA-3'; CTSD Fw 5'-CCCTCCATCCACTGCAAACT-3', CTSD Rv 5'TGCCTCTCCA CTTTGACACC-3', GAPDH Fw 5'-AGCCACATCGCTCAGACAC-3', GAPDH Rv 5'-GCCCAATACGACCAAATCC-3'. Data were measured with the LightCycler®96 system (Roche Diagnostics International Ltd.). Expression of individual genes was compared and normalized using the 2-ΔΔCt method against the level of GAPDH mRNA.

Cell Proliferation Analysis

Dynamic monitoring of cell proliferation was performed with the xCELLigence™ System (Acea Biosciences, San Diego CA, USA). MCF7 and MCF7-ISG12 cells were grown at a density of 7.5×10^3 cells/well in quadruplicate on an E-plate 16 using phenol red-free DMEM supplemented with 5% charcoal/dextran-treated FBS. When the cell cultures reached a cell index of 0.5 the medium was supplemented with vehicle (ethanol 0.01%) or 10 nM E2. Cell growth curves were recorded on the xCELLigence™ RTCA System in real-time every 30 min, for at least 96 h.

ISG12 mRNA Expression Levels in Breast Cancer Tumors and Normal Tissue and Kaplan-Meier Analysis

To compared ISG12 mRNA levels in breast cancer tumors and normal tissue we made use of the Breast Cancer Gene-Expression Miner database (http://bcgenex.centregauducheau.fr/BC-GEM/GEM-Accueil.php?js=1). The results are shown as a violin plot of the log2 of ISG12 mRNA expression (p=0.0001, Dunnett-Tukey-Kramer's test). Relapse free survival (RFS) plots were generated using the gene chip database Kaplan-Meier Plotter (https://kmplot.com).The survival analysis was restricted to ERa status and tamoxifen vs other endocrine treatments. Logrank $P < 0.05$ was considered as statistically significant.

Statistics

The experiments were performed in triplicate and presented as mean ± SD. Student's t-test with the GraphPad prism 8 software were used for statistical analyses. $P<0.05$ was considered as statistically significant.

RESULTS

Identification of ISG12 as an ERα Interacting Protein

To identify cell proteins that recognize ERα, we used its AF1 domain (amino acids 1–180) as bait in a yeast two-hybrid screen of 5×10^6 independent clones of a human mammary gland cDNA library. The cDNA clones isolated from the cDNA library screen were subcloned into the pCMV-3Tag vector and co-transfected into MCF-7 cells with the reporter vector ERE-TK-Luc. Two independent clones had an open reading frame encoding a 122 amino acid protein. Sequence analysis using the BLAST program of the National Center for Biotechnology Information showed that the candidate protein had been previously described by different groups as interferon-inducible protein 27 P27/IFI27/ISG12, hereafter ISG12 (37–39).

To confirm ISG12 is an ERα-associated protein in human cells *in vivo*, we used the technique proximity ligation assay (PLA). Physical interaction between endogenous ERα and ISG12 proteins was determined in human breast cancer cell lines MCF-7, T47D, and ZR-75-1 using the corresponding two primary antibodies raised in different species. Next, the cells were incubated with species-specific secondary antibodies attached to a unique DNA strand (PLA probes). If the PLA probes are located less than 40 nm apart in the cell, the DNA strands can interact forming a circle that can be amplified by DNA polymerase. Hybridization with complementary fluorescent oligonucleotide probes allows the visualization of ERα-ISG12 interactions as an individual fluorescent red dot. The results revealed multiple loci of interactions between endogenous ERα and-ISG12 in the cytoplasm, nucleus and perinuclear region in MCF-7, T47D, and ZR-75-1 cells (**Figure 1A**). Quantification of the number of ERα-ISG12 interaction dots showed no significant difference between MCF-7 and T47-D cells. However, the number of ERα-ISG12 interaction events in ZR-75-1 cells was 50% and 60% lower ($p < 0.05$) than in MCF-7 and T47-D cells, respectively (**Figure 1A**).

ISG12 Down-Regulates ERα Transcriptional Activity

To explore the effect of ISG12 expression on ERα transactivation, we performed transient transfection assays in MCF-7 and T47-D cells. In these experiments, pCMV-3Tag-ISG12 was the source of ISG12, and the vector 2xERE-Tk-LUC was used as the indicator of ERα transcriptional activity. In the two cell lines the baseline

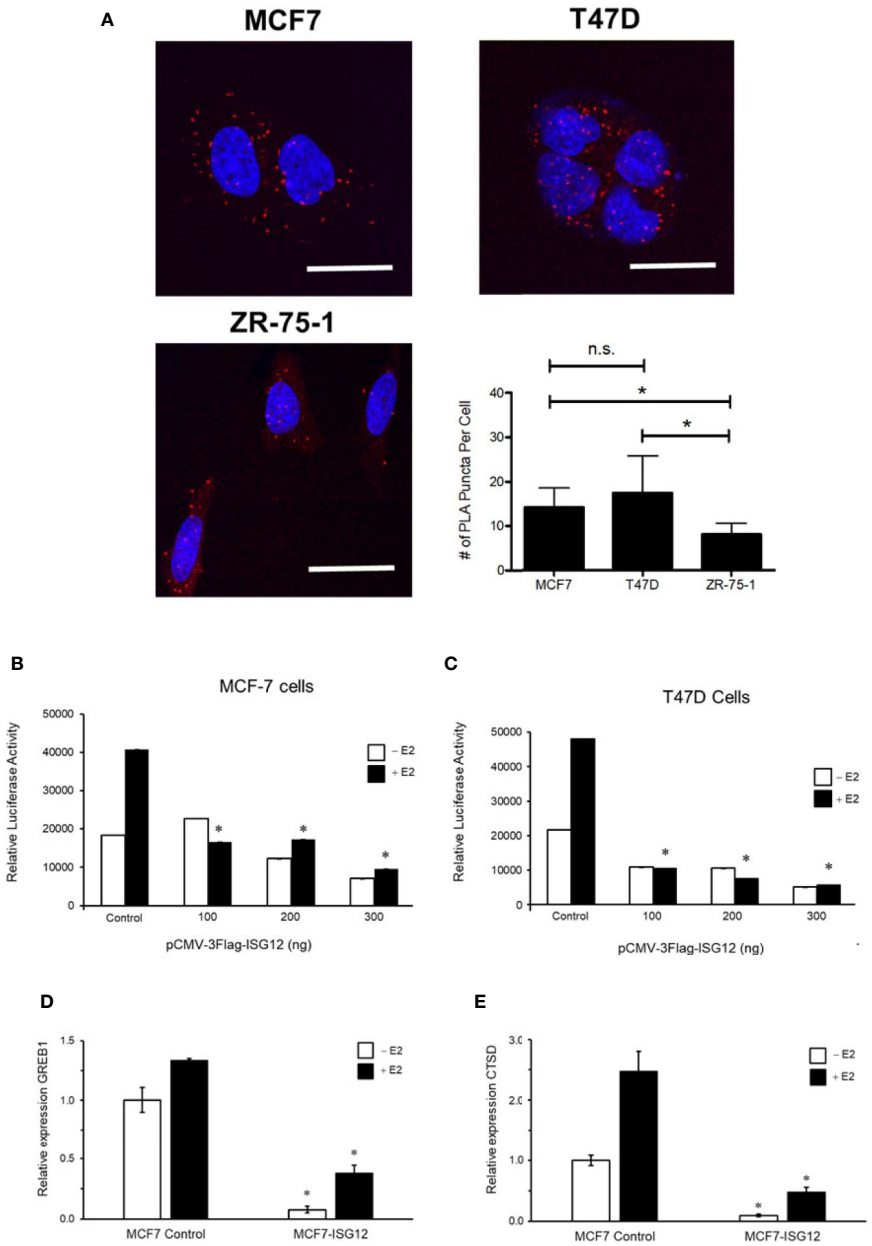

FIGURE 1 | Proximity ligation assays and effect of transient expression of ISG12 on ERα transactivation activity. **(A)** The physical interaction between endogenously expressed ERα and ISG12 in MCF-7, T47-D and ZR-75-1 cells was determined using proximity ligation assays. The figure shows representative confocal microscopy images in which ERα-ISG12 interactions appear as an individual fluorescent red dots. Scale bar, 50 μm. Quantification of ERa-ISG12 interactions in MCF-7, T47-D, and ZR-75-1 cells is represented as mean ± S.E. of two independent experiments. The average number of ERα-ISG12 interactions in ZR-75-1 cells was found to be statistically significant ($p < 0.05$) when compared to the results obtained in MCF-7 and T47-D cells. MCF-7 **(B)** and T47D **(C)** cells were transiently transfected with empty pcDNAvector (control) or with different concentrations (100, 200, 300 ng) of pCMV-3TAG-ISG12 along with ERE-Tk-LUC reporter vector. The effect on ERα transactivation was determined by assay of luciferase activity. Assays were performed in triplicate in three independent experiments in the presence (white bars) or absence (black bars) of E2, and the results are represented as mean ± S.E. of three independent experiments. ISG12 reduces GREB1 **(D)** and CTSD **(E)** mRNA levels. MCF-7 cells were transfected with or without pCMV-3TAG-ISG12 and were stimulated with or without E2 for 24 h. Total RNA isolated from these cell cultures was used to determine GREB1 and CTSD mRNA levels by qPCR. GADPH mRNA was used as an expression control. Results are represented as mean ± S.E. (error bars) of three independent experiments. Differences in ERα activity and GREB1 and CTSD mRNA levels between control MCF-7 cells and MCF7-ISG12 cells were shown to be statistically significant ($p < 0.05$) *$p < 0.05$; n.s., not statistically significant.

luciferase activity increased upon E2 stimulation (**Figures 1B, C**, control). ISG12 overexpression in MCF-7 and T47D cell lines produced a dose-dependent down-regulation of ERα transactivation activity. Transfection with 100, 200 or 300 ng of pCMV-3Tag-ISG12 in the presence of E2 reduced the transcriptional activity of ERα by 60%, 58% and 77% in MCF-7 cells ($p < 0.05$) (**Figure 1B**). The same treatment reduced E2-stimulated ERα activity by 79%, 84%, and 88% in T47D cells ($p < 0.05$) (**Figure 1C**). In the absence of E2, transfection of increasing amounts of ISG12 also reduced the basal ERα activity by 65% and 77% in MCF-7 and T47D cells, respectively (**Figures 1B, C**). To confirm the functional impact of ISG12 in the transcriptional activity of ERα we used qPCR to determine the mRNA levels of GREB1 and cathepsin D (CTSD) in control MCF-7 and ISG12-overexpressing MCF-7 cells incubated in hormone-free medium or in medium supplemented with E2. Control MCF-7 cells stimulated with 100 nM E2 exhibited a 33% increase in GREB1 mRNA levels and 147% increase in CTSD mRNA levels compared to unstimulated MCF-7 cells (**Figure 1D**, MCF-7 control). In contrast, ISG12-overexpressing MCF-7 cells exhibited 90% and 67% reduction in GREB1 mRNA levels in hormone-free and E2-medium with respect to control MCF-7 cells (**Figure 1D**, MCF7-ISG12). CTSD mRNA levels in MCF7-ISG12 cells were also reduced by 90% and 80% in hormone-free and E2 medium, respectively ($p < 0.05$) (**Figure 1E**, MCF7-ISG12). In combination, the PLA and transient transfection assays suggest that in human breast cancer cells ISG12 is an ERα associated protein and that its expression down-regulates the transactivation of this hormone nuclear receptor. Based on the similarities of the PLA and transient transfection results obtained in the different breast cancer cell lines tested we decided to continue the characterization of ISG12 as an ERα-associated protein using a MCF-7 cell line stably transfected with ISG12 (MCF7-ISG12).

ISG12 Reduces ERα Protein Levels in MCF-7 Cells

To continue the characterization of the effect of ISG12 on ERα transactivation, we determined its cellular localization with respect to ERα in control MCF-7 cells and MCF-7 cells stably transfected with pCMV-3Tag-ISG12 incubated in hormone-free medium (**Figure 2A**) or in medium containing E2 (**Figure 2B**). Immunostaining of MCF-7 and MCF7-ISG12 cells with anti-ERα antibody (green) showed that ERα is predominantly localized in the cell nucleus and it seems to be more abundant in the nucleus of cells incubated in the presence of E2 (**Figures 2A, B**, ERα panel). Incubation of MCF-7 and MCF7-ISG12 cells with anti-ISG12 antibody (red) demonstrated ISG12 is expressed in the cytoplasm and nuclear envelope but it seems to be less abundant inside the cell nucleus (**Figures 2A, B**, ISG12 panel). However, MCF7-ISG12 cells exhibited lower nuclear ERα protein levels compared to control MCF-7 cells. Quantification of the ERα signal/nuclear area showed that in MCF7-ISG12 cells the ERα immunofluorescent signal is reduced by 34% and 26% with respect to control MCF-7 cells ($p < 0.05$) incubated in hormone-free and E2 supplemented medium, respectively (**Figure 2C**). To confirm the effect of ISG12 on ERα protein levels we analyzed total protein extracts from MCF-7 and MCF7-ISG12 cells grown with or without E2 by Western blot. The results showed that E2 treatment increased by 20% the ERα protein levels in control MCF-7 cells ($p < 0.05$) (**Figure 3A**, control MCF-7). In contrast, MCF7-ISG12 cells treated with E2 exhibited a 35% reduction ($p < 0.05$) in ERα protein levels with respect to MCF7-ISG12 cells grown in hormone-free medium and a 46% reduction ($p<0.01$) with respect to ERα protein levels in MCF-7 cells treated with E2 (**Figure 3A**, MCF7-ISG12). To determine whether the effect of ISG12 on ERα protein levels is specific, we transfected MCF-7 cells with the vector pCMV-TTP encoding the nuclear hormone corepressor tristetraprolin (TTP) (19) to compare its effect on ERα transactivation and protein levels. The results showed that TTP transfection reduced ERα activity by 80% in MCF-7 cells stimulated with E2 ($p < 0.01$) (**Figure 3B**). However, densitometric quantification of Western blot bands revealed that TTP over-expression did not reduce ERα protein levels (**Figure 3C**). These results suggest that while both TTP and ISG12 expression down-regulate ERα transactivation, only ISG12 reduces ERα protein levels in breast cancer MCF-7 cells.

Next, we focus on the effect of ISG12 expression on ERα located in the cell nucleus where it is responsible for the estradiol-dependent transcriptional regulation. Western blot analysis was performed on protein extracts prepared from isolated nuclei from control MCF-7 and MCF7-ISG12. Our results showed that ERα nuclear protein levels in MCF7-ISG12 cells were 67% lower ($p < 0.01$) than in control MCF-7 cells (**Figure 4A**). To explore whether ISG12 down-regulates ERα transactivation by promoting its nuclear export, we used co-immunoprecipitation assays to determine the interaction of ERα with the nuclear exportin protein chromosomal maintenance 1 (CRM1/XPO1). Nuclear protein extracts for control MCF-7 or MCF7-ISG12 cells gown in hormone free medium or in E2 supplemented medium were immunoprecipitated using anti-CRM1 antibody. The precipitated proteins were separated in acrylamide gels and the interaction with ERα was determined by densitometric analysis of Western blots bands. Our results showed that ISG12 overexpressing MCF-7 cells incubated in hormone free or E2 supplemented medium exhibit a 500% and 300% increase ($p < 0.05$), respectively, in the interaction between CRM1/XPO1and ERα compared to control MCF7 cells (**Figure 4B**, IP: CRM1). The effect of ISG12 on the interaction between ERα and CRM1/XPO1 was confirmed by a reciprocal coimmunoprecipitation assay in which nuclear protein extracts from MCF-7 cells and MCF7-ISG12 cells were immunoprecipitated with anti-ERα antibody and the interaction with CRM1/XPO1was visualized by Western blot using anti-CRM1 antibody (**Figure 4B**, IP: ERα). These experiments showed an 200% and 270% increase ($p < 0.05$) in the interaction between ERα and CRM1/XPO1 in MCF7-ISG12 cells grown in hormone-free and E2 medium with respect to control MCF-7 cells. As a control, 10% of the protein extracts used in each immunoprecipitation assay were analyzed by Western blot using anti- CRM1/XPO1or anti-ERα to confirm the presence of the proteins (**Figure 4B**, Input). In combination, these results indicate that ISG12 promotes the interaction of ERα with the nuclear exportin CRM1/XPO1 and suggest that the ISG12-dependent

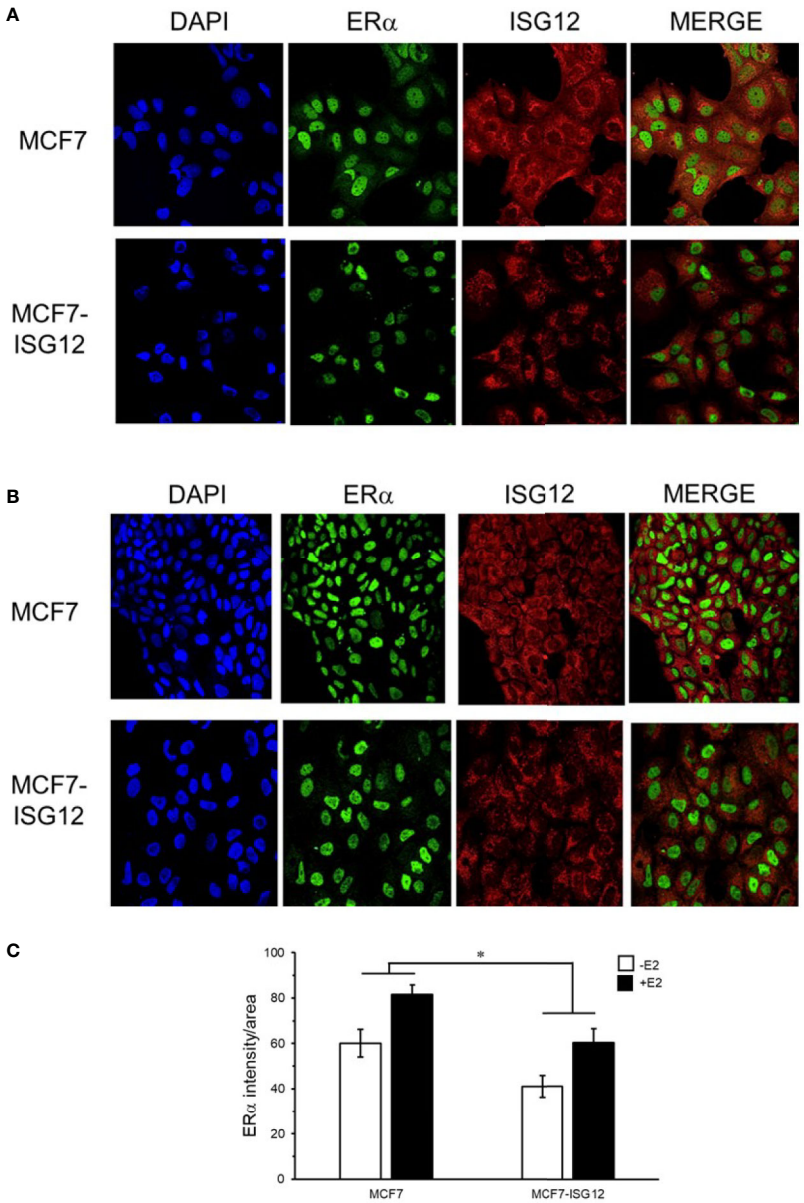

FIGURE 2 | Subcellular localization of ISG12 and ERα in MCF-7 cells and MCF7-ISG12. Control MCF-7 and MCF7-ISG12 cells were cultured in the absence **(A)** or presence **(B)** of E2. The cultures were treated with DAPI to visualize nuclear DNA (blue, panel), anti-ERα antibody (green), and anti-ISG12 antibody (red) as described under *Material and Methods*. **(C)** Quantification of nuclear ERα immunofluorescent signal (ERα signal/area) in control MCF-7 and MCF7-ISG12 cells is represented as mean ± S.E. of three independent experiments. Differences between MCF-7 and MCF7-ISG12 cells were shown to be statistically significant (*$p < 0.05$).

down-regulation of ERα transactivation may be mediated by its export from the nucleus in breast cancer cells.

ISG12 Impairs the Migration of Breast Cancer Cells

To start assessing the physiological impact of ISG12 over-expression in breast cancer we compared the migration ability of control MCF-7 cells and MCF7-ISG12 cells. For these experiments we used the wound-healing assay which allows to determine the migration potential of cancerous cells. Our results showed that control MCF-7 cells stimulated with E2 exhibited a 60% increase in motility at 24 and 48 h compared to MCF-7 cells grown in hormone-free medium ($p < 0.01$) (**Figure 5**, MCF-7 panel). In contrast, the presence of ISG12 impaired the migration of MCF-7 cells. MCF7-ISG12 cells incubated in E2 for 24 and 48 h exhibited an increase of only 45% and 40% ($p < 0.01$) compared to MCF7-ISG12 cells incubated in hormone-free medium (**Figure 5**, MCF7-ISG12 panel).

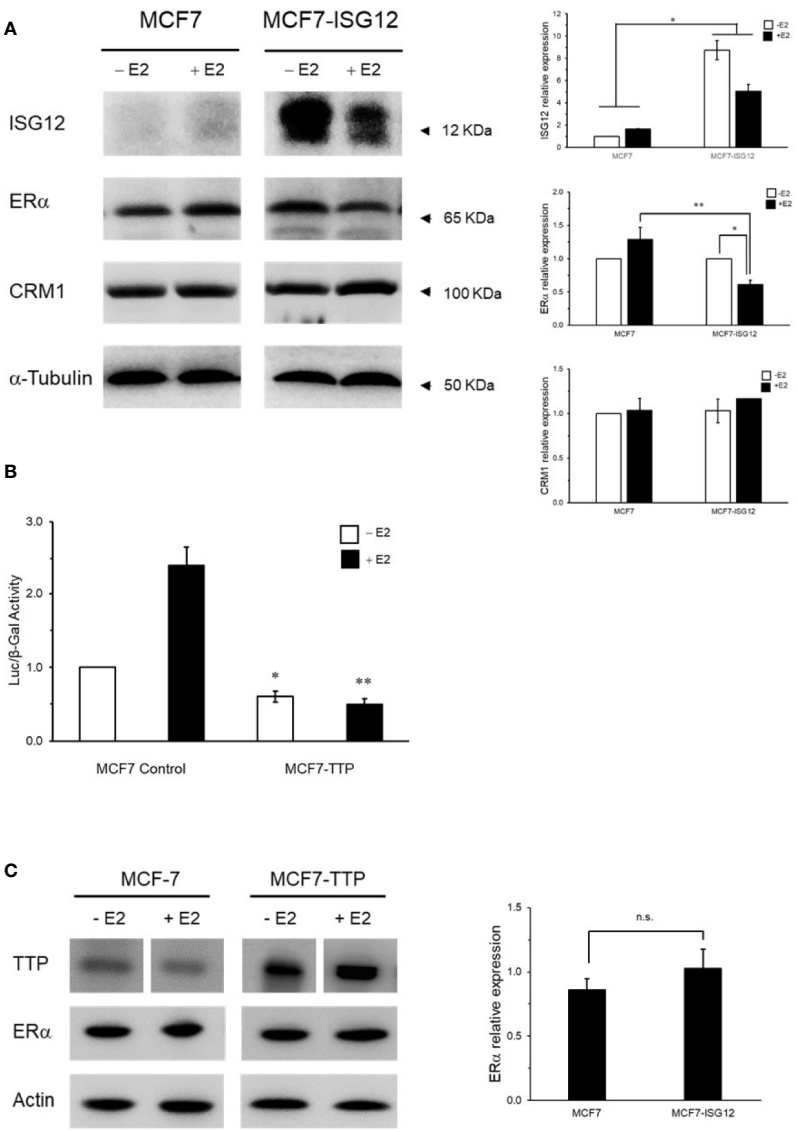

FIGURE 3 | ISG12 and ERα protein levels in breast cancer cells. **(A)** Total protein extracts from control MCF-7 and MCF7-ISG12 cells grown in absence (–E2) or presence (+E2) were resolved by PAGE, and expression levels of ISG12, ERα, nuclear exportin CRM1/XPO1, and α-tubulin, as a loading control protein, were evaluated by Western blot using specific antibodies. Densitometric analysis of Western blot bands is represented as mean ± S.E. of three independent experiments (*$p < 0.05$, **$p < 0.01$). **(B)** The nuclear receptor corepressor TTP was used as a control to determine the specificity of the ISG12 effect on ERα. MCF-7 or MCF-7 over-expressing TTP (MCF7-TTP) were transfected with ERE-Tk-LUC reporter vector incubated in absence (white bars) or presence (black bars) of E2 and ERα transcriptional activity was determined as described in *Material and Methods*. Results are represented as mean ± S.E. of two different experiments and differences in ERα activity between control MCF-7 cells and MCF7-ISG12 cells were shown to be statistically significant ($p < 0.05$). **(C)** Total protein extracts from control MCF-7 and MCF7-TTP cells grown in absence (–E2) or presence (+E2) were resolved by PAGE, and expression levels of TTP, ERα and actin, as a loading control protein, were evaluated by Western blot using specific antibodies as described in *Material and Methods*. Western blot ERα protein bands from control MCF-7 and MCF7-ISG12 cells grown in the presence of E2 were analyzed by densitometry and the results are represented as mean S.E. of three independent experiments.

ISG12 Over-Expression Reduces Proliferation of Breast Cancer MCF-7 Cells

Given that ERα is the major driver of cell proliferation in breast cancer and ISG12 was identified as an over-expressed protein in different breast cancer cell lines, we explored whether MCF7-ISG12 cells proliferate at a different rate than control MCF7 cells. For this assay 7.5×10^3 control MCF-7 or MCF7-ISG12 cells were cultured in the presence of E2, and cell proliferation was determined using the Xcelligence RTCA, ACEA Bioscience (Roche). Although at 12 h, the proliferation of control MCF-7 and MCF-7-ISG12 cells were similar, after 24, 36, 48 and 60 h, the number of MCF7-ISG12 cells was 49%, 34%, 31%, and 45%

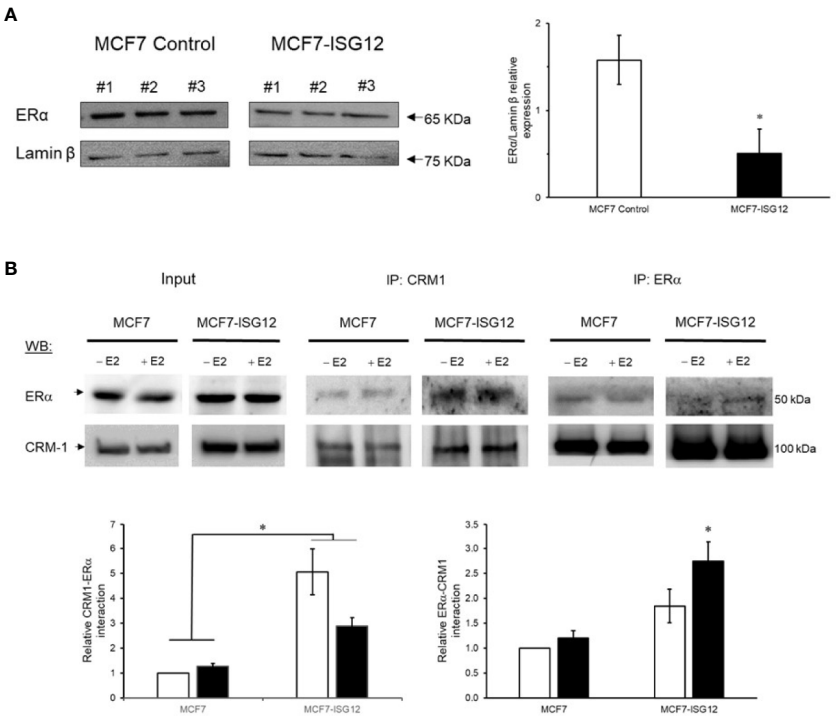

FIGURE 4 | ISG12 over-expression reduces ERα nuclear protein levels. **(A)** Nuclear protein extracts prepared from E2 stimulated control MCF-7 and MCF7-ISG12 cells nuclei were resolved by PAGE, and expression of ERα and lamin β, as a loading control protein, were evaluated by Western blot. Results from densitometric analysis of protein bands from three different experiments are represented as mean S.E. Differences in ERα protein levels between control MCF-7 and MCF7-ISG12 cells were shown to be statistically significant ($p < 0.05$). **(B)** ISG12 increase the interaction between ERα and CRM1/XPO1. MCF-7 and MCF7-ISG12 nuclear protein extracts were immunoprecipitated with anti-ERα antibody (IP: ERα) or anti-CRM1 antibody (IP: CRM1) followed by WB with anti-CRM1 or anti-ERα. Densitometric analysis of protein bands from two different experiments are represented as mean S.E. Differences in CRM1-ERa interaction levels between control MCF-7 and MCF7-ISG12 cells were shown to be statistically significant ($p < 0.05$). Input lanes represents 10% of the nuclear extract used in the capture assays.*$p < 0.05$.

lower, respectively, than the number of control MCF7 cells ($p < 0.05$) (**Figure 6**). These results suggest that ISG12 overexpression reduces the rate of cell division in breast cancer cells.

ISG12 Expression Reduces Spheroid Formation, Estradiol-Dependent Cell Proliferation and Ki67 Expression in 3-D Cell Cultures

To explore further the biological relevance of ISG12 expression on cell proliferation we examined the effect of increasing ISG12 protein levels on 3-D cell cultures. This system allows cells to grow in a microenvironment that mimics cell-cell and cell-matrix interactions and nutrient transport gradient dynamics that exist in living tissues. Control MCF-7 and MCF7-ISG12 cells were grown for 14 days atop a reconstituted layer of Matrigel to form spheroids and incubated in hormone-free medium or in medium containing 100 nM E2 or 1 μM TOT. The 3-D cultures were incubated with anti-Ki67 antibody. The proliferation marker Ki67 reflects the tumor cell proliferation rate as it correlates with progression, metastasis and prognosis in a number of different malignancies and is widely used in routine clinicopathological investigation (40). In this study, the number of Ki67-positive cell nuclei was used as an indicator of cell proliferation.

The spheroids formed by control MCF-7 cells were characterized by exhibiting a compact structure and defined external borders (**Figures 7A, B**, actin and DAPI panels). Incubation with anti–Ki-67 antibody identified that 27% of the total number of cells in control MCF-7 spheroids incubated in hormone free medium were positive for Ki67 (**Figure 7A**, Ki67, vehicle). Stimulation with E2 increased to 43% ($p < 0.05$) the percentage of proliferating Ki67 positive cells in control MCF7 spheroids (**Figure 7A**, Ki67 estradiol panel). As expected, treatment of ERα-expressing control MCF7 spheroids with the anti-estrogen TOT reduced the percentage of Ki-67–positive nuclei in spheroids to 4% ($p < 0.01$) (**Figure 7A**, Ki67 tamoxifen panel). In contrast, ISG12 overexpressing MCF-7 spheroids exhibited a less compact structure and irregular borders (**Figure 7A**, actin and DAPI panels). Anti-Ki67 staining demonstrated that in MCF7-ISG12 spheroids incubated in E2 medium only 10% of the cells were positive for Ki67. Further, no significant differences in the percentage of Ki67 positive nuclei were observed in MCF7-ISG12 spheroids incubated in hormone-free or E2 medium (**Figure 7A**, vehicle

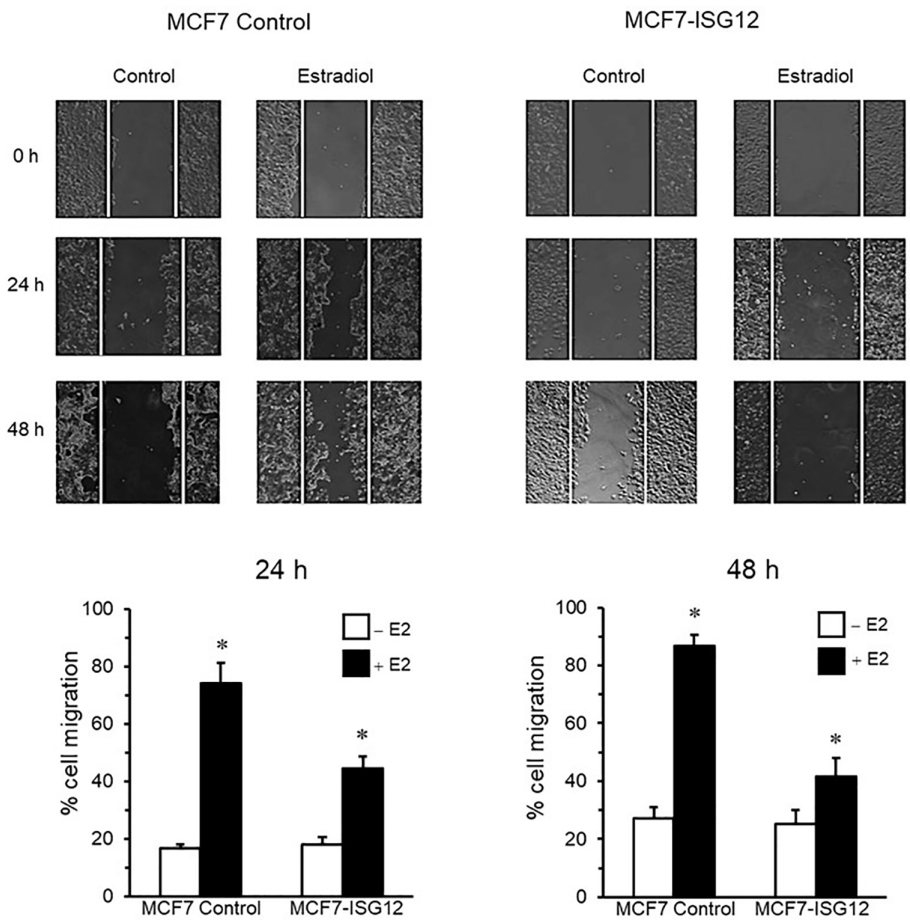

FIGURE 5 | ISG12 over-expression inhibits the migration of MCF-7 cells. Control MCF-7 and MCF7-ISG12 cells were grown to confluence and incubated for 24 or 48 h in the absence (–E2) or presence (+E2) of estradiol. The migration of the cells was determined by the wound-healing assay as described in *Materials and Methods*. Results are represented as mean ± S.E of three independent experiments. Differences in MCF-7 and MCF7-ISG12 cells migration in the presence of E2 were shown to be statistically significant ($p < 0.01$). *$p < 0.01$.

and estradiol panels). In the presence of TOT only 1% of the nuclei was positive for the expression of Ki67 spheroids. These results indicate that ISG12 overexpression impairs spheroid formation and reduces the estradiol-dependent Ki67 expression and cell proliferation in breast cancer 3-D cultures.

ISG12 Expression Reduces Tamoxifen-Induced Apoptosis in 3-D Cell Cultures

In cancer, tumor growth and progression could result from an increase in cell proliferation or inhibition of apoptosis or a combination of both. Because in mammary gland tissue ERα activation affects both proliferation and apoptosis we explored the effect of ISG12 on tamoxifen-induced apoptosis. Control MCF-7 or MCF7-ISG12 spheroids were incubated in hormone-free medium or in medium containing 100 nM E2 or 1 μM TOT for 1 h. The 3-D cultures were incubated with an antibody directed against the cleaved form of PARP1 (Asp214) which is a product of caspases activity and considered a hallmark of apoptosis (41). Cleaved PAPR1 positive cell nuclei were used as an indicator of the number of apoptotic cells. The results showed that less than 1% of cells in control MCF-7 spheroids incubated in hormone-free or E2 supplemented medium were positive for cleaved PARP1 (**Figure 7B**, MCF-7 vehicle and estradiol panels). As expected, in the presence of TOT the number of apoptotic cells in MCF-7 spheroids increased to 45% ($p < 0.01$) (**Figure 7B**, MCF-7 tamoxifen panel). In MCF7-ISG12 spheroids treated with vehicle or E2 less than 1% of the cells were positive for cleaved PARP1. In contrast, treatment with TOT increased the number of apoptotic cells in MCF7-ISG12 spheroids to 18% ($p < 0.01$). These results suggest that ISG12 expression reduced by almost 50% the efficiency of TOT to induce apoptosis compared to control MCF-7 3-D cell cultures.

To confirm the ISG12 inhibition of TOT-induced apoptosis we determined the viability of 2-D and 3-D cultures of control MCF-7 and MCF7-ISG12 cells treated with 5 μM TOT for 5 or 7 days. In 2-D cultures, TOT treatment for 5 and 7 days reduced the viability of control MCF-7 cells by 65% and 90% ($p < 0.05$), respectively compared to control cells (**Figure 7C**, white bars). Instead, the viability of 2-D cultures of MCF7-ISG12 cells incubated in the

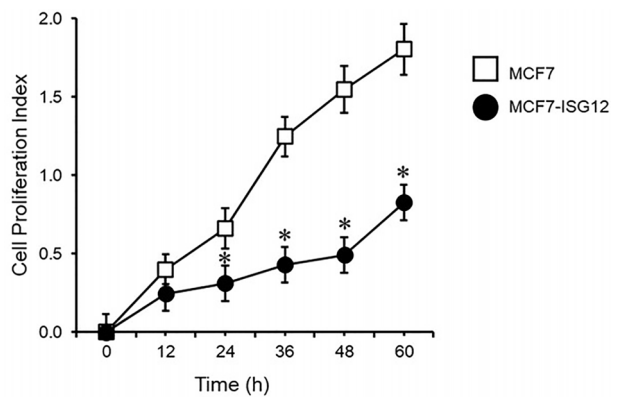

FIGURE 6 | ISG12 impairs the proliferation of MCF-7 cells. Control MCF-7 and MCF7-ISG12 cells were grown in the presence of estradiol and proliferation was recorded on the xCELLigence RTCA System in real-time every 30 min, for 60 h as described in *Materials and Methods*. Results are represented as mean ± S.E of six independent experiments. *p < 0.01.

presence of TOT showed a reduction of only 10% at 5 days and an increase in the cells viability of 8% at 7 days of treatment ($p < 0.05$) compared to control MCF-7 cells (**Figure 7C**, black bars). When we exposed 3-D cell cultures to TOT the viability of control MCF-7 spheroids was reduced by 20% and 25% after 5 and 7 days of treatment ($p < 0.05$) (**Figure 7**, black bars D). In contrast, the viability of ISG12 over-expressing spheroids was not affected by TOT (**Figure 7**, black bars D). These results in combination with Ki-67 and cleaved PARP1 immunostaining experiments suggest that ISG12 overexpression impairs the estradiol signaling pathway that is responsible for both the E2-dependent proliferation and the TOT-induced apoptosis in breast cancer cells.

Correlation Between ISG12 mRNA Expression Levels and Relapse-Free Survival in Breast Cancer Patients

To explore the relationship between ISG12 expression with breast cancer tumorigenesis, we made use of the Breast Cancer Gene-Expression Miner database (http://bcgenex.centregauducheau.fr/BC-GEM/GEM-Accueil.php?js=1) to compare the ISG12 mRNA

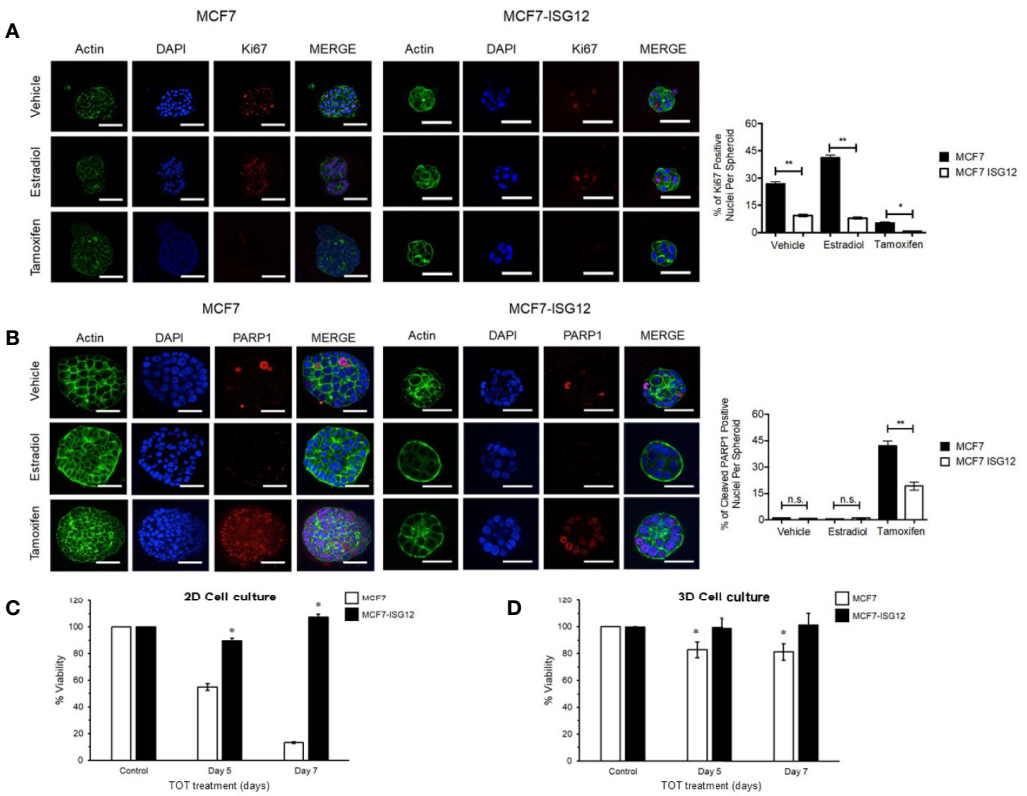

FIGURE 7 | ISG12 inhibits estradiol-dependent proliferation and tamoxifen-induced apoptosis in MCF-7 3-D cultures. Control MCF7 and MCF7-ISG12 cells were grown for 14 days in matrigel to form spheroids and incubated in hormone-free medium (vehicle) or in medium containing 100 nM E2 or 1 μM TOT. The structure of spheroids was visualized by incubation with DAPI (blue) and anti-actin antibody (green). To determine the effect of ISG12 on cell proliferation **(A)** and apoptosis **(B)**, the 3-D cultures were incubated with anti-Ki67 **(A)** or anti cleaved PARP1 (PARP1) antibodies. The number of Ki67 or PARP1 positive nuclei in spheroids were taken as indicators of cell proliferation or apoptosis, respectively. Results are represented as mean ± S.E. of three independent experiments and were shown to be statistically significant (*$p < 0.05$; **$p < 0.01$). The effect of TOT on cell viability of monolayer **(C)** and 3-D cultures **(D)** of control MCF-7 (white bars) and MCF7-ISG12 cells (black bars) was determined as described in *Material and Methods*. Results are shown as mean ± S.E. and the differences between MCF-7 and MCF7-ISG12 spheroids were shown to be statistically significant ($p < 0.05$). Scale bar, 50 μm. n.s., not statistically significant.

levels in tumors, adjacent-tumor tissue and normal tissue in breast cancer patients. The results showed that ISG12 mRNA levels are increased up to 140-fold in tumors compared to tumor-adjacent tissue or healthy tissue (p = 0.0001, Dunnett-Tukey-Kramer's test) (**Figure 8A**). Next, we used the KM-ploter database to compare the expression levels of ISG12 mRNA in breast cancer tumors. The results were ranked from low to high based on ISG12 mRNA median values. Kaplan-Meier curves were generated to compare relapse-free survival (RFS) between the low and high expression groups. The results showed that patients with ERα positive and high ISG12 mRNA expression tumors had significantly worse relapse-free survival than patients with low ISG12 expression levels (**Figure 8B**). In patients with ERα negative tumors the RFS rate did not show a significant association to ISG12 expression levels (**Figure 8C**) suggesting that the effect of ISG12 on RFS in breast cancer patients depends on the expression of ERα. Interestingly, when we focused on ERα positive breast cancer patients that had received tamoxifen as part of their treatment we found that individuals with high ISG12 mRNA expression levels had a significantly worse relapse-free survival rate than patients with low ISG12 expressing tumors (**Figure 8D**).

DISCUSSION

In this work we identified ISG12 as a novel ERα-associated protein using a two-step selection protocol consisting in a yeast two-hybrid screen followed by transient transfection assays in

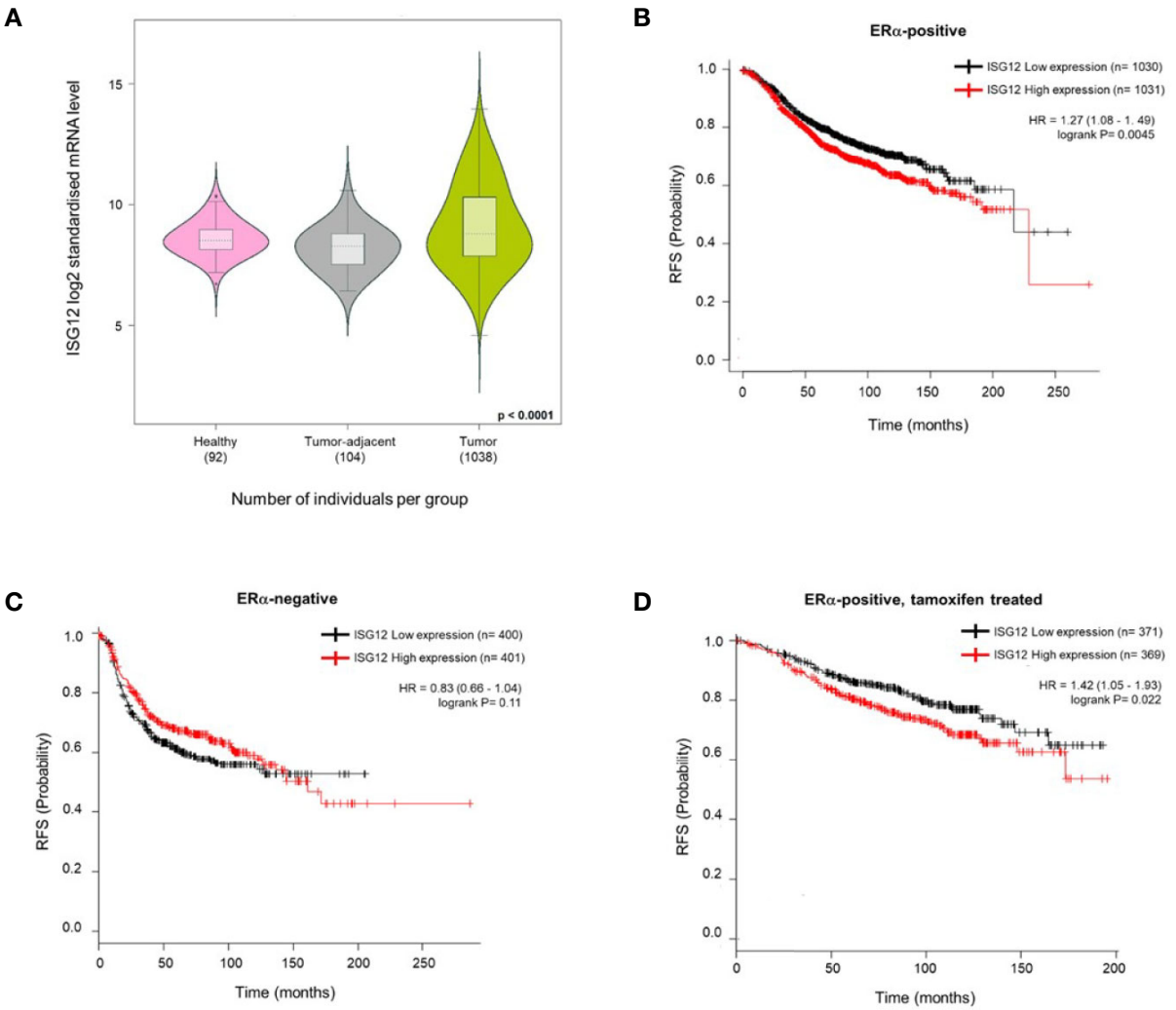

FIGURE 8 | ISG12 expression in breast cancer tumors and Kaplan-Meier analysis of the association between ISG12 expression and relapse-free survival (RFS) in breast cancer patients. **(A)** ISG12 mRNA expression levels in normal and breast cancer tissue was determined using the Breast Cancer Gene-Expression Miner database. The results are shown as a violin plot of the log2 of ISG12 mRNA expression (p = 0.0001, Dunnett-Tukey-Kramer's test). **(B)** Patients with ERα-positive breast cancer tumors and high ISG12 expression levels had poor RFS. **(C)** patients with ERα-negative breast cancer tumors had no significant association between ISG12 expression and RFS. **(D)** Patients with ERα-positive breast cancer tumors and high ISG12 expression levels that received tamoxifen as part of their treatment had poor RFS.

human breast cancer cells. We had previously used this experimental strategy to identify nuclear receptor coregulators including NHERF2 and TTP (19, 20). ISG12 is 122 amino acid protein that belongs to a family of hydrophobic proteins designated FAM14 (42), characterized by possessing a highly conserved 80 amino acid domain known as the ISG12 motif (43). ISG12 was originally identified as a cell factor localized in the nuclear envelope and whose expression is induced by estradiol and interferon in different human breast cancer cell lines (37). More recent studies have reported that ISG12 is over-expressed at the mRNA or protein levels in primary invasive breast carcinomas, breast cancer bone metastasis, oral squamous cell carcinoma, psoriatic epidermis, chronic eczema and cutaneous squamous cell cancers (37, 38, 44). The function and impact of ISG12 expression and over-expression in human cells is not completely understood, but functional and molecular studies suggest it may have different roles in cell physiology.

Using Proximity ligation assays (PLA), a technique that allows the detection of proteins and proteins interactions with single molecule resolution, we confirmed that endogenously expressed ISG12 is an ERa associated protein in human MCF-7, T47-D, and ZR-75-1 cells. The overexpression of ISG12 was shown to attenuate ERα transactivation through different experimental approaches. First, exogenously expressed ISG12 reduced both the estradiol dependent and independent ERα transcriptional activity in a dose-dependent manner in transient transfection assays in human breast cancer MCF-7 and T47D cells. Second, ISG12 overexpressing MCF-7 cells showed a reduction in the mRNA levels of ERα target genes cathepsin D and GREB1 compared to control MCF-7 cells.

The mechanism responsible for the ISG12 effect on ERα transcriptional activity is different from that exhibited by nuclear receptor coregulators. For example, coactivators, like SRC-1, GRIP1/TIF2, and NHERF2 are localized to the nuclear compartment where they interact with the AF2 region of nuclear receptors as part of large coactivator protein complexes that possess histone acetyl transferase activity (20, 45). In the same way, the corepressors NCOR, SMRT, and TTP also require to be translocated inside the cell nucleus to interact with nuclear receptors and repress their transactivation activity as part of corepressor protein complexes that exhibit histone deacetylase enzymatic activity (19, 46–48).

The regulatory function of ISG12 seems to be atypical because, unlike *bona fide* corepressors, it is not highly expressed in the cell nucleus and PLA and coimmunoprecipitation experiments showed it co-localizes more abundantly with ERα in the cytoplasm and perinuclear region than in the cell nucleus. Further, immunostaining of MCF-7 cells and Western blot analysis of total cell protein extracts and nuclear protein extracts showed that ISG12 overexpression is accompanied by a reduction in nuclear ERα protein levels in MCF7 cells. To test the specificity of these results we transfected MCF-7 cells with the nuclear receptor coregulator TTP. We had previously shown that TTP represses the transactivation activity or different steroid nuclear receptors including ERα, progesterone receptor, androgen receptor and glucocorticoid receptor without affecting their protein levels in breast cancer cells (49). Our results showed that TTP transient transfection into MCF-7 cells reduced ERα transactivation and Western blot analysis of protein extracts confirmed that TTP expression in MCF-7 cells did not reduce ERα protein levels suggesting that the ISG12 effect on its nuclear protein levels is specific.

Our results suggest that ISG12 reduces ERα nuclear protein levels by facilitating its interaction with CRM1/XPO1. In human cells the nuclear localization and expression levels of RNA molecules, transcription factors, oncoproteins and tumor suppressor proteins is the result of a delicate balance between import to the nucleus and export to the cytoplasm through the nuclear pore complex of a cell. CRM1/XPO1 is the major receptor for the export of proteins, including ERα and other hormone nuclear receptors, out of the nucleus (31, 35, 50). In human cells the CRM1/XPO1-dependent export of ERα has been shown to regulate its nuclear protein levels and to reset the steroid signal transcription pathway by preventing the nuclear accumulation of transcriptionally inactive forms of ERα that after their export from the nucleus are degraded by the proteasome (35). The functional impact of ISG12 on ERα was demonstrated by our experiments that show that ISG12 overexpression reduces both the E2-dependent and E2-Independent ERα transactivation activity and the expression of genes transcriptionally regulated by estradiol. These changes likely reflect the reduction in the nuclear protein levels of transcriptionally active ERα in MCF-7 and T47-D cells.

Our results on the effect of ISG12 on the physical interaction between ERα and CRM1/XPO1 partially replicate those previously reported by Papac-Milicevic et al. (51) This group showed that ISG12 localized in the nuclear envelop interacts with the orphan nuclear receptor NR4A1 and promotes its nuclear export in a CRM1/XPO1 dependent manner reducing the expression of its target genes. These findings and the ISG12-dependent downregulation of ERα transactivation suggest that ISG12 forms part of a general regulatory mechanism that modulates the transactivation activity of multiple hormone nuclear receptors by facilitating their exit from the cell nucleus *via* the CRM/XPO1 complex.

It has been suggested that the dysregulation of the cellular balance of nuclear receptor associated proteins is linked to the development of different forms of cancer. For example, increased expression the coactivators SRC-1 and NHERF2 or the loss of expression of corepressors such as NCoR and TTP correlate with cell proliferation, tumor development and progression (19, 20, 34, 52). Similarly, changes in the expression levels of proteins that affect ERα nucleocytoplasmic translocation such as CRM1/XPO1 or prosaposin have also been associated to breast cancer tumor development (3). In particular CRM1/XPO1 overexpression in breast cancer tumors is associated to poor prognostic characteristics including larger tumor size and positive lymph node metastasis (53).

In recent years different research groups have documented the association between increased ISG12 expression and different forms of cancer. The impact of ISG12 in tumor development is not clearly understood because the observed effects of ISG12

over-expression are quite diverse and, in some cases, antagonistic in nature. For example, in ovarian cancer, psoriatic skin, cutaneous squamous cell cancers and cholangiocarcinoma patients ISG12 over-expression has been associated to epithelial proliferation, epithelial–mesenchymal transition, cell cycling and tumorigenicity (38, 54–56). In contrast, other studies have linked ISG12 to control of the innate immune response and regulation of IFN-induced apoptosis (42, 57, 58). These studies have determined ISG12 overexpression using different cancer cell lines in culture or by analyzing cDNA microarray databases, and it is not known whether these different outcomes are result from tissue-specific mechanisms or by differences in ISG12 protein expression levels.

In this work we have studied the effect of ISG12 under conditions of protein overexpression in breast cancer cells and the results suggest that increased ISG12 levels lead to an augmented export of ERα from the nucleus. It is possible that the reduction in the nuclear ERα protein levels impairs the steroid signal transduction pathway which in breast cancer cells is responsible for the E2-dependent cell proliferation and TOT-induced apoptosis (59). This hypothesis seemed to be confirmed by the observation that in the presence of increased levels of ISG12 E2 and TOT were less effective to induce cell proliferation or to reduce the number of viable cells, respectively in 2-D and 3-D cell cultures.

One of the main causes of mortality in breast cancer patients is the development of estradiol-independent and tamoxifen-resistant tumor growth. Although this is believed to be a multifactorial phenomenon our results suggest the possibility that the ISG12 mRNA overexpression reported in human breast carcinomas may contribute to the impaired hormonal response in breast cancer cells.

To explore a possible relation between ISG12 overexpression levels and development of breast cancer we analyzed public mRNA microarray databases. These experiments showed that breast cancer patients that received treatment with TOT and whose tumors exhibited high ISG12 mRNA levels had a significant reduction in RFS with respect to patients with low expression levels of ISG12 that also received this anti-estrogen therapy. Our results cannot exclude the possibility that the apparent effect of ISG12 on RFS in breast cancer patients could be associated to the dysregulation of the nuclear protein levels of yet to be identified tumor suppressors and other transcription factors whose nuclear export is mediated by the CRM/XPO1 system. However, our findings on the effect of ISG12 on the response of breast cancer cells to E2 and TOT suggest the need to explore further whether ISG12 protein overexpression could impair the cellular response to E2 and TOT which is often observed in tumor recurrence and metastatic breast cancer (60–62).

In summary, this study has identified ISG12 as a novel ERα-associated protein that participates in the nuclear to cytoplasm export of this hormone nuclear receptor by facilitating its interaction with the exportin CRM1/XPO1. In normal cells ERα nuclear export plays an important role in the control of its nuclear protein levels and in preventing the nuclear accumulation of inactive ERα proteins which in the cytoplasm are eventually degraded by the proteasome. The characterization of ISG12 as a facilitator of the interaction between CRM1/XPO1 and ERα will help to a better understanding of the impact of nuclear to cytoplasm transport on the regulation of ERα transactivation. The impairment in the cellular E2-dependent proliferation and TOT-induced apoptosis by ISG12 over-expression suggest the possibility that this protein affects proliferation, migration and response to hormonal treatment in breast cancer cells. Further studies will be necessary to explore the relationship between increased ISG12 protein levels in breast cancer patients with tumor progression, metastatic disease and to explore the potential of ISG12 expression levels in the development of new diagnostic and therapeutic strategies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MC-B performed qPCR, WB, and Co-IP experiments and participated in the writing of the article. AP-V performed cell transient transfection, luciferase reporter assays, bioinformatic and statistical analysis and participated in the writing of the article. VG-R and RC-R performed MCF-7 and MCF7-ISG12 Immuno-staining, cell proliferation and wound-healing assays and stable transfection assays. AC-Q contributed in cell proliferation assays. LA-R and OV-C designed and performed immunostaining and confocal microscopy experiments of MCF-7 and MCF7-ISG12 3-D cultures and ISG12 PLA assays. MG-M collaborated in nuclear CRM1 co-IP experiments. GR-G and TB-G performed TTP cell transfection assays and TTP effect on ERα protein levels. LC-V performed TOT effect on 3-D cultures. AZ-D contributed to conception and design of study and analysis of results. AL-D-R contributed to conception, design of study and analysis of results, performed yeast two-hybrid cDNA library screen, and wrote the final draft of the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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Multifaceted Control of GR Signaling and Its Impact on Hepatic Transcriptional Networks and Metabolism

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Glucocorticoids (GCs) and the glucocorticoid receptor (GR) are important regulators of development, inflammation, stress response and metabolism, demonstrated in various diseases including Addison's disease, Cushing's syndrome and by the many side effects of prolonged clinical administration of GCs. These conditions include severe metabolic challenges in key metabolic organs like the liver. In the liver, GR is known to regulate the transcription of key enzymes in glucose and lipid metabolism and contribute to the regulation of circadian-expressed genes. Insights to the modes of GR regulation and the underlying functional mechanisms are key for understanding diseases and for the development of improved clinical uses of GCs. The activity and function of GR is regulated at numerous levels including ligand availability, interaction with heat shock protein (HSP) complexes, expression of GR isoforms and posttranslational modifications. Moreover, recent genomics studies show functional interaction with multiple transcription factors (TF) and coregulators in complex transcriptional networks controlling cell type-specific gene expression by GCs. In this review we describe the different regulatory steps important for GR activity and discuss how different TF interaction partners of GR selectively control hepatic gene transcription and metabolism.

Keywords: Glucocorticoid receptor, chromatin, transcription, metabolism, liver

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INTRODUCTION

Any living organism must adapt and respond to the surrounding environment to maintain its existence. For multicellular organisms such as mammals, this includes daily transitions between different physiological conditions including sleep/awake, fasted/fed, and physical inactivity/activity. Moreover, occasional response to environmental changes such as confinement, predator stress, extreme temperatures, inflammation and prolonged lack of food is critical for survival. Glucocorticoids (GCs) serve as important endocrine signaling molecules controlling many molecular signaling pathways that enable cells in the organism to respond to different extrinsic cues. This is particularly evident for cellular responses in the arousal state including the transitions mentioned above. Importantly, pathophysiological conditions leading to dysfunctional GC signaling have dramatic effects on many important biological functions including development, inflammatory response, reproduction, cognitive function, anxiety, circadian entrainment, cardiovascular regulation and cellular metabolism in a tissue-specific manner (1). For example,

uncontrolled GC secretion observed in Cushing's syndrome leads to metabolic complications such as type 2 diabetes and osteoporosis, which are also observed in situations of prolonged treatment with GCs. In contrast, conditions of low GC production, seen in Addison's disease, are associated with muscle weakness, low blood pressure and weight loss (2).

Glucocorticoids exert their actions primarily by binding to the glucocorticoid receptor (GR or Nr3c1), which is expressed in most cells in mammals. Yet, GCs have highly tissue/cell-specific effects regulated by multiple mechanisms. As a DNA-binding transcription factor (TF), GR is primarily involved in the control of gene expression, with transcription of GR target genes in a given cell being controlled by three overall mechanisms (**Figure 1**). First, activity of GR is directly correlated with the amount of GC molecules available in the cell. This is controlled by adrenal GC synthesis and local availability of GCs in the cell. Second, expression of active GR in the nucleus determines the molecular response to GCs. This is regulated by GR turnover (synthesis and breakdown), expression of different GR isoforms, posttranslational modifications (PTMs) and nuclear translocation. Third, genomic action of GR is controlled by cell type-specific accessibility of GR response elements (GRE) in the genome in synergy with cell-specific TFs, coregulators and regulatory RNAs. In this review we will discuss all three regulatory aspects of GR signaling with a specific focus on GR interaction with the genome. We will primarily refer to studies from mouse liver tissue to discuss recent insights to hepatic gene regulatory networks and metabolism controlled by GCs. This will specifically be related to the hepatic transcriptional response to the circadian rhythm, feeding and fasting.

REGULATION OF GLUCOCORTICOID SECRETION AND AVAILABILITY IN THE CELL

Glucocorticoids (cortisol in humans; corticosterone in rodents) are steroid hormones secreted circadianly by the adrenal cortex. Their daily levels peak immediately before the active phase (early morning for humans; early evening for rodents) in anticipation of a waking state, but also in quick response to external stimuli such as stress, hypoglycemia and exercise (3, 4). The hypothalamic-pituitary-adrenal (HPA) axis controls and maintains GC secretion into the bloodstream (**Figure 1A**). The hypothalamus

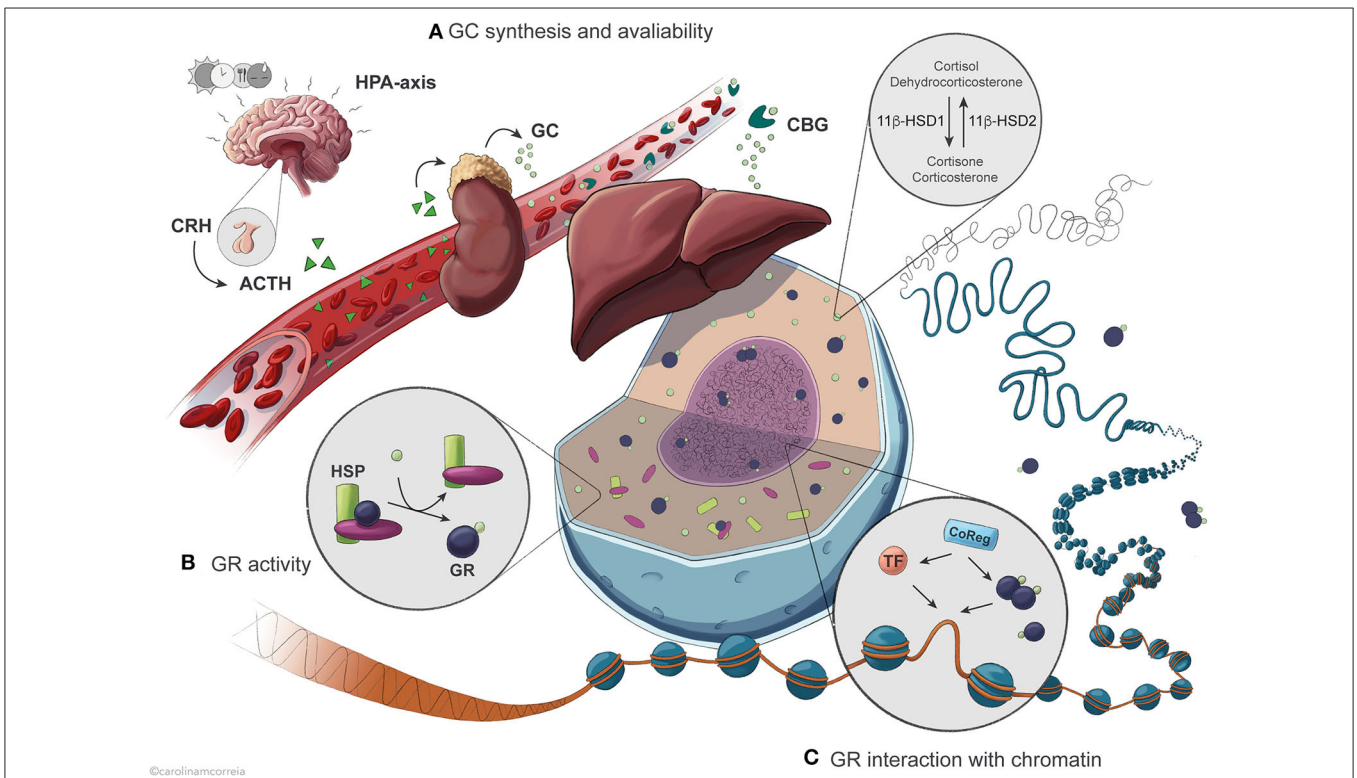

FIGURE 1 | Overview of the regulatory levels affecting GR activity in the control of hepatic transcription. **(A)** Circadian and ultradian synthesis of GCs is controlled by the HPA axis in response to external stimuli including feeding, stress, light and circadian timekeepers. Availability of active GCs is further influenced by binding to the serum protein CBG and by intracellular conversion catalyzed by the enzyme 11β-HSD1/2. **(B)** Once in the cell, GCs are bound by the GR with an affinity that is conditioned by association with chaperone complexes containing HSPs, expression of specific GR isoforms and GR protein turnover. **(C)** GR exerts its action after translocation to the nucleus, where it binds GRE sequences in the DNA to regulate transcription of target genes as a result of dynamic interaction with different TFs and coregulators.

produces corticosteroid-releasing hormone (CRH), stimulating the pituitary gland to secrete adrenocorticotropic hormone (ACTH), which in turn promotes GC secretion by the adrenal gland (5). As many other hormones, including growth hormone and insulin (6, 7), GCs are secreted in an ultradian pattern with pulsatile secretion once every 60 to 90 min, as a result of feedback and feedforward mechanisms between ACTH, CRH and GC secretion keeping GC levels in a physiological range (4, 8, 9). The circadian secretion of GCs results partly from oscillations in ACTH secretion, but mostly from varying adrenal sensitivity to ACTH (3–5). In the blood, GCs circulate in association with corticosteroid-binding globulin (CBG) or, to a lower extent, albumin, and only a small fraction remains unbound in most vertebrates. As only free GCs diffuse into the target cells, CBG modulates GC bioavailability (10–12). Disruption of CBG expression in mice leads to reduced total serum GC (13) and as CBG and albumin are synthesized by the liver, it is possible that hepatic regulation of GC-binding proteins modulates the levels of available GC.

Additionally, non-adrenal production of cortisol has been described in visceral adipose tissue and liver via the conversion of inert cortisone catalyzed by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in humans (dehydrocorticosterone to corticosterone in rodents), and reversely by 11β-HSD2 (14, 15) (**Figure 1A**). Liver activity of this enzyme is particularly relevant to the whole-body non-adrenal production of cortisol; however, HPA axis feedback mechanisms likely blunt any systemic effects (15). Therefore, activity of 11β-HSD1 mostly contributes to locally maintaining intracellular levels of active GCs in the liver and visceral adipose tissue, fine-tuning the highly variable GC levels. This enzyme thus regulates the availability of receptor-active GCs in the cell, modulating access to GR and amplifying GC effects (16–18). In mice, absence of 11β-HSD1 leads to an inability to produce active GCs from the inert form, resulting in compensatory activation of the HPA axis, increased basal corticosterone levels and failure to fully elicit a hepatic gluconeogenic response to fasting, similarly to absence or impairment of GR (19). Dysregulation of 11β-HSD1 expression and activity is associated with apparent hypercortisolemia, disrupted metabolism and HPA axis function, obesity, type 2 diabetes and metabolic syndrome; however, the specific contribution of the enzyme to these processes is still controversial (16, 18).

CIRCADIAN CONTROL OF GLUCOCORTICOID LEVELS

The circadian synthesis and secretion of GCs by the adrenal glands is controlled by both the local molecular clock and the central clock in the suprachiasmatic nucleus (SCN) via a sympathetic neuronal pathway, and can be blunted by stress stimuli (3, 4). The SCN is important for GC rhythmicity, as it regulates the hypothalamic-hypophysial portions of the HPA axis affecting CRH secretion (20–22). During light-induced HPA axis-independent GC secretion, the SCN directly activates the adrenal glands via the adrenal sympathetic nerves, suggesting that GCs can act as SCN-gated mediators of the light stimuli to entrain metabolic-responsive peripheral clocks (5). The ubiquity of GR expression and the marked circadian secretion of GCs imply that these are efficient SCN-driven synchronizers of peripheral clocks and, specifically in the liver, are fundamental for the circadian expression of metabolic genes, even with contribution from other hormonal signals and entrainment factors (3, 4, 23). However, GCs do not affect the central clock, since GR is not expressed in the SCN (3, 23).

Unlike the SCN, the phase of peripheral clocks can be modulated by feeding, and even uncoupled from the SCN (3). As a metabolic organ, the liver is particularly responsive to feeding patterns, which can lead to desynchronization of its peripheral clock from the central clock (24, 25), an entrainment partly mediated by GCs (26–29). The interplay between eating behavior and GCs can be observed during day-restricted feeding of mice (opposite to their normal feeding pattern), leading to secretion of GCs with two distinct peaks instead of a single one, with one being feeding-responsive (before feeding time, in the early morning) and the other light-entrained (before the normal active period, in the early evening) (3, 4, 27, 30). Misalignment also occurs as a result of the disruption of normal activity patterns due to jet lag, shift work, sleep disorders or social jet lag, and associates with the development of metabolic disorders, such as diet-induced obesity and non-alcoholic fatty liver disease (31).

GR STRUCTURE, SPLICE VARIANTS AND PTMs IN THE MODULATION OF GR ACTIVITY

The effects of GCs are mediated by GR through its three functional domains: a hydrophobic C-terminal ligand-binding domain (LBD) containing a ligand-dependent trans-activation portion (τ_2, or AF2), a zinc-finger DNA-binding domain (DBD) located adjacently, and an N-terminal trans-activation domain (τ_1, or AF1) (32–34). There is extensive alternative splicing and translation of human GR, impacting cell-specific GC actions. Alternative splicing originates multiple isoforms varying primarily in the DBD and the C-terminal LBD/AF2, while multiple translational start sites give rise to GR proteins with different lengths of the AF1 domain. The expression of some GR isoforms is evolutionarily conserved, but while many have shown biological relevance in humans (35), isoforms in rodents are less characterized. In humans and rodents, GRα (referred to simply as GR henceforth) is considered the canonical GR isoform that mediates most actions of GCs and is the primary isoform expressed in most tissues. Alternative splicing of the GR primary transcript in humans and rodents can give rise to additional GR isoforms, including GRβ, which has a truncated C-terminus, resulting in an inactive AF2, with compromised ability to bind GCs. Thus, GRβ is considered dominant negative (36, 37). Although expressed to a lower level than GRα, GRβ is considered a functional TF in a number of tissues, including the liver (36, 38). Additional isoforms include the widely expressed GRγ, which exhibits similar affinities to both GCs and DNA as GRα, but has a compromised transactivation potential and is

associated with GC resistance. Expression of GR is also affected by the activity of miRNA molecules that bind to the 3′ UTR of GR transcripts, affecting their stability and preventing their translation (37). Additionally, lncRNAs such as Gas5 repress ligand-activated GR activity by binding to its DBD as a decoy GRE in starvation conditions, leading to suppression of GC-stimulated mRNA expression of key gluconeogenic enzymes *G6Pase* and *Pck1* during fasting (39).

In addition to the coregulatory function of specific GR isoforms, the activity of hormone-bound GR in different tissues can be modulated by specific sets of PTMs (40). For example, upon hormone binding, ligand-selective phosphorylation of the GR affects GR-mediated transcriptional activity and recruitment of coregulators, and is thus involved in directing and modulating GR action as a repressor or activator, namely via crosstalk from other signaling pathways such as in GSK3β-mediated phosphorylation (40–45). The relevance of PTMs on the GR protein and their effects on GR function are also illustrated by the protein-protein interactions between clock components and GR leading to suppression of GR activity via acetylation of a lysine residue by the CLOCK protein, potentiated by the presence of BMAL1 (46). Additionally, modifications such as GC-dependent phosphorylation reduce GR stability and half-life by tagging it for ubiquitination and subsequent degradation, and also influence its subcellular localization (37, 43, 44, 47–49). Other PTMs affecting GR function include SUMOylation, which reduces protein stability and regulates transcriptional activity, as well as nitrosylation and oxidation, both associated with reduction of GC-binding (37).

REGULATION OF GR TRANSLOCATION TO THE NUCLEUS

Inactive GR is located in the cytoplasm, monomerically associated with a multimeric chaperone complex important for GR stability, folding and translocation (**Figure 1B**). The maturation of the complex involves a stepwise ATP-dependent assembly from the initial GR-HSP70-HSP40 complex, to the recruitment of HSP90 and Hop facilitating the assembly of a final high GC affinity complex consisting of GR, HSP90, p23, and FKBP51 (50). Circulating GCs enter the cells via diffusion across the cell membrane and interact with GR. Upon ligand-binding, a FKBP51-FKBP52 switch exposes the GR nuclear localization signals, which are recognized by importins and nucleoporins, facilitating the translocation of activated GR through a nuclear pore via microtubules (50, 51). Disruption of FKBP52 leads to reduced expression of GR target genes in the liver and augmented hepatic steatosis as a result of diet-induced obesity (52), also observed in liver-specific GR knock out (L-GRKO) mice (26), demonstrating a functional role of the multimeric chaperone complex for hepatic GR function. In general, the subcellular location of GR follows the diurnal GC concentration (53). However, both ligand-bound and unbound GR shuttle dynamically between the nucleus and the cytoplasm with a variable rate, consequently regulating GR activity. Aberrantly high cytosolic pH and chemical stress can lead to dissociation of HSP90 and increased nuclear import of GR. GR nuclear translocation can also be regulated by context-specific PTMs, e.g., phosphorylation of GR by kinases like MAPKs, CDK, and GSK3 (50). In the liver, factors including HDAC6 and REV-ERBα have been found to affect GR translocation, thus affecting GR activity (53, 54).

GENOMIC ACTIONS OF GR: GENERAL CONCEPTS

Following nuclear translocation, GR accumulates at specific gene regulatory regions (e.g., enhancers) depending on the DNA sequence, occupancy of other TFs, organization of nucleosomes and higher order chromatin structures (**Figure 1C**). GR residence time at specific regions of chromatin lasts seconds, whereas freely diffusing unbound GR occupies chromatin in milliseconds (55) (**Figure 2A**). This enables GR to efficiently probe tens of thousands of putative enhancers within a short time frame and initiate transcription of hundreds of genes within minutes of activation by hormone (56). Also, the dynamic nature of chromatin interaction is shared by transcriptional coregulators known to interact with GR (57), both likely playing an important kinetic role in GC-regulated gene expression, including the duration and frequency of transcriptional bursting (58). As a result of the pulsatile secretion pattern, GC concentration in the serum is highly dynamic, allowing a rapid transcriptional response that can be translated into a fast biochemical response (59). For example, transcriptional bursting has been linked to a fast-acting metabolic switch in hepatic glucose metabolism, where expression of gluconeogenic genes such as *G6pc* and *Pck1* is rapidly decreased in response to feeding (60), the latter being regulated by GCs (61, 62).

Direct and Indirect GR Interaction With the DNA Template

Genomic occupancy of GR is facilitated by direct GR binding to GREs on DNA as a monomer, homodimer or tetramer (63) (**Figure 2B**), with the tetrameric structure being suggested as the final active form of GR (64). GR binds directly to the canonical DNA motif consisting of inverted repeats separated by 3 bps (nGnACAnnnTGTnCn) or to half-sites of these inverted repeats (nGnACA) (63, 65) and degenerate versions of these (66). In addition, GR can bind other inverted repeats separated by 0-2 bps ($CTCC(n)_{0-2}GGAGA$) (67, 68), termed negative GREs (nGRE). Besides binding directly to DNA, GR can occupy enhancers by tethering to DNA-bound TFs by protein-protein interactions (63, 65).

Binding of GR to canonical DNA motifs as homodimers and tetramers is generally associated with GC-mediated transactivation (63, 69–71). Also, studies suggest that GR association with GR half-sites is linked to active gene expression (63, 65). Once GR is associated with enhancers, GC-induced transactivation involves recruitment of transcriptional coactivators to facilitate chromatin remodeling, histone hyperacetylation and mediator recruitment which leads to recruitment and/or increased activity of RNA polymerase II

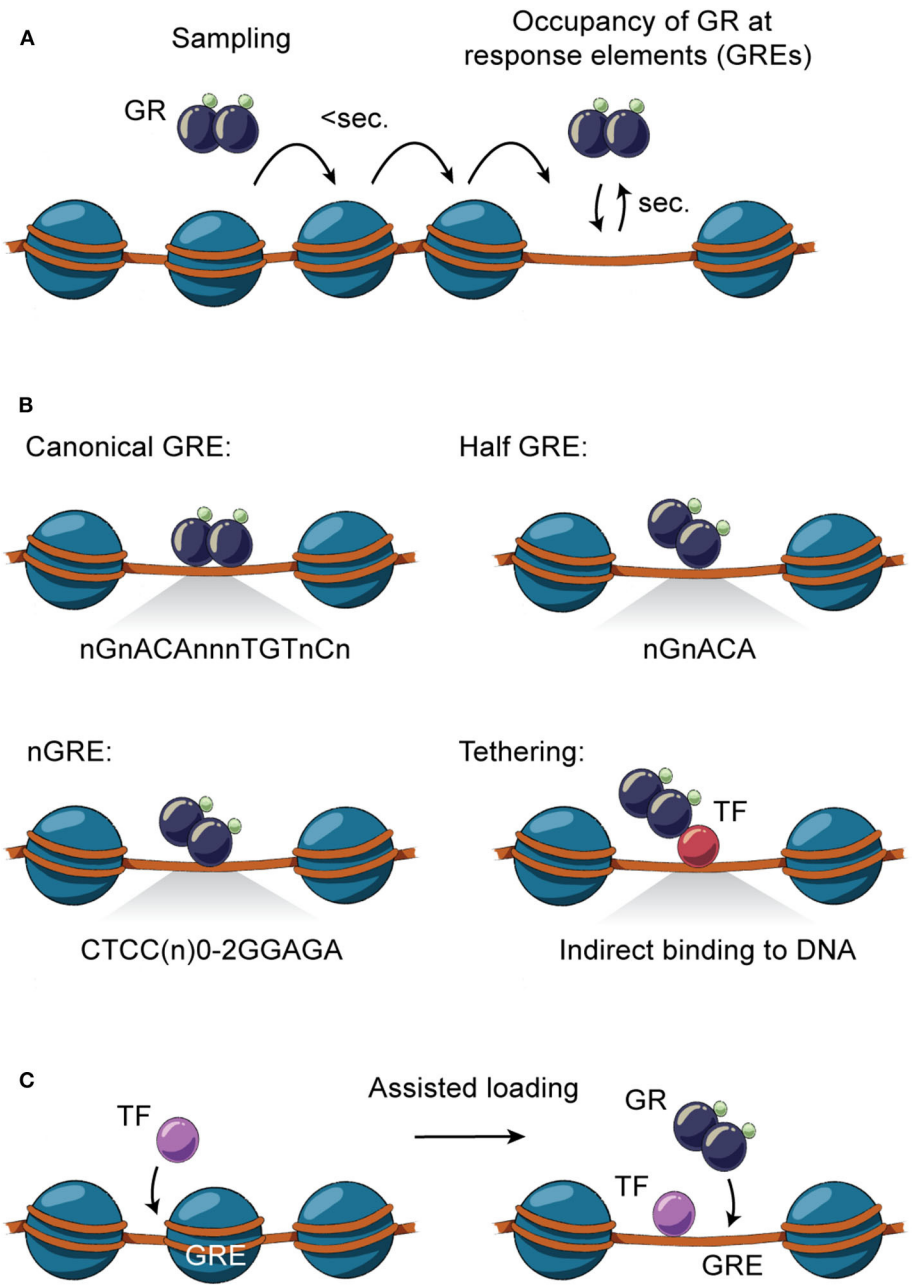

FIGURE 2 | Direct and indirect GR-DNA interactions. **(A)** GR interacts dynamically with DNA. Freely diffusing GR occupies chromatin with residence time in milliseconds, whereas GR binding at specific regions of chromatin is measured in the order of seconds. **(B)** GR interacts directly with DNA by binding to canonical GRE (nGnACAnnnTGTnCn), half-sites (nGnACA) and nGRE (CTCC(n)$_{0-2}$GGAGA) or indirectly by tethering to DNA-bound TFs by protein-protein interactions. **(C)** TFs can assist the loading of GR, or vice versa, by facilitating an accessible chromatin environment at the regulatory site.

at juxtaposed gene promoters (56, 72–74). In contrast, GC-mediated transrepression has been widely discussed, and hence several different models have been presented, including direct binding of GR to nGRE motifs, interaction with DNA sequences bound by other TFs, tethered GR binding to transactivating TFs, redistribution of monomeric GR, sequestering of transactivating coregulators and/or GR-regulated expression of negative modulators of transcription (75). Even though nGREs have been associated with transcriptional repression (67, 68, 76), their role has been debated (63, 72, 74). For example, recent studies found no enrichment of nGREs at enhancers juxtaposed to GC-repressed genes (74). In contrast to enhancers induced by GC, repressed enhancers show marginal canonical GR binding motifs, suggesting that GR binds other DNA motifs (77) or

tethers to other TFs (78). This type of GR interaction with DNA is generally believed to be mediated by monomeric GR, based on structural studies of the GR DBD and mice expressing a mutant GR (GR^{dim}) unable to achieve DBD dimerization (69, 76–79). Although mice expressing GR^{dim} indeed show reduced GR transactivation ability in the liver and maintain transrepressive activity (70), studies have suggested that GR^{dim} forms dimers in the nucleus through another dimerization surface of the LBD (80). This suggests that binding to GR half-sites or other DNA motifs may be mediated by GR dimers, where possibly only one part of the dimer binds directly to DNA (**Figure 2B**). Cistromic analysis of GR and GR^{dim} in the liver and in macrophages suggests extensive GR binding to chromatin through GR half-sites, which in many cases colocalizes with lineage-determining TFs driving cell-specific gene transcription (63, 70). Accordingly, GC treatment has been suggested to induce pronounced GR redistribution from GR half-sites to canonical GREs leading to reduced transcription of genes controlled by lineage-specific TFs (63). Introducing a mutation that completely disrupts direct GR binding to DNA ($GR^{\Delta Zn}$) leads to a perinatal lethal phenotype similar to knock out of GR, emphasizing an essential function of direct binding to DNA. Interestingly, studies of mouse embryonic fibroblasts isolated from $GR^{\Delta Zn}$ mice show that direct GR-DNA interaction is essential for both transcriptional activation and repression by GCs, arguing that tethering is not a dominant mechanism for GR transrepression (81). Thus, genomic action of GR is primarily mediated by multimeric or monomeric actions involving direct interaction with the DNA template.

GR Interaction With Chromatin

GR binding to DNA is not solely dependent on the DNA sequence of the GRE. As GR binding sites are part of enhancer regions organized in higher order chromatin structures, occupancy of GR to specific regions of the genome is determined by a number of interdependent factors. This includes selective chromatin accessibility, epigenetic modifications of the histones, and the presence of other signal-dependent TFs, lineage-determining TFs and transcriptional coregulators (56). In the mouse liver, GR binds at least 11,000 distinct regions which are primarily located in intronic and intergenic distal regions (26, 61, 63, 72, 82). The vast majority of the GR binding sites are accessible prior to GC stimulation (pre-accessible chromatin) and only some are *de novo* remodeled following GR recruitment (72). Similar findings are observed for other cell types (56, 83, 84), demonstrating that selective GR occupancy of chromatin is largely determined by the accessibility of GREs. This pre-programmed chromatin landscape is shaped by cell-specific TFs and interacting coregulators that facilitate an accessible chromatin environment thereby assisting the loading of other TFs to the chromatin (discussed below; **Figure 2C**) (85). Accordingly, when comparing the liver cistrome across a number of well-described GC-responsive cell types, more than 80% of GR binding sites are unique to the liver and only 0.5% of the binding sites in the liver are shared with other cell types (72). This correlates with the findings that GR-occupied enhancers active in one cell type are inaccessible and nucleosomal in another cell type (73). GR has also been found to facilitate binding of other TFs to enhancers in the liver by establishment of accessible chromatin (72). In fact, binding of GR to genomic regions with different levels of chromatin accessibility has been linked to the type and strength of the GRE motif, with weaker motifs being found at nucleosome-depleted enhancers, compared to more nucleosomal dense sites (73).

Control of Gene Transcription by Recruitment of Coregulators and Chromatin Remodeling

Upon GR binding to chromatin, the local nucleosome-sparse region expands and the accessibility of the chromatin is further increased trough recruitment of chromatin remodeling complexes such as SWI/SNF and additional TFs (86–88). In addition, GR facilitates recruitment of widely expressed coactivators including histone acetyl transferases CBP, P300, GRIP1, PCAF and SRC-2 and components of the Mediator complex such as MED1 and MED14 (56, 66, 73, 89, 90). Moreover, other important GR coactivators have been identified in the liver, including CRTC2 (91), SIRT1, PGC-1α (92), ASCOM complex (93) and SETDB2 (94). On the other hand, GR has been found to interact with corepressors including SMRT (95), HDAC1 (96), CtBP (97), SMAD6-HDAC3 (98), CRY1 (99) and recently TAZ (100), although these interactions are not necessarily associated with transcriptional repression. The wide variety of coregulator interactions allows transcriptional fine-tuning of specific genes in a given cell in a concerted response to cellular signals and circulating GC levels.

Local recruitment of GR and associated coregulators to specific enhancers is translated to a transactivation potential by assembly into higher order enhancer-enhancer and enhancer-promoter condensates (101), facilitating localized increased concentration of the transcriptional machinery (102). Interestingly, interaction between promoters and enhancers occupied by GR is mostly established prior to GC stimulation (103, 104), suggesting that GC treatment does not necessarily lead to new chromosomal interactions but rather increases existing interactions between GR-occupied enhancers and GC-regulated target genes (74). Importantly, availability of GCs has been shown to be central for this differential interaction, suggesting that rapid regulation of gene transcription in response to changes in GC levels not only involves dynamic loading of GR and coregulators on the genome but also differential regulation of enhancer-promoter interaction (103).

GR OPERATES IN TRANSCRIPTIONAL NETWORKS TO CONTROL HEPATIC GENE EXPRESSION

The general GR working model described above illustrates that cell-specific GR actions are orchestrated by auxiliary lineage-determining and signal-dependent TFs. As any given cell expresses multiple cell-specific TFs that shape the accessible chromatin landscape, it is evident that GR-GC action in a given cell is controlled by signaling pathways regulating the activity

and expression of these TFs. For example, the liver receives a variety of context-dependent signals controlling specific signaling pathways including circadian cues, insulin, glucagon, growth hormone and free fatty acids, that collectively shape and are shaped by the GC response in hepatocytes. These different signals are integrated in spatial and temporal TF signaling networks that regulate and fine-tune the hepatic transcriptional response. GR interaction with different TFs and the importance of these interactions for transcriptional regulation have been investigated for decades (105). Recently, several key genome-wide studies in mouse liver tissue have demonstrated that GR interacts with a large repertoire of TFs and that these interactions are diverse, bidirectional, dynamic and highly context- and cell-specific (**Table 1**). The interactions between GR and TFs can be classified as direct or indirect. Direct interactions cover protein-protein interactions or concurrent and co-localized binding to regulatory sites in the chromatin (**Figure 3A**), impacting coregulator recruitment, and consequently enhancer activity (**Figure 3B**). Indirect interactions involve TF cascades, where the expression of one TF regulates the expression of another TF (**Figure 3C**).

Composite TF Interactions and Assisted Loading

At composite sites, GR binds to GREs and can functionally interact with other TFs bound to a neighboring site in the same regulatory region, co-operatively regulating enhancer activity. These binding sites can be overlapping or closely located on the DNA strand and involve GREs, half GREs and/or nGREs (**Figure 3A**). Many liver-expressed TFs have been found or suggested to co-occupy GR binding sites (**Table 1**). ChIP-seq experiments have confirmed the composite binding of CREB1, FOXO1, FOXA, HNF4α, HNF6, C/EBPα, C/EBPβ, PPARα, E47, STAT5, and REV-ERBα at several GR-occupied enhancers (26, 61, 63, 72, 82, 107, 108, 110, 114). In the liver, ChIP-seq data suggests that GR binds GRE half-sites together with lineage-determining TFs including HNF4α, C/EBPβ, HNF6, and FOXA (63, 72). In addition, AP-1 and SP1 motifs have been found to be enriched at GR binding sites (122) and the AhR binding site contains a GRE (123), suggesting that these TFs could work together with GR at specific sites to regulate transcription (124). However, further investigations are needed to determine the relevance of AP-1, SP1, and AhR on GR activity in the liver.

Several confirmed composite GR-TF interactions have been found to impact GR activity and hepatic metabolism, including C/EBPβ, E47, STAT5, and LXRβ, which are required for GR recruitment to specific sites (26, 72, 110, 113), in accordance with the model for assisted loading. For example, GR and E47 co-occupy many promoters and enhancers, working in synergy to regulate GC-induced metabolic genes. Studies using liver-specific E47 knock-out mice emphasize the importance of E47 in the recruitment of GR, FOXO1, and the mediator complex to composite sites. This cooperation affects glucose, fatty acid and lipid metabolism, which is demonstrated by E47 knock-out mice being protected from GC-induced hyperglycemia, dyslipidemia and hepatic steatosis (110). Another example is the C/EBP-facilitated assisted loading of GR. C/EBP has been found to occupy and prime the majority of GR target sites in the liver, making the chromatin accessible for GR binding. Disruption of C/EBP binding attenuates GR recruitment and GR-induced chromatin remodeling at composite sites (72). The concept of assisted loading is also found reversely, with GR assisting the loading of TFs including C/EBP and CREB1 at a subset of sites (**Figure 2C**) (72, 107). For example, GR-mediated assisted loading of CREB1 at a subset of CREB1 target enhancers doubles the number of CREB1 bound sites and increases chromatin accessibility, eventually leading to increased hepatic glucose production during fasting (107).

Protein-Protein Interactions: Heterodimerization and Tethering at Chromatin

As mentioned above, multiple GR isoforms can be generated from the primary transcript and protein processing. Thus, GRα/β heterodimers can be formed on chromatin, impacting the activity of occupied enhancers (125–127) (**Figure 3A**). In fact, GRβ has been shown to have metabolic relevance in the liver. For example, feeding induces GRβ expression within 7 h, likely in response to insulin (36). This is supported by observations that hepatic GRβ expression increases in diet-induced obese mice (128). Overexpression of GRβ in mouse liver reduces expression of known GRα target genes such as *Pck1* and *Ppara*, associated with disrupted gluconeogenesis and increased hepatic lipid accumulation and inflammation, respectively (128, 129). Moreover, the GRβ-mediated increase in lipid accumulation is also seen in L-GRKO mice (26, 130), suggesting that GRβ may function as a negative regulator of GRα in hepatic fatty acid metabolism. Importantly, GRβ expression in a GRα-negative background leads to expression of a specific set of genes not regulated in the presence of GRα (129), suggesting that GRα and GRβ regulate each other's activities by mechanisms involving accessibility to chromatin, cooperation with TFs and coregulators and indirect regulation of enhancer activity (**Figures 3A–C**). Likewise, GR has been found to form a heterodimer with the mineralocorticoid receptor (MR) (34) in a number of different tissues and cells, including the hippocampus and mammary cells. Here, the GR-MR complex binds to GREs and regulates gene expression (131, 132). Although a GR-MR complex has not, to our knowledge, been shown to be functional in the liver, it has been suggested that GR-MR could regulate hepatic expression of *G6Pase* (133) (**Figure 3B**). However, further investigations are needed.

Besides heterodimerization on DNA, GR has been suggested to form other protein-protein interactions on chromatin which tether GR to enhancers independently of its DBD. This includes interaction with COUP-TFII, STAT5, PPARα and the molecular clock components BMAL1, CLOCK, and REV-ERBα, influencing GR activity and hepatic metabolism (82, 106, 115, 116, 119). For example, COUP-TFII protein interaction with GR is important for GC-induced promoter activity and hepatic *Pck1* gene expression (106). Also, GR is suggested to be recruited to a

TABLE 1 | Examples of hepatocyte expressed transcription factors interacting with GR on chromatin.

Transcription factor		Signals regulating TF activity	Interactions with GR	Model	References
Metabolism					
C/EBPα	CCAAT enhancer binding protein alpha		Co-localization	Mouse liver	(72)
C/EBPβ	CCAAT enhancer binding protein beta		Co-localization. C/EBPβ-mediated assisted loading of GR	Mouse liver	(63, 72)
COUP-TFII	Orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor II	9-cis-retinoic acid All-trans-retinoic acid	Protein-protein interaction. Co-localization on chromatin	H4IIE and HepG2	(106)
CREB1	CAMP responsive element binding protein 1	Glucagon	GR-mediated assisted loading. Co-localized binding	Mouse liver	(61, 107–109)
E47			Co-localization on chromatin. E47 is important for GR recruitment.	Mouse liver	(110)
FOXA	Forkhead box A1		Half-site tethering	Mouse liver	(63)
FOXA2	Forkhead box A2		FOXA2-mediated assisted loading of GR. Co-localization at site	Mouse liver and primary mouse hepatocytes	(63, 109)
FOXO1	Forkhead box O1	Insulin	Co-localization on chromatin and protein-protein interaction	Mouse liver H4IIE	(61, 110, 111)
LXRα	Liver X receptor alpha	Oxysterols	Competes with GR for binding at target sites	HepG2	(112)
LXRβ	Liver X receptor beta	Oxysterols	Facilitates GR binding to selected GREs	Mouse liver	(113)
HNF6	Hepatocyte nuclear factor 6		Half-site tethering	Mouse liver	(63)
PPARα	Peroxisome proliferator activated receptor alpha	Fatty acids, eicosanoids, phospholipids, polyphenols	Co-localization on chromatin	Primary mouse hepatocytes	(114)
Circadian clock					
BMAL1	Brain and muscle ARNT-like 1	Circadian	Protein-protein interaction. GR is tethered to BMAL1-CLOCK complex. Co-localization on chromatin	Mouse liver	(26, 115)
CLOCK	Circadian clock regulator	Circadian	Protein-protein interaction. GR is tethered to BMAL1-CLOCK complex. Co-localization on chromatin	Mouse liver	(26, 115)
CRY1/CRY2	Cryptochrome circadian regulator 1/2	Circadian	Co-localization on chromatin through tethering. Protein-protein interaction	HepG2 cells Mouse liver	(26, 99, 116)
PER1/2	Period circadian regulator 1/2	Circadian	Co-localization on chromatin	Mouse liver	(26)
REV-ERBα/β	Nuclear receptor subfamily 1 group D member 1/2	Circadian, hem	Protein-protein interaction. Co-binding to sites. REV-ERBα-mediated assisted loading of GR	Mouse liver	(26, 82)
RORα/γ	RAR related orphan receptor A/C	Circadian	Co-localization on chromatin	Mouse liver	(26)
Development and growth					
HNF1α	Hepatocyte nuclear factor 1 alpha		Co-localization at sites	Mouse liver PLC/PRF/5 cells	(117, 118)
HNF4α	Hepatocyte nuclear factor 4 alpha	Linoleic acid	Co-localization at sites	Mouse liver	(63, 117)
STAT5	Signal transducer and activator of transcription 5	Growth hormone. Cytokines	Protein-protein interaction. Co-localization at sites. STAT5 tethers GR to sites. STAT5 induces GR recruitment to sites	Mouse liver	(26, 119, 120)
General					
HSP90	Heat shock protein 90		GC-dependent co-localization on chromatin	Rat hepatoma HTC cells	(121)
p23	Prostaglandin E Synthase 3	Prostaglandin E Synthase 3	GC-dependent co-localization on chromatin	Rat hepatoma HTC cells	(121)

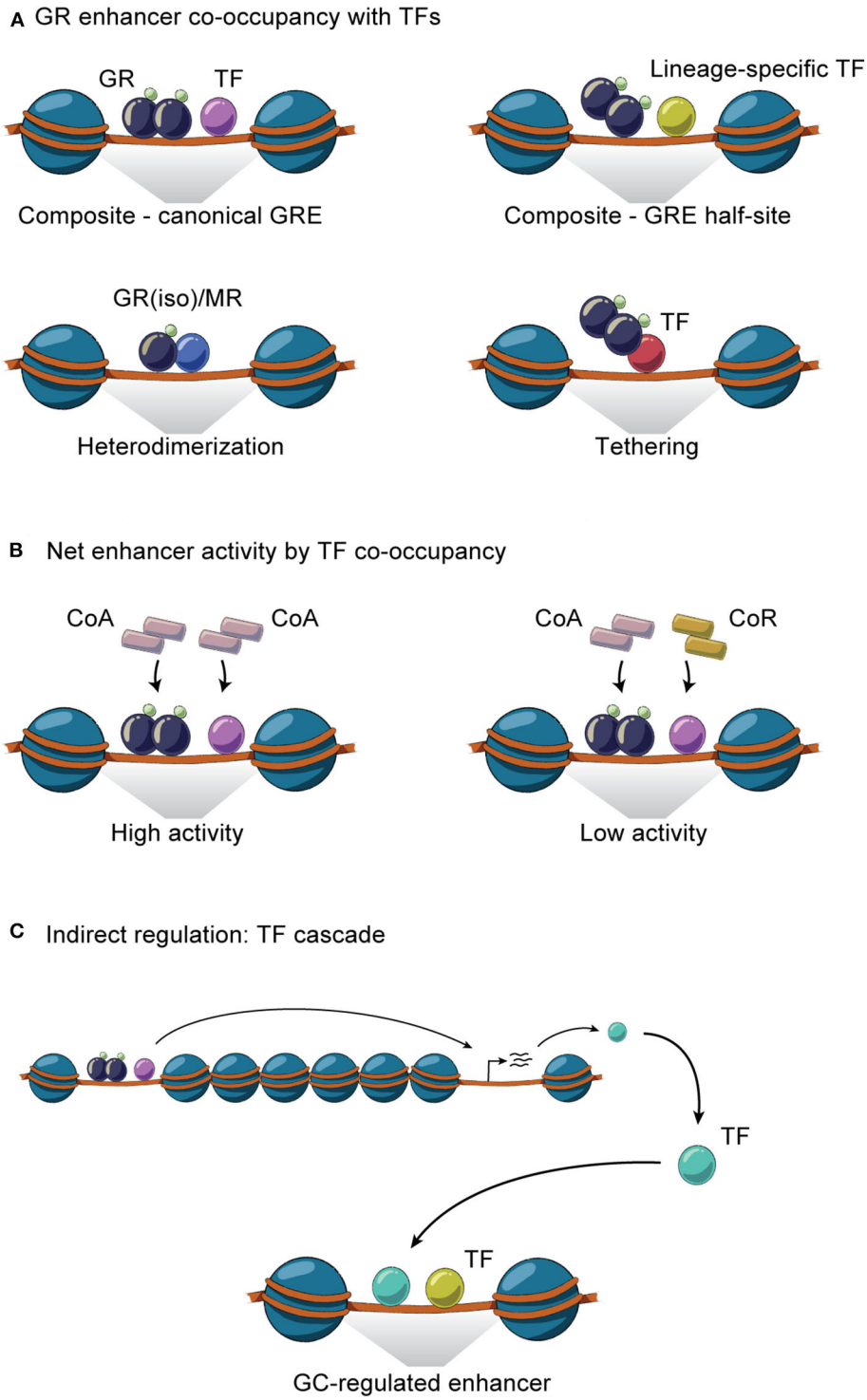

FIGURE 3 | GR interaction with TFs on chromatin. **(A)** GR and TFs co-occupy enhancers through homodimeric or monomeric GR binding together with TFs at composite sites, by heterodimerization and through tethering. **(B)** GR- and TF-mediated recruitment of coactivators (CoA) and/or corepressors (CoR) to co-occupied regulatory sites controls the net enhancer activity. **(C)** Indirect GR-TF interaction involves TF cascades, where the expression of GR regulates the expression of TF or vice versa.

subset of sites via tethering to DNA-bound PPARα to regulate metabolic genes in the liver including *Pdk4* (114). Moreover, GR tethering to the BMAL1-CLOCK complex is suggested to repress hepatic *Rev-erbα* expression (115), demonstrating how GR and the molecular circadian clock interconnect to regulate shared gene programs.

Controlling Enhancer Activity by Co-occupancy of Multiple TFs

The transcriptional effect of multiple TF interactions at enhancers can be evaluated by looking at the expression of juxtaposed target genes or at localized histone acetylation and mediator recruitment. In the case of TF cooperation at individual enhancers, activation of several TFs will result in synergistic effects on enhancer activity and gene expression. In contrast, TFs working independently at the shared enhancer would result in gene expression corresponding to a sum of the contribution from each TF. For example, composite GR-PPARα sites have been found to synergistically affect the expression of fatty acid oxidation and ketogenic genes while GR-CREB1 sites synergistically regulate gluconeogenic genes (107). Likewise, synergistic and additive regulation has been reported for genes controlled by GR and FOXO1 in co-occupancy (61). These cooperative effects likely reflect increased recruitment of coactivators to a given set of enhancers involved in transcription of a specific gene (**Figure 3B**).

In contrast to the synergistic action of composite GR-TF binding sites to increase enhancer activity, several studies have suggested negative regulation between GR and TFs occupied at a given enhancer. Such negative regulation can be understood as a competition between the TFs for a given DNA sequence. For example, in the liver, LXRα binds GREs together with its heterodimerization partner RXRα, thereby potentially competing with GR for binding to the same sites leading to differential regulation of genes involved in glucose metabolism (112). Another example is the GR isoform competition model, which seeks to explain how dominant negative GRβ functions as a negative regulator for GRα at some sites. Similarly, GR has been suggested to compete with AP1 at AP1 motifs with embedded GR half-sites (77). However, these competitional models do not agree with the dynamic nature of GR and most other TFs as these factors bind transiently to chromatin with residence times in a matter of seconds (55, 134), possibly allowing multiple factors to interact with the same site (135). Thus, GR-TF competition at composite sites is likely not a competition for the same response element. Instead, the negative regulation likely reflects the different coregulators recruited to the response element. Composite binding of different TFs recruiting coregulators of opposite activity or competition between limited amount of avaliable coregulators for binding to the specific TFs would balance the transcriptional response. For example, corepressors and coactivators have been suggested to bind GR in equilibrium, balancing GR activity (136), which has also been suggested for other nuclear receptors in the liver, including the thyroid hormone receptor (137).

Regulating TF Networks by GR

The direct interaction between GR and other key TFs on chromatin in the liver can take different forms, as described above, to jointly regulate hepatic gene expression. However, indirect GR-TF interactions involving TF cascades are equally important, though more challenging to investigate, with several potential interaction steps (**Figure 3C**). Important indirect pathways have been studied in the liver. For example, GR binds GREs near core clock genes to induce transcription of *Per1*, *Bmal1*, *Cry1*, *Dbp* (138, 139). This in turn controls a range of circadian-regulated genes. In regards to energy metabolism, GR interacts with several key factors in TF cascades connecting and impacting different signaling pathways. For example, glucagon-mediated activation of CREB1 induces the transcription of YY1, which then induces the transcription of GR. This interaction cascade is important in hepatic gluconeogenesis (140). Moreover, GR induces the transcription of Klf9, which has been linked to the downstream induction of PGC1α expression and of hepatic gluconeogenic genes (141). GR interaction with PGC1α has furthermore been suggested to regulate mitochondrial oxidative phosphorylation (142). Additionally, GR induces the transcription of PPARα upon long-term fasting, initiating hepatic fatty acid oxidation and the ketogenic gene program (107).

GR REGULATORY NETWORKS IMPACT MULTIPLE ASPECTS OF HEPATIC METABOLISM

The emerging studies in complex gene-regulatory networks controlled by GR and controlling GR activity emphasize the importance of the context-dependent action of GCs in tissues like the liver. Accordingly, genetic disruption of GR in the liver impacts a range of metabolic pathways leading to dysregulated glucagon synthesis, lipid metabolism, gluconeogenesis, urea metabolism and bile acid synthesis and uptake (26, 143–146). For example, L-GRKO mice and GR^{dim} mice show dysregulated glucose, fatty acid and bile acid metabolism (26, 144, 146). Reduced expression of key gluconeogenic genes including *Pck1*, *G6Pc,* and *Pfkfb3* in L-GRKO mice is linked to fasting hypoglycemia (26, 144–146), and around half of newborn albumin-alpha-fetoprotein-driven L-GRKO mice die within 48 h after birth, possibly due to hypoglycemia (120, 146). L-GRKO mice are more sensitive to insulin than WT littermates and liver glycogen content in L-GRKO mice is reduced (145). These effects of L-GRKO on glucose metabolism could in part be explained by the interaction with TFs such as CREB1, FOXO1, FOXA2, PPARα, E47, STAT5, LXRα, LXRβ, and circadian regulators (26, 61, 82, 107, 109, 110, 112–114) (**Figure 4**). Yet, the effects of L-GRKO on glucose metabolism seem to be partially compensated by increased gluconeogenesis in the kidney (145) and by a shifted hormonal balance involving reduced plasma concentration of insulin and increased glucagon levels, compared to WT mice (146).

Hepatic GR disruption also leads to decreased fat mass (145) and lower plasma triglyceride levels (26, 146), while free fatty acid plasma levels are similar in fasted and fed L-GRKO mice and WT mice (146). Recently, L-GRKO mice were reported to accumulate triglycerides in the liver and to develop hepatic steatosis (26), although this is controversial (130), but may be explained by the promoter controlling CRE expression. Many TFs have been found to work together with GR to regulate fatty acid and lipid metabolism including STAT5, PPARα, FOXO1,

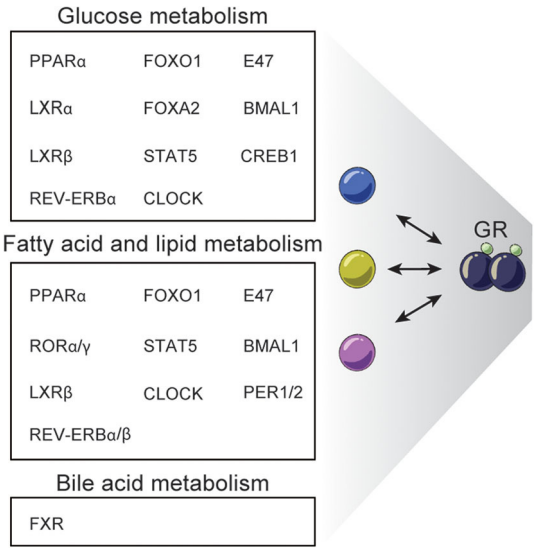

FIGURE 4 | Examples of TFs interacting with GR regulating hepatic metabolism. GR interacts with different TFs to regulate specific processes in hepatic glucose, fatty acid, lipid, and bile acid metabolism.

E47, LXRβ, CLOCK, REV-ERBα/β, CRY, BMAL1, RORα/γ, and PER1/2 (26, 82, 110, 111, 113, 114) (**Figure 4**).

Finally, disruption of hepatic GR function leads to dysregulated systemic bile acid homeostasis. Specifically, mice with hepatic GR knock down by shRNA have a reduced amount of bile acid in the gallbladder, elevated serum bile acid levels, impaired bile acid uptake/transport and are more susceptible to develop gallstones when fed on cholesterol-rich diet. Moreover, these mice do not undergo the normal changes in bile acid levels in the serum, liver and intestines in the fast-refeeding transition (144). GR^{dim} mice fed a lithogenic diet have elevated fasting serum bile acid levels and decreased gallbladder bile acid volume. These effects have been associated with interaction between GR and FXR, a key TF regulating bile acid metabolism (97, 144). GR deficiency reduces the expression of the classical FXR-target gene *Shp* encoding the SHP repressor, leading to increased expression of the rate-limiting enzymes in bile acid synthesis Cyp7a1 and Cyp8b1 (144). Additionally, dex-induced GR recruits the co-repressor CtBP to block FXR activity at shared sites related to bile acid gene metabolism, e.g. *Shp* promoter (97) (**Figure 4**).

EXAMPLES OF KEY HEPATIC GENE REGULATORY NETWORKS CONTROLLED BY GR

GR Crosstalk With FOXO1

The daily change from the inactive fasting phase to the active feeding phase requires a major transcriptional reprogramming of the liver. This is particularly relevant at the transition between the unfed and fed states, which takes place around zeitgeber time (ZT) 12 (i.e., 6 p.m.) in nocturnal animals such as mice. The interaction between GR and the insulin-regulated TF FOXO1 is involved in driving this transcriptional transition. Pre-prandial high GC and low insulin levels are associated with GR and FOXO1 binding to chromatin, respectively, and regulation of target genes. In fact, in this fasted state, more than half of all FOXO1 binding sites are co-occupied with GR regulating gene expression. Conversely, the post-prandial increased insulin and reduced GC lead to reduced FOXO1 and GR occupancy, respectively, and reduced transcriptional regulatory activity. Importantly, more than 80% of feeding-repressed genes in the liver are associated with a nearby enhancer bound by GR, FOXO1 or both (61). One example of a metabolic gene coregulated by GR and FOXO1 in the liver is *Angptl4*, associated with the regulation of glucose and lipid metabolism. In a fasted state, GR and FOXO1 bind a specific GRE and forkhead box transcription factor response element (FRE), respectively, located in the regulatory region of *Angptl4*. GCs induce, while insulin abolishes, the occupancy of both factors at the region (111). Besides the direct interaction between GR and FOXO1 at enhancers in the liver, GR has been found to induce the expression of *Foxo1* gene in the liver and in this way indirectly regulate target genes (147). Furthermore, FOXO1 binding has been found at the promoter of GR, suggesting that the indirect interaction is bidirectional (148).

GR Crosstalk With PPARα

Like GR, PPARα is important for the hepatic response to fasting. The role of PPARα in regulating metabolism and inflammation as well as the importance of crosstalk between PPARα and other TFs, including GR, have been covered in detail in previous reviews (1, 149). The GR-PPARα interactions in the liver include co-localization to chromatin and coregulation of genes involved in lipid and glucose metabolism (150). More specifically, in co-ligand treatment of primary murine hepatocytes, 13% of GR peaks are co-bound with PPARα (114). Furthermore, other studies have found that, during fasting, GR and PPARα have a synergistic effect on genes involved in ketogenesis and fatty acid oxidation; however, the GR-PPARα interaction has been suggested to be indirect as GR induces the expression of PPARα and time-course experiments show a gradual effect of GR on PPARα activity (107).

GR Crosstalk With STAT5

STAT5 is activated by the growth hormone through the growth hormone receptor-JAK2 signaling pathway and by cytokine signaling. In the liver, STAT5 is known to regulate genes involved in body growth, cell cycle, lipid, bile acid, drug and steroid metabolism (151). The STAT5 and GR signaling pathways are connected as exemplified by the reduced body size in mice with inactivated hepatic GR showing impaired growth hormone signaling (120). Furthermore, the importance of STAT5 and GR signaling is demonstrated in liver-specific STAT5 and STAT5 GR double mutant mice exhibiting hepatic steatosis and, for the double mutant, also hepatic carcinoma (130). The STAT5 and GR crosstalk at multiple levels. STAT5 and GR form protein-protein interactions in hepatocytes, which have been found to be important for postnatal growth and maturation-related gene expression. Mice expressing a point mutation in the GR DBD

(GR^{dim} mice), previously suggested to reduce GR DNA-binding and GR dimerization (69, 79), have an unaltered ability to interact with STAT5 (120). These GR^{dim} mice have normal body size, suggesting that the joint GR-STAT5 regulation of growth genes happens through tethering of GR to the STAT5 bound sites or through half GREs in conjunction with STAT5 binding sites (119, 120). However, as mentioned above, more recent studies have found that GR^{dim} is able to dimerize and bind DNA (80), suggesting a reassessment of GR and STAT5 interaction type at shared sites.

Recently, it has been shown that high-fat diet feeding of mice leads to reprogramming of the hepatic GR cistrome primarily during the active feeding phase. Many sites with high-fat diet-induced increased GR recruitment are associated with increased STAT5 co-occupancy. These co-occupied sites showed increased enhancer activity and were associated with genes involved in fatty acid, lipid and glucose metabolism. Hepatocyte-specific STAT5 and GR KO mice demonstrated that STAT5 facilitated the recruitment of GR at gained sites, whereas GR had no effect on STAT5 recruitment. It is still unknown whether the increased STAT5 activity in obese mice is a response to altered growth hormone or cytokine signaling or if it originates from nutritional adaptations in the chromatin landscape (26).

GR Crosstalk With Molecular Clock Components

In the liver, the effect of exogenous GCs on gene regulation is highly dependent on the time of administration. For example, in mice about eight times more genes are differentially regulated by GCs at daytime compared to nighttime. Pathway analysis shows a strong time-dependent regulation of genes in glucose and lipid metabolism (82), which has also been observed in studies looking at endogenous GC effects (26). Hence, timing of GC administration according to the endogenous GC levels has shown positive effects. Administration of GCs at ZT12, as opposed to ZT0, leads to less hepatic lipid accumulation and behavioral changes. This time-differential effect of GC is suggested to be caused by a disrupted circadian regulation of GC-target genes with administration at ZT0, which is supposedly more critical compared to an over-activation of GR at ZT12 (152).

This diurnal oscillation of GC action stems from cooperativity and multiple interactions between GR and the molecular clock components in the liver. For example, GR and central clock components including BMAL1, CLOCK, REV-ERBα/β, PER1, PER2, CRY1, CRY2, and RORα/γ co-occupy different genes involved in clock function and in metabolism (26, 82, 99). The cooperativity also involves different physical interactions between GR and clock factors on the chromatin level, regulating the expression of other clock factors and metabolic genes (see **Table 1**). For example, GR physically interacts with CRY1/2 in a GC-induced manner and, in the post-prandial phase, CRY1/2 represses GR activity on e.g. the expression of *Pck1*. CRY1/2 deficient mice have constitutively high GC levels and exhibit glucose intolerance, suggesting reduced suppression of HPA axis and increased GR activity in the liver (99).

It has been long known that GC and GC-activated GR influence the expression and circadian phase-shifts of several clock factors, including *Per1*, *Dbp* and *Cry1* (23, 30, 139). In fact, GR is recruited to the promoters and enhancers of all central clock genes, suggesting a gene regulatory function of GR (26). Reversely, molecular clock elements also affect GR function, as exemplified by the previously mentioned binding of REV-ERBα to HSP90 (53) and the acetylation of GR by CLOCK (46), both leading to suppression of GR action.

The interaction between GR and members of the molecular clock and its influence on hepatic metabolism can be further exemplified focusing on a single molecular factor. REV-ERBα is one of the key transcriptional repressors in the molecular transcriptional clock, contributing to the characteristic circadian expression in many tissues, including the brain and metabolic tissues like the liver, muscle, pancreas and adipose tissue. In the liver, REV-ERBα is involved in the daily regulation of glucose and lipid metabolism (153). REV-ERBα represses clock genes by binding to RevDR2/RORE DNA elements and recruiting the corepressor complex NCoR-HDAC3. On the other hand, REV-ERBα regulates many metabolic genes by tethering to cell-type specific TFs. Hepatic REV-ERBα tethers to e.g., HNF6 and recruits HDAC3 for active repression of lipogenic genes (154, 155). GR has been found to interact with REV-ERBα on different levels. REV-ERBα interacts physically with GR and, together with HNF4α and HNF6, binds regulatory regions controlling gene expression in mouse liver. REV-ERBα was found to be important for efficient GR recruitment to chromatin during the day, presumably by maintaining histone acetylation at binding sites (82). Moreover, indirect interactions between GR and REV-ERBα have also been observed. REV-ERBα inhibits GR protein expression and nuclear localization (53), and GR inhibits REV-ERBα RNA expression (156) by forming a complex with CLOCK and BMAL1, where GR may be tethered to the regulatory site of the REV-ERBα gene (115).

PERSPECTIVES IN DISEASE AND CLINICAL USE OF GCs

Glucocorticoids have immunosuppressant and anti-inflammatory properties, making them an effective treatment for allergies, inflammatory and autoimmune diseases. The anti-inflammatory effects mediated by GR are conducted by the immune cells, with the macrophages having a particularly important role in the repression of inflammatory genes [reviewed in (157)]. However, by administering GCs systemically, there is a risk of eliciting undesirable side effects on other tissues and cellular processes, such as hepatic metabolism, which is highly impacted by GR regulation. In this review, we described the multiple layers of regulation of GR function, from the control of hormonal availability to the modulation of GR expression at both mRNA and protein levels, as well as PTMs and interactions with different proteins and TFs affecting the transcriptional activity of GR. In depth knowledge of the multifaced control of GR activity provides a unique opportunity to tailor GC treatment and prevent metabolic-related side effects.

One strategy could involve administration of different GR ligands affecting interacting coregulators to modulate transcriptional regulation by GR (8). Another strategy could be to selectively activate or inhibit specific and relevant GR-mediated

regulatory pathways, where treatments involving a combination of different TF ligands could have potential. For example, co-administration of GC and LXR agonists attenuates the transcriptional activity of GR on a subset of genes in glucose and lipid metabolism, suggesting co-treatment with LXRβ agonists might reduce metabolic side effects in patients with autoimmune or inflammatory diseases (112). However, the function of LXRs on GR target sites is debated (113), and the mechanisms behind the positive and negative effects of LXRs on GR should be elucidated. Also, the antagonistic effect of activated PPARα on GR-mediated transcription of metabolic genes to circumvent GC side effects seems promising (150), with potentials and challenges recently discussed in another review (1). Additionally, the natural ultradian GC release and subsequent dynamic activation of GR contrasts with the constant exposure to GCs during pharmacological therapies. The development of new synthetic GCs and pulsatory administration strategies could potentially minimize side effects by mimicking physiology (58, 59, 103). Finally, pharmacological chronotherapy involving GCs seems promising in several inflammatory disorders, with outcomes improving when GC administration is consistently timed (4). This timed GC-administration has been shown to be beneficial in, for example, patients with rheumatoid arthritis (158).

CONCLUDING REMARKS

The multifaceted regulation of GC action and GR activity discussed in this review highlights the complexity of transcriptional regulation by ligand-dependent TFs. The cooperation with signal-dependent and lineage-specific TFs makes GC-dependent gene regulation very responsive to environmental cues and is thus essential to understand for future optimized usage of GCs in the clinic. Specifically, a deeper understanding of the regulatory mechanisms underlying GR action would be fundamental for future development of safer and more effective therapies for disorders where GC secretion and signaling is involved. The recent genomics studies into the GR interactome show promise in the elucidation of the complex GR-TF networks and could contribute to a shift toward future tailored pharmacological strategies including spatio-temporal drug delivery and personalized medicine.

AUTHOR CONTRIBUTIONS

SP and CC wrote the manuscript with supervision from LG. All authors contributed to the article and approved the submitted version.

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PPARγ S273 Phosphorylation Modifies the Dynamics of Coregulator Proteins Recruitment

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The nuclear receptor PPARγ is essential to maintain whole-body glucose homeostasis and insulin sensitivity, acting as a master regulator of adipogenesis, lipid, and glucose metabolism. Its activation through natural or synthetic ligands induces the recruitment of coactivators, leading to transcription of target genes such as cytokines and hormones. More recently, post translational modifications, such as PPARγ phosphorylation at Ser273 by CDK5 in adipose tissue, have been linked to insulin resistance trough the dysregulation of expression of a specific subset of genes. Here, we investigate how this phosphorylation may disturb the interaction between PPARγ and some coregulator proteins as a new mechanism that may leads to insulin resistance. Through cellular and *in vitro* assays, we show that PPARγ phosphorylation inhibition increased the activation of the receptor, therefore the increased recruitment of PGC1-α and TIF2 coactivators, whilst decreases the interaction with SMRT and NCoR corepressors. Moreover, our results show a shift in the coregulators interaction domains preferences, suggesting additional interaction interfaces formed between the phosphorylated PPARγ and some coregulator proteins. Also, we observed that the CDK5 presence disturb the PPARγ-coregulator's synergy, decreasing interaction with PGC1-α, TIF2, and NCoR, but increasing coupling of SMRT. Finally, we conclude that the insulin resistance provoked by PPARγ phosphorylation is linked to a differential coregulators recruitment, which may promote dysregulation in gene expression.

Keywords: PPARgamma, coregulator interaction, Ser273 phosphorylation, insulin resistance, coactivator, corepressor, nuclear receptors

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INTRODUCTION

Peroxisome proliferator-activated receptor gamma (PPARγ) is closely linked to energy homeostasis regulation, playing important role in adipogenesis, lipid and carbohydrates metabolism, insulin sensitivity, cell proliferation, and inflammatory processes. This nuclear receptor (NR) acts as a metabolic sensor of dietary lipids and is a crucial metabolism modulator (1, 2), regulating diabetes through cytokines and hormones, such as TNFα and leptin genes (2–4). Like other NR superfamily members, PPARγ is activated by natural ligands, such as fatty acids and their metabolites, and by synthetic ligands such as the insulin sensitizers Thiazolidinediones (TZDs), as Rosiglitazone and Pioglitazone, drugs commonly used in type 2 diabetes treatment.

The canonical transcriptional activity of PPARγ occurs through its interaction with several cofactors, which activate or suppress gene transcription. In the absence of ligands, the inactive conformation of helix 12 (H12) of PPARγ ligand binding domain (LBD), favors the binding of corepressor proteins, such as silencing mediator of retinoid and thyroid hormone receptor (SMRT) and the nuclear receptor corepressor 1 (NCoR). These proteins form a corepressor complex with histone deacetylases (HDAC) repressing target gene transcription (5). In the presence of ligands, the receptor undergoes a conformational change, that reallocates H12, forming a charge clamp between H3 and H12 (6). This conformation leads to corepressors dissociation and coactivators recruitment, forming a coactivator complex by the recruitment of other proteins, as well as histone acetyltransferases (HAT) and other general transcription factors, promoting the transcription of the target gene (7). Beyond this canonical transcriptional activity, PPARγ can also be regulated by post-translational modifications (PTMs), as acetylation, phosphorylation, SUMOylation, and ubiquitination (8, 9). These fine-tuning adjustment is part of the cell tissue-specific modulation (9, 10) and can dramatically alter the receptor function, as well as its binding to coregulators (11). By all these PTMs, the PPARγ phosphorylation is one of the most studied, and may promote different receptor's behavior, depending on the residue in which it occurs, and on the enzyme that performs the phosphorylation and/or dephosphorylation (12, 13).

Most of PPARγ phosphorylation were described on its N-terminal domain. The phosphorylation of Y78 is regulated by SRC proto-oncogene, nonreceptor tyrosine kinase (c-SRC), and Protein-tyrosine phosphatase 1B (PTP-1B), and affects the inflammatory response and insulin sensitivity (14). The phosphorylation in S112 by Mitogen-Activated Protein Kinases (MAPKs) pathway (12, 13), and by the Cyclin-Dependent Kinase 7 (CDK7) and 9 (CDK9) (15, 16) intensifies the interaction between PPARγ and the circadian clock protein PER2 (Period Circadian Regulator 2) (17), decreasing PPARγ activation trough reduction of both coactivator binding (12) and ligand binding affinity (18). In addition, S133 and T296 residues were also identified as targets to Extracellular Signal-Regulated Kinase (ERK)/Cyclin-Dependent Kinase 5 (CDK5) phosphorylation pathway (19).

Particularly, one special obesity-mediated phosphorylation that targets PPARγ ligand binding domain (LBD) has been associated with insulin resistance (20, 21). This phosphorylation, performed by the CDK5 at PPARγ S273 (or S245 in isoform 1), does not alter the adipogenic activity of PPARγ, but deregulates a subset of genes, that presented altered expression in obesity and diabetes, such as adiponectin and adipsin (20, 21). It is known that this phosphorylation does not changes the occupancy of PPARγ in the chromatin (21), but the mechanism that corelates this phosphorylation to the deregulation of these specific genes is still unknown. Various PPARγ ligands are capable of inhibit this phosphorylation. Among them is the insulin-sensitizer class of drugs TZDs, which owns familiar anti-diabetic actions but presents negative side effects due to its strong agonism. On the other hand, some partial agonists, such as MRL24 (20), SR1664 (21), GQ-16 (22), UHC1 (23), F12016 (24), L312 (25), Chelerythrine (26), and AM-879 (27), have been identified to inhibit this PTM without the agonist activity. Structural data analysis showed that PPARγ ligands that inhibit S273 phosphorylation do not make direct contact with this residue, but induces structural modifications in PPARγ:CDK5 interaction interface. Such ligands fit into binding pocket promoting an interaction network that protects S273, blocking its phosphorylation (28). Therefore, the most recent strategy of PPARγ modulation have been target the partial agonism of receptor, aiming S273 phosphorylation inhibition.

Mastery and manipulation of the mechanisms involved in this phosphorylation pathway can be a promising approach in the improvement of metabolic disorders therapies. Also, it is known that phosphorylation may contribute to increased coactivator and decreased corepressor activity (29). For example, it is reported that the Thyroid Hormone Receptor 3-Associated Protein (THRAP3), directly interacts with PPARγ specifically when S273 is phosphorylated, acting as a specialized coregulator that docks on certain phosphorylated transcription factors (30). Moreover, the corepressor NCoR was reported as an adaptor protein that enhances the ability of CDK5 to associate with and phosphorylate PPARγ (31).

Here, we demonstrate that the dysregulation caused by Ser273 phosphorylation might occur through the differential recruitment of coregulatory proteins, causing differences in the target genes expression. By using five coregulators reported to interact with PPARγ in adipogenesis, the PGC1-α, TRAP220 and TIF2 coactivators, and the SMRT and NCoR corepressors (32–35), we evaluated how the PPARγ S273 phosphorylation modifies its interaction with coregulators. Our results show that both the presence and absence of phosphorylation at S273 can alter PPARγ activation and its binding profile with some coregulators. The absence of phosphorylation can lead to an increased activation of PPARγ due to a higher interaction with coactivators and decreased interaction with corepressors. Additionally, we found that the CDK5 presence also disrupts this coregulator harmony. Finally, we also hypothesize that additional interfaces may be formed in coregulator:PPARγ interaction due to differential PPARγ phosphorylation states.

MATERIALS AND METHODS

Plasmids for Cell Assays

Cell assays were performed using the following plasmids: pBIND-PPARγ harboring a chimeric protein composed of Gal 4 DBD and

the PPARγ LBD region (aa 238-503), pGRE-LUC (containing the upstream activating sequence of Gal 4 followed by a firefly luciferase reporter gene), pRL-TL (which constitutively express Renilla reniformis luciferase, used as transfection control for vector normalization). All the coregulators constructs were inserted into the commercial vector pM (Clontech), which contains the Gal 4 DBD. The Gal- PGC1-α (containing mouse PGC1-α from 136 to 340 amino acids) and Gal-TRAP220 (ID1 + ID2 containing human TRAP220 from 404 to 654 amino acids) are plasmids belonging to the Laboratory of Spectroscopy and Calorimetry (LEC, LNBio/CNPEM, Brazil). Gal-TIF2 [harboring three interaction domains (IDs) of human TIF2 from 624 to 869 amino acids], Gal-SMRT (ID1 + ID2, containing human SMRT from 982 to the C terminus), Gal-NCoR (ID1 + ID2 + ID3 containing mouse NCoR from 1629 to the C terminus), and VP16-PPARγ (harboring the chimeric protein of the LBD region of PPARγ with the transactivation domain of the VP16 Human herpes simplex virus 2) were kindly provided by Dra. Albane Le Maire from Centre de Biochimie Structurale (CBS, CNRS, France). The plasmid pCDNA-CDK5 (which encodes the CDK5 and P35 proteins) were kindly provided by Professor Sang K. Park of Pohang University of Science and Technology.

Mutations

To evaluate whether S273 phosphorylation would change both activation of PPARγ and its interaction with coregulators, we mutated this residue (target of phosphorylation) in order to mimic the phosphorylated serine and the inhibition of phosphorylation. Mutations of pBIND-PPARγ and VP16-PPARγ at S273 to alanine (PPARγ S273A), used as a constitutive dephosphorylation PPARγ form, and to aspartic acid (PPARγ S273D), used to mimic phosphorylation were performed using Quick Solution of QuickChange site-directed mutagenesis kit (Promega) with pFU DNA polymerase (Promega).

This same strategy was applied to generate Gal-PGC1-α, Gal-TIF2, Gal–SMRT, and Gal-NCoR derivatives harboring mutated IDs. In order to inactivate each ID, for coactivators two specific leucine were substituted by alanine, as Gal-PGC1-α domain LKKLL was mutated to LKKAA (residues 142-146, Gal4-PGC1-α ID1m), Gal-TIF2 had the ID1(residues 641-645) changed from LLQLL to LLQAA (Gal-TIF2 ID1m), the ID2 (residues 689-694) changed from LHRLL to LHLAA (Gal-TIF2 ID2m), and the ID3 (residues 744-749) changed from LRYLL to LRYAA (Gal-TIF2 ID3m). For corepressors, the specifics isoleucine were replaced by alanine, as Gal-SMRT had the ID1 (residues 2094-2098) changed from ISEVI to ISEAA (Gal-SMRT ID1m), and the ID2 (residues 2296-2300) changed from LEAII to LEAAA (Gal-SMRT ID2m), and Gal-NCoR had the ID1 (residues 2073-2077) changed from ICQII to ICQAA (Gal-NCoR ID1m), the ID2 (residues 2277-2281) changed from LEDII to LEDAA (Gal-SMRT ID2m), and the ID3 (residues 1932-1937) changed from IDVII to IDVAA (Gal-SMRT ID3m). The used primers are listed in the **Supplementary Material** and all the mutations and constructs were verified by DNA sequencing.

Reporter Gene Assays

COS-7 and 293T cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% Bovine Fetal Serum (FBS), 1% antibiotics (penicillin and streptomycin) and 0.37% sodium bicarbonate and kept in a humid incubator, at 37°C and 5% CO2. Plasmids transfection were performed using 400ng of each plasmid and the JetPEI (Polyplus) transfecting agent in 3:1 ratio. After 24 h of transfection, 1 μM of Rosiglitazone was added to the wells, which was incubated for more 24 h. The cells were lysed and assayed for reporter expression. Luciferase was measured using the Dual-Luciferase® Reporter Assay System kit (Promega). Luminescence reading was performed on the GloMax®-Multi Detection System reader. In each case, we normalized results by co-expressed Renilla luciferase signal. We carried out each transfection in triplicate and repeated each assay three to eight times (36).

To measure possible changes in PPARγ activation in different phosphorylation states, transactivation assays were performed on 293T cells with transient transfection of plasmids pBIND-PPARγ, pBIND-PPARγ S273A, pBIND-PPARγ S273D, pGRE-LUC, pRL-TL as transfection control, and pCDNA3-CDK5. To measure the interaction between coregulators and PPARγ, and possible differences due to different receptor phosphorylation states, mammalian two-hybrid assays were performed in Hek293T cells for corepressor assays and COS-7 for coactivators assays. The plasmids used were: VP16-PPARγ, VP16-PPARγ S273A, VP16-PPARγ S273D, Gal-Coregulators (PGC1-α, Gal-TRAP220, Gal-TIF2, Gal-SMRT, Gal-NCoR, and its mutated derivatives), pGRE-LUC, pRL-TL as transfection control, and pCDNA-CDK5.

The luminescence value was corrected by transfection control (luciferase Firefly/Renilla) and the value of each tested condition was divided by the luminescence value of the experimental control to obtain the activation rate. As negative control of transactivation assays empty pCDNA3.1 vector was used. For mammalian two hybrid assays, the luminescence value of each tested condition was divided by the baseline condition of the experiment, which for the corepressors is the corepressor tested without the presence of PPARγ, and for the coactivators it is the empty Gal4 vector to obtain the interaction rate (37, 38). Data analysis was performed with GraphPad Prism, by two-way ANOVA, comparing the groups treated with Rosiglitazone and untreated of each PPARγ derivative by Bonferroni's test, with values of $p < 0.05$.

Adipocyte Differentiation

3T3-L1 cells were cultured in DMEM medium containing 50 units/ml of penicillin and streptomycin, 3.7 mg/L of sodium bicarbonate and 10% (v/v) of neonatal bovine serum in T125 bottles (Sarstedt). Cells were plated in 6-well plates (Corning®) at a density of 2.8×10^5 cell/well and cultivate until reaching 100% confluence. To induce differentiation, cells were initially treated for 48 h with a differentiation induction medium (DMEM medium containing 50 units/ml of penicillin and streptomycin, 3.7 mg/L sodium bicarbonate and 10% SFB, 1 μM dexamethasone, 0.5 mM IBMX (3-isobutyl-1-methylxanthine) and 1 ug/ml of insulin). Forty-eight hours later the medium was changed by maintenance medium (containing DMEM containing 50 units/ml of penicillin and streptomycin, 3.7 mg/L of sodium bicarbonate and 10% of SFB and 1 ug/ml of insulin). Maintenance medium was renewed every 48 h for 7 days. The control condition received no treatment, except

for the differentiation cocktail. The treated conditions were: Rosiglitazone (1μM), Roscovitine (10μM) and Rosiglitazone (1μM) + Roscovitine (10 μM). These component concentrations were maintained and renewed every 2 days, along with the change of medium. After the 7 days of treatment, Trizol® RNA extraction was performed as previously described in (39), followed by cDNA preparation with High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The cells were also stained with Oil Red O according to described in (40), and their absorbance at 520 nm was measured by a spectrophotometer. Data analysis was performed with GraphPad Prism, by one-way ANOVA, comparing the different treatments, values of $p < 0.05$.

qPCR

For the real-time amplification, we used the SYBR® Green PCR Master Mix (ABI) kit with 0.2 to 0.6 μM of primers in a final reaction volume of 12 μl. The amplifications were performed in the 7500 Real-Time PCR System (Applied Biosystems) thermal cycler with the following protocol: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min (data collection). The specificity of each reaction was tested using dissociation curves with temperature variation from 65°C to 95°C, with an increase of 0.5°C every 15 s, with continuous fluorescence measurement. The amplifications were performed in triplicates, the negative reaction controls with no-template (NTC), were performed at each amplification to ensure the absence of reaction contamination.

The relative normalized expression calculation was determined by the 2-ΔΔCq method (41), which considers a 100% efficiency for the amplifications, confirmed by the primer efficiency test. Tbp and Rpl27 reference genes were used to normalize the reactions. The data were statistically compared using the Kruskal-Wallis test (non-parametric), followed by the Dunn *post hoc* test, using the Prism 5.01 software (GraphPad Software, San Diego, CA, USA).

Protein Expression and Purification

pET-28a_PPARγLBD (aa207-aa477), pET-28a_PPARγS237D, and pET-28a_PPARγS237A expression and purification was performed as previously described in (27). pET-15_NCoR (aa2059-aa2297) expression was performed in Escherichia coli BL21 (DE3) strain. Cells were growth in Luria-Bertani medium (LB), at 37°C, until OD600nm = 0.8 and were induced with 1mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) and 10μM ZnCl2, at 22°C for 16 h, 200RPM. Then, bacteria were harvested by centrifugation (20 min at 16,000 rcf at, 4°C), and the pellet was resuspended in lysis buffer (20 mM Tris–HCl pH 7.5, 300 mM NaCl, 5% glycerol, 2 mM β-mercaptoethanol, 100 mM PMSF and 1 mg lysozyme). After 1 h at 4°C, the extract was sonicated on ice bath and the soluble fraction was separated by centrifugation at 36,000 rcf, for 1 h at 4°C.

pGEX-2T_SMRT (aa2041-aa2359) expression was performed in modified Escherichia coli BL21(DE3) strain (42). Cells were growth in LB medium, at 37°C, until OD600nm = 0.88 and were induced with 1mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) at 18°C for 16 h, 200RPM. Then, bacteria were harvested by centrifugation (20 min at 16,000 rcf at 4°C), and the pellet was resuspended in lysis buffer (10 mM NaH_2PO_4 pH 7.4, 140 mM NaCl, 2.7 mM KCl, 1mM β-mercaptoethanol). After 1 h at 4°C, the extract was sonicated on ice bath and the soluble fraction was separated by centrifugation at36,000 rcf, for 1 h at 4°C. The supernatant was incubated with previously equilibrated Glutathione Sepharose 4B GST-tagged resin (GR Healthcare) for 3 h. After that, resin solution was transferred to a plastic column and flow through was collected. The resin was washed with (10 mM NaH_2PO_4 pH 7.4, 140 mM NaCl, 2.7 mM KCl, 1 mM β-mercaptoethanol) and fractions were eluted with elution buffer (60mM Tris pH 8.0, 10mM reduced glutathione, 1 mM β-mercaptoethanol).

The coactivators pET-28a_PGC1-α (aa138-aa341) and pGEX-2T_TIF2 (aa563-aa757), were expressed in in Escherichia coli BL21 (DE3) strain. Cells were growth in Luria-Bertani medium (LB), at 22° C, until OD600 nm = 0.8 and were induced with 1 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) for 16 h at 200RPM. Then, bacteria were harvested by centrifugation (20 min at 16,000 rcf at, 4°C), and the pellet was resuspended in lysis buffer (PGC1-α: 20 mM Hepes pH 7.4; 1 M NaCl; 2 mM β-mercaptoethanol; 80 ug lysozyme; 1 mM PMSF. TIF2: 20mM Hepes pH 8; 300 mM NaCl; 5% glycerol; 80 ug lysozyme; 1 mM PMSF). After 1 h at 4°C, the extract was sonicated on ice bath and the soluble fraction was separated by centrifugation at 36,000 rcf, for 1 h at 4°C. PGC1-α affinity purification was performed in TALON® Superflow™ histidine-tagged protein purification resin, the extract was incubated for 2 h, then eluted wit elution buffer (10 mM Tris-Cl pH 8; 10 mM reduced glutathione; 100 mM NaCl). TIF2 affinity purification was performed in previously equilibrated Glutathione Sepharose 4B GST-tagged resin (GR Healthcare) incubated for 16 h, then eluted in elution buffer (200 mM hepes pH 8; 300 mM NaCl; 5% glycerol; 10 mM reduced glutathione; 1 mM DTT). For our purpose, these two proteins did not undergo gel filtration purification.

Fluorescence Anisotropy Assay

Affinity purified coregulators were labelled with FITC (fluorescein isothiocyanate), in a proportion of 500 ul of coregulator/control affinity elution with 50 ul of 20mM FITC at 4°C for 3 h. The probe excess was removed by a desalting column (HiTrap, 5 ml, GE). To evaluate the affinities between coregulators and PPARγ, serial dilutions of purified PPARγ wild-type (wt) or S273A and S273D mutants (200 μM to 6 nM) were performed using the elution buffer of each coregulator (see Protein Expression and Purification section), in three replicates, in black 384-wellplates (Greiner). The coactivator conditions were incubated also with Rosiglitazone (3× molar excess). In order to measure any unspecific interaction, we performed the same experiment with control expressions of non-induced protein extracts. These extracts were incubated in GST and cobalt resins, labeled with FITC in the same proportion (50 ul in 500 ul of extract elution) and the affinity with PPARγ we and mutants were measured. For each fluorescence curve, the mixtures were submitted to fluorescence anisotropy measurements using ClarioStar® plate reader (BMG) (emission of 520 nm and excitation of 495 nm). Data were analyzed using the software

OriginPro 8.6 and Kd were obtained from fluorescence data fitted to binding curves using Hill model.

Pull-Down

To confirm PPARγ:Coregulators interaction, extracts of 2L of His tagged PGC1-α (aa138-aa341) and NCoR(aa2059-aa2297) protein expression were incubated with 300 ul of TALON® Superflow™ histidine-tagged protein purification resin (GE Healthcare) for 2 h in agitation. The same amount of GST-tagged TIF2 (aa563-aa757) and SMRT (aa2041-aa2359) extracts were incubated with 300 ul of Glutathione Sepharose® High Performance (GE Healthcare) for 16 h in agitation. After initial incubation, resins were washed with 3mL of lysis/wash buffer of each protein (previously described in session 2.6). Then, the resins contained tagged proteins were incubated with purified tag-free PPARγ (aa207-aa477) and PPARγS237A (aa207-aa477) for 2 h at 4°C in agitation. The conditions with the coactivators were added with rosiglitazone which was incubated with PPARγ and PPARγS237A for 20min before being incubated with the resin. After 2 h, the resins were washed with 3mL of lysis wash buffer proper to each coregulator protein. Then, they were eluted in 100 ul of elution buffer of each protein (previously described).

Western Blotting

To confirm pull-down formed complexes, 50ug of each eluted complex were electrophoresed on 12% polyacrylamide gels and transferred into nitrocellulose membranes (Amersham™ Protran®). Membranes were blocked with 3% skim milk in Tris-buffered saline containing 0.1% Tween-20 (T-TBS) for 4h, and then incubated for 16 h at 4°C with the primary antibodies followed by a 2 h incubation with secondary antibodies. Proteins were analyzed using anti-PPARγ (Cell Signaling #2050S), and anti Phospho-CDK Substrate Motif (Cell Signaling #9477).

In Vitro Phosphorylation Assay

CDK5 mediated phosphorylation of PPARγ and of the PPARγ complexes with PGC1-α, TIF2, SMRT and NCoR from pull down assays were measured by luminescent detection of ADP produced in the *in vitro* phosphorylation reaction, as it was described in (27, 28). We used ADP-Glo™ kinase assay (Promega) following manufacturer's instructions, in which 15 μM of purified PPARγ LBD and the pull down purified complexes PPARγ + PGC1-α, PPARγ + TIF2, PPARγ + SMRT and PPARγ + NCoR were incubated with 25 ng of purified CDK5/p35, at room temperature for 15 min, in the kinase assay reaction buffer (200mM Tris-HCl, pH 7.4, 100mM MgCl2 and 0.5 mg/ml BSA, SignalChem kinase assay buffer III) added 10 μM of ATP, in 12.5 μl of reaction volume. After the kinase reaction, ADP-Glo™ Reagent was added and the reaction was incubated at room temperature for 40 min. Then, the samples were denaturated at 95°C for 30 s. After this step, the Kinase Detection Reagent was added, and the samples were incubated at room temperature for 30min. Luminescence signal was recorded using GloMax-Multi + Detection System (Promega) microplate luminometer. Statistical analysis was performed with GraphPad Prism, by t-test, with values of p-values < 0.05.

RESULTS

The Absence of Phosphorylation Increases PPARγ Activation

To measure possible differences in the PPARγ activation due to S273 phosphorylation, we performed gene reporter assay comparing the activation of PPARγ wt, PPARγ S273A, a phosphorylation-defective mutant, and PPARγ S273D, a structural phosphomimic mutant. The lack of phosphorylation of PPARγ S237A and the phosphorylation of PPARγ co-transfected with CDK5 were confirmed by PPARγ immunoprecipitation, followed by western blotting against phosphorylated CDK-5 substrate analysis (**Supplementary Figure 1**). In addition, we measured the PPARγ wt activation in the presence of the CDK5, enzyme responsible for PPARγ S273 phosphorylation. The Rosiglitazone induced PPARγ activation in similar way for both wt and phosphorylated conditions (PPARγ wt, PPARγ S273D, and PPARγ + CDK5), presenting a fold activation of 115, 110, and 100, respectively (**Figure 1**). Interestingly, these results imply that the phosphomimic mutant behaves close to PPARγ wt inside the cells, in the presence and the absence of CDK5 (PPARγ + CDK5), validating the use of this mutant to mimic PPARγ phosphorylation situations. Moreover, PPARγ wt possibly is phosphorylated in this specific cellular assay conditions.

On the other side, PPARγ S273A mutant presented the highest absolute value of Rosiglitazone-induced activation among all the mutants (**Figure 1**); however, its activation fold was the lowest (90-fold). This lower activation ratio reflects the increased basal activation of this mutant (no treatment) that doubled in comparison to PPARγ wt basal activation. These results suggest that the inhibition of S273 PPARγ phosphorylation increases this receptor's activation, which may be associated to an enhanced

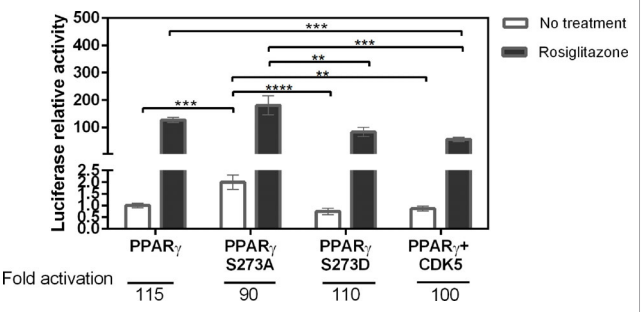

FIGURE 1 | Activation of PPARγ in different phosphorylation states. Transactivation assay with reporter gene in mammalian cells (Hek293T) was used to evaluate the activation profile of PPARγ wt and its mutants in the presence and absence of the Rosiglitazone. The PPARγ S273A mutant prevents the occurrence of phosphorylation, the PPARγ S273D mutant is a structural phosphomimic. The CDK5 enzyme is responsible for the phosphorylation of PPARγ in S273. It is possible to observe that phosphorylation prevention increases activation of PPARγ. Eight assays were performed in biological triplicate with n = 24. Statistical analysis: one-way ANOVA. p ≤ 0.01** p ≤ 0.001***; p ≤ 0.0001****. The phosphorylation inhibitor mutant had greater activation relative to the other conditions.

dissociation of corepressors and/or to an improvement on coactivators recruitment.

The Absence of S273 Phosphorylation Increases Both Coactivators Coupling and the Corepressors Dissociation

To evaluate if phosphorylation could increase coactivator and/or decrease corepressor interaction with PPARγ, we perform mammalian two-hybrid assays comparing PPARγ interaction with the selected coregulators (PGC1-α, TRAP220, TIF2, NCoR and SMRT). Firstly, we measured the PPARγ binding preferences with the chosen coregulators (**Figure 2**). The results show that within the coactivators, PGC1-α had the highest interaction with PPARγ (7-fold), followed by TRAP220 (4-fold). Interestingly, our construct of TIF2 did not presented significant changes in its interaction due to ligand responsiveness, suggesting low PPARγ binding due to agonist effect. Among the corepressors, both showed a similar dissociation rate, in the presence of the ligand, of 65% and 64% respectively for SMRT and NCoR. In addition, the initial interaction rate (No treatment) of SMRT is higher, suggesting a preferential binding to PPARγ.

Furthermore, we measured the coregulators interaction with PPARγ in various phosphorylation states. Despite having different interaction rates, TIF2 and PGC1-α coactivators (**Figures 3A, C**), presented similar interaction profile with the PPARγ wt, PPARγ S273A, and PPARγ S273D, both presenting higher interaction with phosphorylation-defective mutant (S273A). Moreover, the interaction with the phosphomimic mutant (S273D) presented similar behavior to the wt receptor, indicating that these coactivators binding are sensitive to S273 phosphorylation and suggesting an increased binding of these coactivators in absence of PPARγ phosphorylation. This interaction profile agrees with the activation profile seen in **Figure 1**, confirming that the lack of phosphorylation might increase coactivators binding. Nevertheless, the TRAP220 did not show interaction changes with the receptor in any phosphorylation state, suggesting that its binding to the receptor occurs independently of the PPARγ phosphorylation state (**Figure 3B**).

Regarding the corepressors, both were influenced by PPARγ dephosphorylation, as they presented the lowest interaction with S273A mutant (**Figures 3D, E**), and a decrease in dissociation ratio after Rosiglitazone addition. In contrast, the PPARγ wt and the S273D mutant presented similar interaction activity with corepressors, for both SMRT (**Figure 3D**) and NCoR (**Figure 3E**), opposite behavior observed for the coactivator's recruitment. Combined, these results confirm that the phosphorylation inhibition reduces the recruitment of NCoR and SMRT and, at the same time, increases the recruitment of PGC1-α and TIF2.

In addition, these PPARγ:coregulator interactions were confirmed by pull-down assays (**Figure 4A**). We used tagged coregulators protein, as the bait to purify excess of PPARγ and of PPARγS273A by affinity chromatography, generating PPARγ: coregulator complexes. Although very useful to confirm the existence of these complexes, this assay did not provide enough accuracy to quantify the differences in affinities between the four

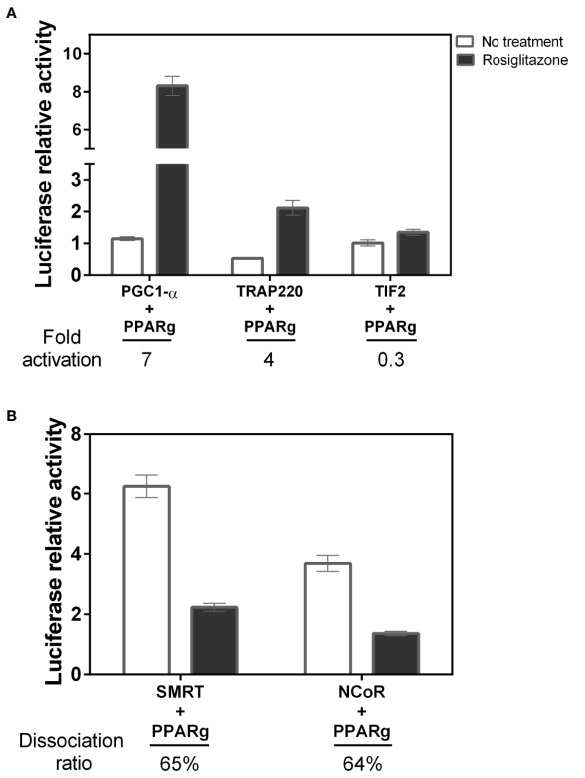

FIGURE 2 | Affinity of different coregulators with PPARγ. **(A)** The interaction with the PGC1-α coactivator was the highest among the coactivators studied, followed by TRAP220 that maintains the high activation due to the ligand. The TIF2 coactivator did not have a large increase in the presence of the ligand, suggesting low interaction after ligand binding. B) Among the corepressors the SMRT had a higher affinity than NCoR. Four assays in biological triplicate were performed to coactivators n=12, and three for corepressors CoRs n=9. **(B)** Among the corepressors the SMRT had higher affinity than NCoR Error bars, SEM. (n=9).

coregulators chosen and the different PPARγ phosphorylation states. However, qualitatively, it is possible to observe that PPARγ binds to all the coregulators in this assay, but the expression of these coregulators in *E. coli* system is variable in terms of protein content and different affinity comparisons are not possible to perform.

To confirm these differential interactions, we perform a fluorescence anisotropy assay within the coregulators that were responsive to S273 phosphorylation (**Figures 4B–E**). In this assay, coregulators were expressed in *E. coli*, purified by affinity column and labeled with FITC. Our results show that PGC1-α binds better to the S273A mutant (**Figure 4B**) (Kd = 46.9 ± 10) in comparison to S273D mutant (Kd = 153.5 ± 44.4, respectively), confirming our previous results (**Figure 3A**). TIF2 presented very low affinities to binding to all the PPARs, which is reflected by the low amplitude of the anisotropy binding curve and by Kds not determined because curves did not achieved saturation (**Figure 4C**), This result confirms that shown in two hybrid assays (**Figure 2A**). Besides this, a preference for the S273 mutant is suggested due to the binding curve shape. Both

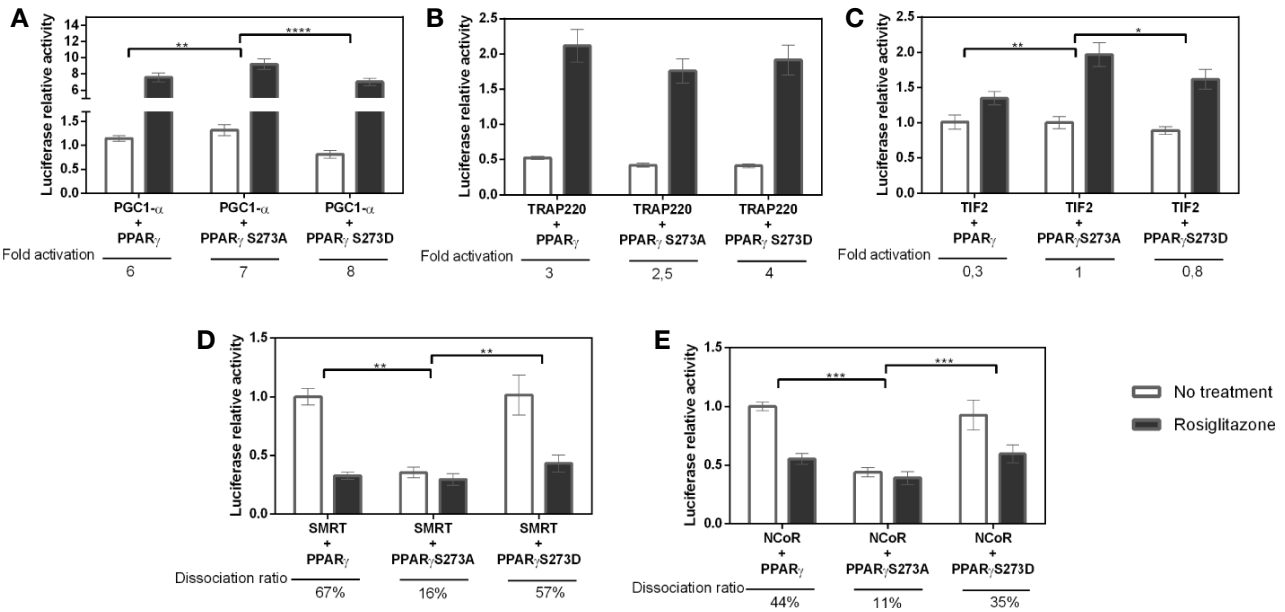

FIGURE 3 | Interaction between PPARγ and coregulators in different phosphorylation states. Interaction measured by mammalian two-hybrid assays were performed in COS-7 cells for **(A)** PGC1-α, **(B)** TRAP-220, and **(C)** TIF-2 coactivators and 239T cells for **(D)** SMRT and **(E)** NCoR corepressors. Error bars, SEM. (n=15) Statistical analysis: two-way ANOVA. P values: $p \leq 0.05^{*}$ $p \leq 0.01^{**}$ $p \leq 0.001^{***}$; $p \leq 0.0001^{****}$. The coactivators PGC1-α and TIF2 presented increased interaction with PPARγS273A mutant, while the corepressors presented decreased interaction with the same receptor mutant.

corepressors presented better affinities with phosphomimic mutant S273D (**Figures 4D, E**), and, as also shown in two hybrid assays, SMRT presented better affinity in comparison to PGC-1 (Kd = 4.06 ± 1.01 uM, and Kd = 55.8 ± 2.9 uM). Together, these data demonstrate strong binding preferences among PPAR mutants, which confirms our two-hybrid assays (**Figure 3**) results. It is important to mention that this is the first time that bigger constructions of coregulators were assayed in this kind of fluorescence assays, while the most common data about this kind of interaction is presented in the literature using the ID peptides of these molecules. Despite that, the Kds may not be compared to the found ones.

The Phosphorylation State Alters Adipogenesis Profile but Not Necessarily Coregulators Gene Expression

To investigate whether the differential coupling of coregulators is due to differential protein recruitment or to changes in coregulators gene expression, we performed gene expression analysis on differentiated 3T3L1 cells (**Figure 5**). The cells were treated with Rosiglitazone, PPARγ agonist, known for increasing its adipogenic capacity (43) and for PPARγ phosphorylation inhibition (20); with Roscovitine, a CKD5 inhibitor which has already been shown to significantly suppress CDK5-mediated phosphorylation, improving the expression of most of the genes regulated by PPARγ S273 phosphorylation (44, 45); and by both ligands. In this assay, the two compounds were used as a treatment during adipogenesis to evaluate whether CDK5 inhibition phosphorylation capacity would modify the expression profile, of the chosen coactivators and the adipogenic capacity of PPARγ.

First, we observed that adipogenesis were reduced in Roscovitine and Roscovitine+Rosiglitazone treatments, as it is shown in **Figures 5A, B.** Only Rosiglitazone effectively induced adipocyte differentiation, which is evidenced by the size of the lipid droplets coloured by Oil Red O, and by the Oil Red O absorbance measurements, suggesting that Roscovitine impairs white adipocyte (WAT) differentiation. As it was reported, Roscovitine can induce browning of adipose cells, turning the characteristic bigger lipid droplet in WAT in smaller and multiple lipid droplets that are usual in brown adipose tissue (BAT) (45).

The gene expression results confirm that the differences in PPARγ:coregulators interaction were not due differential availability of coregulators in different PPARγ phosphorylation state. Therefore, it confirms the hypothesis of differential interaction profiles that leads to differential activation. Among all the assayed coregulators, we observed a decreased expression of PGC1-α, while TIF-2, NCoR and SMRT kept the same expression rates in all the treatments. In another words, PGC1-α was the only coregulator downregulated by Roscovitine treatment, even when this compound was associated to Rosiglitazone. Interestingly, as it was previous shown, the PGC1-α is the PPARγ most recruited coactivator after Rosiglitazone treatment (**Figure 2A**), and, that this interaction increased in the absence of PPARγ phosphorylation (**Figure 3A**). However, CDK5 inhibition seems to decrease this gene expression, suggesting a fine regulation in this coactivator recruitment, which should be specific and strong enough to overpass the limiting expression rates of it.

Additionally, the other coregulators did not presented differences in gene expression rates in all the treatments,

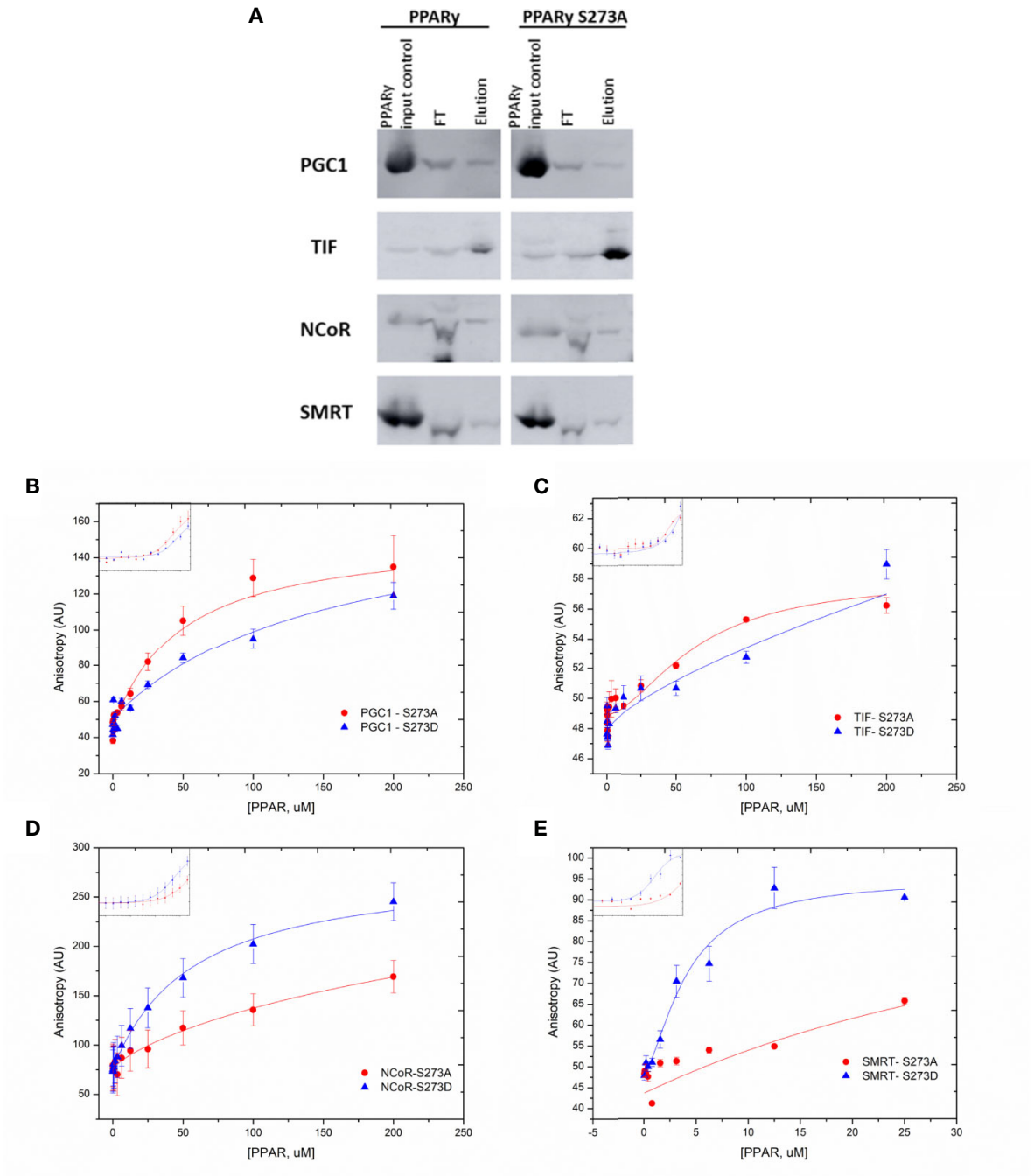

FIGURE 4 | Differential PPARg: coregulator interactions. **(A)** Western blotting analysis of PPARg: coregulator complexes. Tagged coregulators protein extracts were used as bait to bind PPARγ and PPARγS273A by affinity chromatography. The confirmation of complex formation is showed using an antibody against PPARγ in pull-down eluted samples. **(B–E)** Fluorescence anisotropy measurements obtained from the titration of PPARγ wt, S273A and S273D mutants into fluorescein-labeled coregulators. **(B)** PGC1-α anisotropy measurements. **(C)** TIF2 anisotropy measurements. **(D)** NCoR anisotropy measurements. **(E)** SMRT anisotropy measurements. The experimental controls and kd values are in **Supplementary Material**.

suggesting that, for TIF-2, NCoR and SMRT, changes in PPARγ binding, even in different PPARγ phosphorylation states, are probably caused by different interaction modes, and not due to increased or decreased availability of these proteins. Moreover, we also observed that the PPARγ regulated genes Cd36, Adipoq and Leptin were upregulated by rosiglitazone, while Adpsin was downregulated by Rosiglitazone + Roscovitine, and that TNF-α did not changed expression profile in all the treatments. These

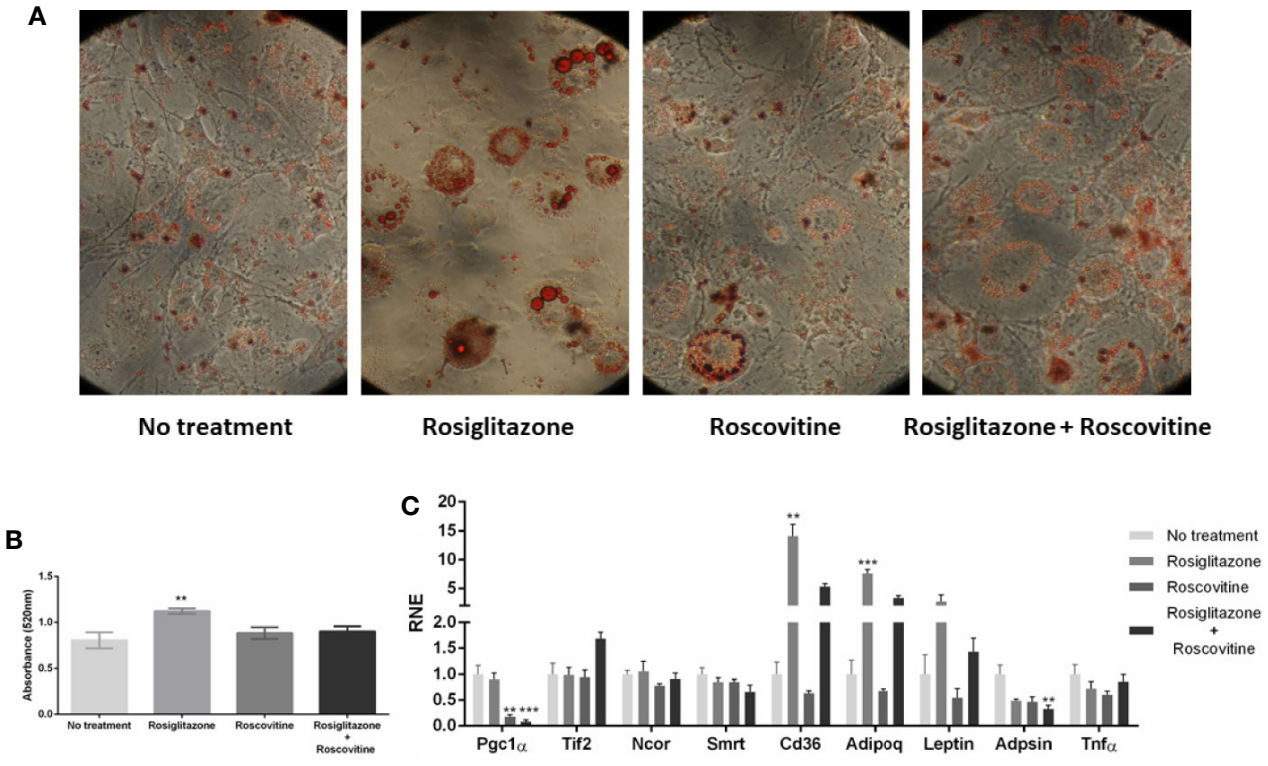

FIGURE 5 | Adipocyte differentiation in different states of PPARγ phosphprylation. **(A)** Images of the 8th day of treatment for differentiation into adipocytes. 3T3-L1 cells after 7 days of treatment with differentiation cocktail stained with Oil Red O in 40× lens. During the differentiation process, were added1 μM of Rosiglitazone, 10 μM of Roscovitine, or both treatments. **(B)** Absorbance measurement of differentiated cells into adipocytes. After each treatment, the cells were stained with Oil Red O and the absorbance was measured by a spectrophotometer. Statistical differences were measured by one-way ANOVA, comparing the different treatments, values of $p < 0.05$/** 0.001/*** 0.001. The treatment with Rosiglitazone showed greater absorbance, therefore a higher level of differentiation. **(C)** Gene expression of genes of the studied coregulators, and some of the PPARγ regulated genes that were reported be dysregulated by S273 phosphorylated state (Cd36, Adipoq, Leptin, Adpsin, and Tnf-α). The statistical analysis was performed by Kruskal-Wallis test (non-parametric), followed by the Dunn *post hoc* test comparing the untreated condition with each one of treated conditions. P values: $p \leq 0.01$**; $p \leq 0.001$***.

results suggest improved adipogenesis after agonist treatment (20, 43), phospho-protective effects against adipogenesis after Roscovitine treatment (45, 46), and no inflammation induced responses in all the conditions, as expected.

IDs Preferences for PPARγ-Coregulator Interaction

Additionally, to identify preferential binding of PPARγ to each coregulator ID, we performed mammalian two-hybrid assays with coregulators using wt and ID defective constructs of coregulators (**Figure 6A**), by mutating their active IDs. Hence, the coactivators IDs, which have the LXXLL motifs recognized as the ID, had their last two leucine replaced by alanine residues, resulting in the LXXAA motif. The corepressors domains had the IXX(V/I) motif modified by the substitution of isoleucine or valine residues for alanine, resulting in IXXAA motif.

Our searching for the preferential IDs for PPARγ wt - CoAs binding reveal a panel of ID binding preferences. Firstly, each TIF 2 ID contributes differently to the PPARγ interaction. The ID1 absence (**Figure 6B**) increased the interaction between TIF2 and PPARγ, indicating that its presence may be disrupting the binding of TIF2 to the PPARγ, possibly by competition between the IDs or unfavorable conformation of the coactivator structure when the ID1 is present (**Figures 6C, D**). The ID2 mutation (**Figure 6C**) does not altered the CoA-PPARγ binding, which means that this ID does not contribute for PPARγ-TIF2 interaction. However, the lack of ID3 (**Figure 6D**) drastically reduced the interaction with PPARγ, demonstrating that this ID possibly is the most important for PPARγ-TIF2 binding. Concerning PPARγ–PGC1-α binding, the mutation on the unique PGC1-α ID (**Figure 6E**) decreased the Rosiglitazone-induced interaction with PPARγ, as expected.

We also checked the preferential IDs in the PPARγ wt - CoR binding. Our results show that the lack of SMRT ID1 (**Figure 6F**) did not provoked any significant differences in the interaction with the receptor, as the efficiency of dissociation of this CoR in the presence of the ligand was also maintained. However, mutation of SMRT ID2 (**Figure 6G**) reduced the PPARγ-SMRT binding about 6-fold in comparison with SMRT wt, showing that this ID possibly is the most important in the

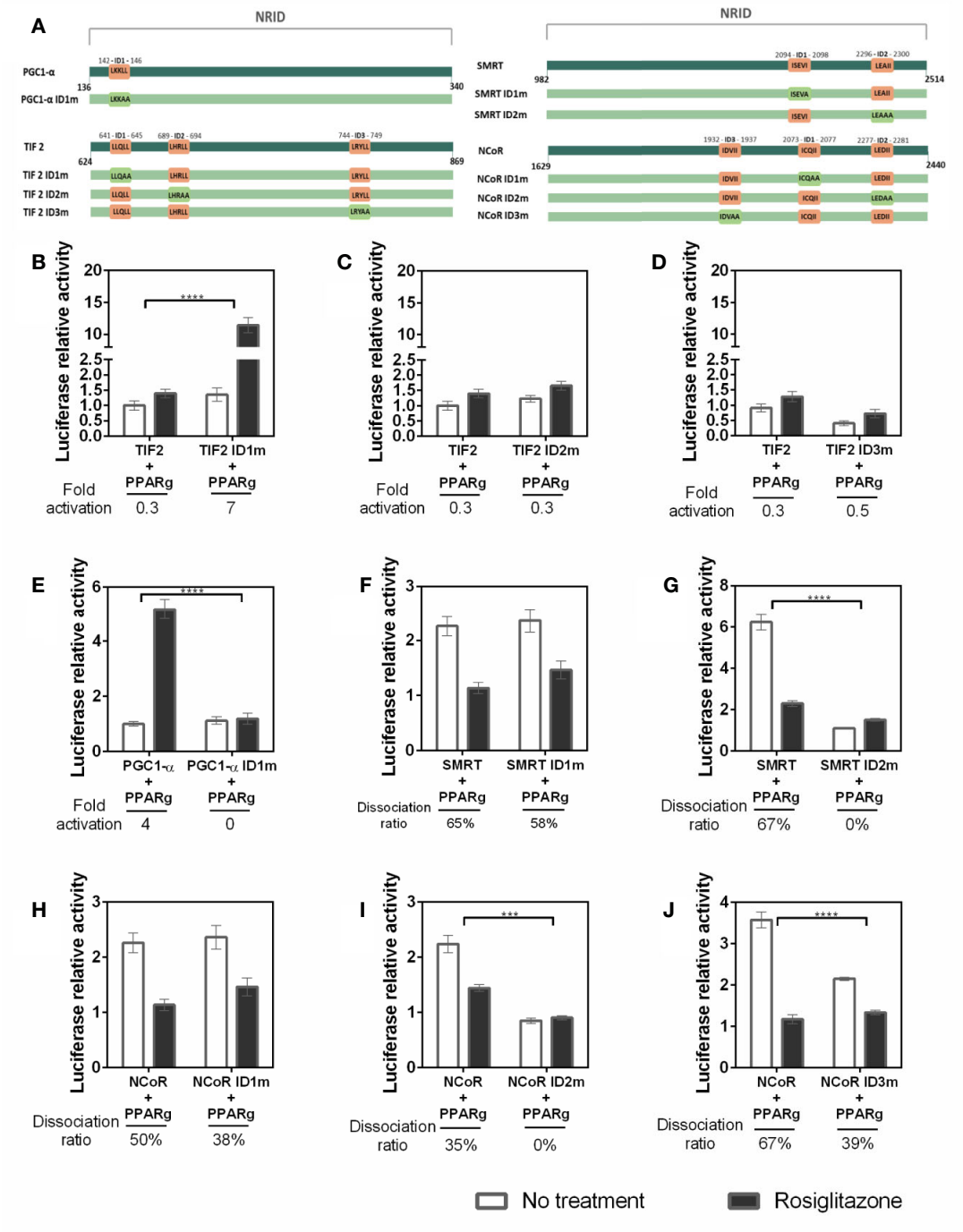

FIGURE 6 | Affinity of the coregulators IDs with PPARγ. **(A)** Representative image of the IDs in the sequences used and their respective mutations. In dark green are the original sequences, in light green are the mutated sequence. The original IDs sequence are in orange squares and the mutations on IDs are presented on light green squares. **(B–J)** Mammalian two hybrid assays were performed to evaluate whether the mutation on each interaction domain (ID) of the coregulators alters the interaction with PPARγ. **(B)** Comparison between interaction with TIF2 coactivator wt and PPARγ, and the ID1 of TIF2 mutated (TIF2 ID1m) and PPARγ. **(C)** ID2 of TIF2 coactivator mutated. **(D)** ID3 of TIF2 coactivator mutated. **(E)** ID1 of PGC1-α coactivator mutated. **(F)** ID1 of SMRT corepressor mutated. **(G)** ID2 of SMRT corepressor mutated. **(H)** ID1 of NCoR corepressor mutated. **(I)** ID2 of the NCoR corepressor mutated. **(J)** ID3 of the NCoR corepressor mutated. Error bars, SEM. (n = 15) Statistical analysis: two-way ANOVA. p-value: P values: $p \leq 0.001$***; $p \leq 0.0001$****. For the TIF coactivator the withdrawal of ID1 improves the interaction, in this case we can say that ID1 interferes with the interaction PPARγ-TIF2 and ID3 of the same coactivator is the most important for the interaction. In the case of PGC1-α, we only have 1 ID and when it is not present the interaction is broken. For SMRT and NCoR, ID 2 is important, ID1 does not change the interaction and ID3 of NCoR seems to contribute to the interaction with PPARγ.

PPARγ-SMRT interaction. For NCoR, the lack of ID1(**Figure 6H**) also did not significantly change its interaction with PPARγ, as it was observed for SMRT, but the NCoR ID2 absence (**Figure 6I**) abolished the PPARγ-NCoR interaction, pointing to the importance of this ID in the corepressor-receptor interaction, as it was also seen for SMRT. Finally, the absence of NCoR ID3 (**Figure 6J**) decreases the PPARγ-NCoR interaction, but the reduction found was lower than the found for ID2, suggesting that both ID2 and ID3 contributes in the PPARγ-NCoR interaction, but ID2 is likely the most important one.

The IDs Preferences for PPARγ Binding Change Due the Phosphorylation State

To evaluate whether the PPARγ S273 phosphorylation state modifies the PPARγ-coregulators interaction profile we also performed the mammalian two hybrid assays with the PPARγ S273 mutants and coregulators with IDs mutants. Our results show that changes in TIF2 IDs (**Figures 7A–C**) presented considerable variation in the interaction with the different PPARγ phosphorylation states. The absence of ID1 (**Figure 7A**) increased the responsiveness of PPARγ wt to the Rosiglitazone ligand (as it was shown in **Figure 6B** and in the first bar of **Figure 7A**). However, when the phosphorylation is inhibited (PPARγ S273A) the PPARγ-TIF2 interaction decreased, and, in the phosphorylation-mimicking condition (PPARγ S273D) no significant differences between PPARγ wt was observed. Inversely, the absence of ID2 (**Figure 7B**) increased the interaction of TIF2 with the receptor when the phosphorylation is inhibited (PPARγ S273A) and decreased this interaction with the PPARγ wt and in the phosphorylation mimetic receptor (PPARγ S273D). Mutation on ID3 of TIF2 dramatically decreased receptor interaction under all conditions (**Figure 7C**). Together, these indicate that the TIF2 ID3 is the most important for the PPARγ interaction, and IDs 1 and 2 are affected by S273 phosphorylation. ID1 may be important to help in the protein-protein interaction for non-phosphorylated

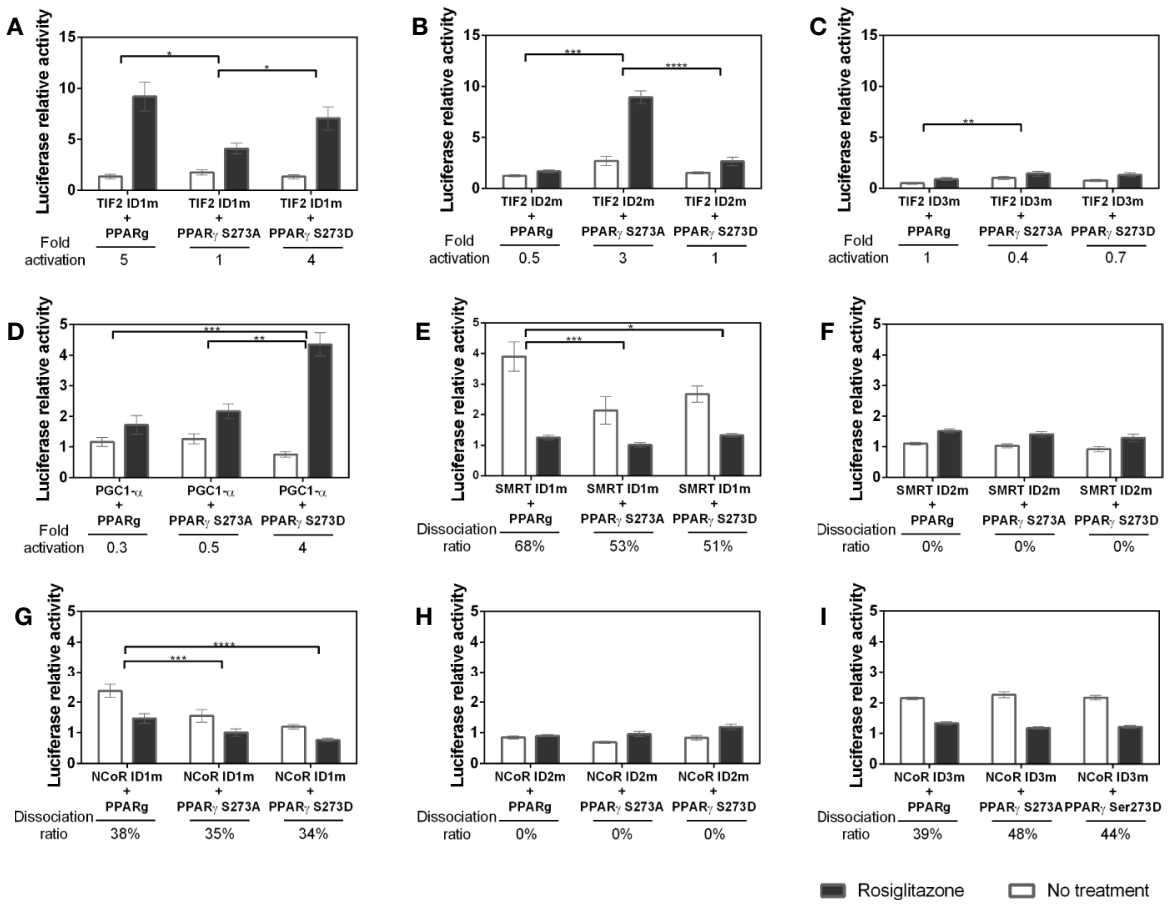

FIGURE 7 | Interaction between coregulators and PPARγ in different phosphorylation states. Mammalian two hybrid assays were performed to evaluate whether the S273 mutation in the receptor interferes with its interaction with the coregulators. The PPARγ S273A mutant prevents the occurrence of phosphorylation and the PPARγ S273D mutant is a phosphomimic. **(A–C)** Interaction between TIF2 mutants and PPARγ in different phosphorylation states. **(D)** Interaction between PGC1-α mutant and PPARγ in different phosphorylation states. **(E, F)** Interaction between SMRT mutants and PPARγ in different phosphorylation states. **(G–I)** Interacton between NCoR mutants and PPARγ in different phosphorylation states. Error bars, SEM. (n = 15) Statistical analysis: two-way ANOVA. P values: $p \leq 0.05$*; $p \leq 0.01$**; $p \leq 0.001$***; $p \leq 0.0001$****.

PPARγ, and the ID2 may be important for the phosphorylated PPARγ interactions.

The lack of ID1 in PGC1-α (**Figure 7D**) show similar interaction of this CoA with PPARγ wt and PPARγ S273A. However, the phosphorylation (PPARγ S273D) substantially increased the interaction with PPARγ in the presence of ligand, unveiling that this coactivator may bind to an additional region of the receptor uniquely when it is phosphorylated.

The mutation of SMRT ID1 presented decreased interaction with both conditions of PPARγ mutants (**Figure 7E**). This suggests that the structural changes provoked by S273 affect the interaction with this ID. The ID2 mutation (**Figure 7F**) decreased the interaction between PPARγ and SMRT in all states of phosphorylation. This profile was already observed in **Figure 6G** and are consistent with other studies that demonstrate that this is the most important ID for receptor interaction (47, 48). Moreover, no significant difference was observed between the mutation of this ID and PPARγ phosphorylation.

NCoR ID1 mutation (**Figure 7G**) was also able to reduce the interaction with both mutants, S273A and S273D. Mutation on ID2 (**Figure 7H**), as the SMRT ID2m, presented the lower interaction with PPARγ in all conditions. The result shows that there is a reduction in the interaction between NCoR with inactive ID2 independent of the state of receptor phosphorylation, but due to the PPARγ preference for binding *via* this ID. The ID3 mutation (**Figure 7I**) showed no difference due the phosphorylation state, which indicates that this ID is irrelevant in the interaction corepressor-receptor due to phosphorylation/dephosphorylation of PPARγ.

CDK5 Modifies PPARγ-Coregulator Interaction

Finally, to evaluating the preferential coregulators IDs for PPARγ binding and the changes in this preference caused by receptor's phosphorylation state, we performed some assays in the presence of CDK5, to check if this enzyme would modify the interaction profile with the different coregulators. These assays allow us to estimate what occurs in the cell at the beginning of phosphorylation, while in the previous assays, using S273 mutants, we evaluate the result of phosphorylation in the PPARγ-coregulators binding.

Our results show that PGC1-α, TIF2, and NCoR assays (**Figures 8A, C, D**, respectively) decreased receptor interaction in presence of CDK5. The PGC1-α-PPARγ decreased from 5-fold in absence of CDK5 to 2-fold. TIF2 decreased PPARγ binding from 1.5-fold to 0.7-fold, indicating that the interaction with the receptor was missed, and NCoR interaction decreases from 4 to 2-fold. Meanwhile, the SMRT corepressor (**Figure 8E**) displayed opposite behavior, increasing interaction with PPARγ in the presence of CDK5, indicating that

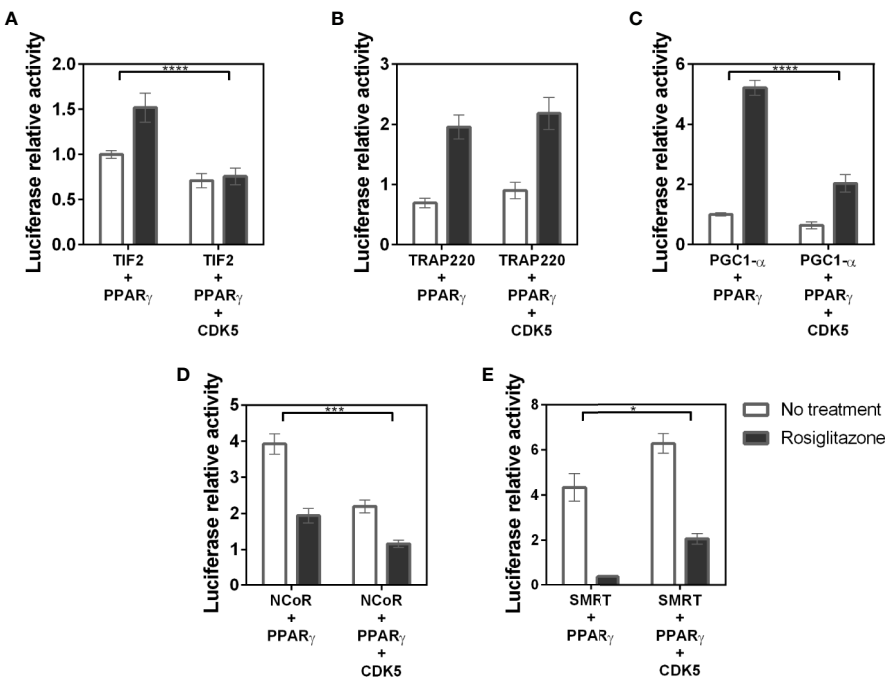

FIGURE 8 | Interaction between coregulators and the PPARγ receptor in the presence of the CDK5 enzyme. Mammalian two hybrid assays to evaluate if the presence of the CDK5 enzyme, responsible for the phosphorylation of S273 in the receptor, interferes with PPARγ:coregulators interaction. **(A)** Interaction of the TIF 2 coactivator with PPARγ in the absence and presence of CDK5. **(B)** Interaction of the TRAP220 coactivator with PPARγ in the absence and presence of CDK5. **(C)** Interaction of the PGC1-α coactivator with PPARγ in the absence and presence of CDK5. **(D)** Interaction of the NCoR corepressor with PPARγ in the absence and presence of CDK5. **(E)** Interaction of the SMRT corepressor with PPARγ in the absence and presence of CDK5. Error bars, SEM. (n = 15) Statistical analysis: two-way ANOVA. P values: $p \leq 0.05$*; $p \leq 0.001$***; $p \leq 0.0001$****. PGC1-α, TIF2 and NCoR showed dissociation of the receptor in the presence of CDK5 while SMRT increased the association with the receptor.

the enzyme may play some roles as PPARγ-corepressor coupling, as previously suggested (31). Interestingly, for TRAP220, the CDK5 presence did not change the PPARγ-coactivator interaction, as it was shown for the PPARγ mutants. All these results allow us to infer that the enzyme may alter the interaction profile by competing or coupling coregulators to the PPARγ binding site, depending on the coregulator, and that TRAP is not affected by PPARγ phosphorylation.

Still to confirm that CDK5 presence disturbs the PPARγ interaction with coregulators we perform *in vitro* phosphorylation assay with heterologous expressed PPARγ, and PPARγ-coregulators complexes formed in the pull-down assays (**Figure 9**). The phosphorylation of PPARγ by CDK5 was used as the control, set up as 100% of phosphorylation, and the increase or decrease of the PPARγ phosphorylation due to coregulator presence was compared with this condition. Our results show that PPARγ:SMRT complex presented an increase of 164% in phosphorylation rate, confirming our cellular assays (**Figure 8E**) that showed that CDK5 presence increases SMRT interaction with PPARγ. Moreover, as also shown in our cellular assays, the other three complexes presented reduced interaction in CDK5 presence, been PPARγ:PGC1-α complex the one which presented the major interaction disruption, decreasing 52% when added CDK5 in the system. In addition, TIF2 presented the lower interaction difference (11%), possibly due to its weak interaction with PPARγ even in absence of CDK5. PPARγ:NCoR complex presented a 17% of reduction of phosphorylation rate, indicating that NCoR may compete with CDK5-PPARγ for docking.

DISCUSSION

Previous studies have reported that Ser273 phosphorylation of PPARγ LBD is related to obesity-induced development of insulin resistance (14, 20, 21). A key question to understand the mechanisms of action of this pathway is to elucidate how this phosphorylation influences the PPARγ activation. Our results showed that both phosphorylation status and CDK5 presence can indeed alter the PPARγ activation (**Figure 1**). Moreover, our results show that these differences on activation are due the differential interaction with coregulator proteins (**Figures 2**–**4**).

As it is well known, the formation of protein-protein complexes and subsequent transcriptional regulation is completely dependent on the structure (49, 50). PTM-dependent interactions occur through structural changes that creates binding sites for a range of IDs (51). Our results showed the PPARγ binding to coregulators occurs and presented different preferences of binding (**Figures 3** and **4**), that may be modified by phosphorylation. Additionally, our results show that these binding preferences dependent of PPARγ phosphorylation state is not due to differential expression of the coregulators or

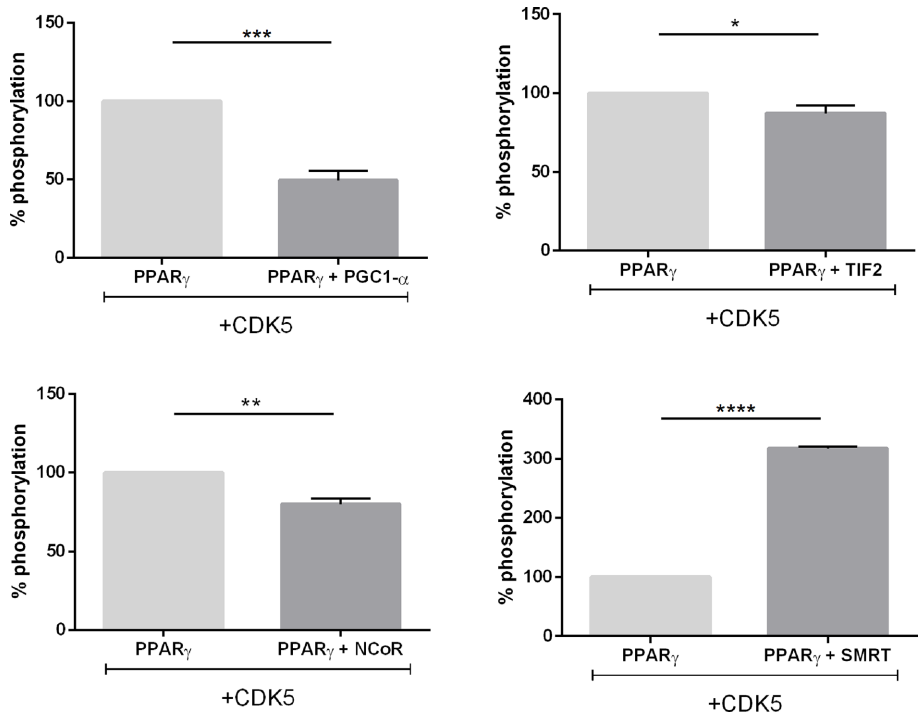

FIGURE 9 | In vitro phosphorylation assay. Luminescence signal produced as consequence of the ADP production *in vitro* reaction containing CDK5/p35 kinase, ATP and PPARγ, and the complexes with coregulators. All the luminescence signals were normalized by PPARγ condition which is 100% of phosphorylation. Error bars, SEM, (n = 3). Statistical analysis: one-way ANOVA. P values: $p \leq 0.05$*; $p \leq 0.01$**; $p \leq 0.001$***; $p \leq 0.0001$****. The complex PPARγ + SMRT presented increased luminescence while the other three complexes presented decreased luminescence.

guided by increased availability of a determined coregulator when phosphorylation is suppressed (**Figure 5**). On the contrary, the decreased expression of PGC1-α when phosphorylation is inhibited did not change the higher preference of the receptor for this coactivator (52, 53).

Through cellular assays, we demonstrate that the coactivator TRAP220 was not responsive to Ser 273 phosphorylation nor to the presence of the CDK5 enzyme (**Figures 3** and **6**). One possible explanation for this lack of responsiveness is that, although it has 3 different IDs, this coactivator probably binds to PPARγ only by the canonical interface formed by PPARγ H12 relocation and H3, H4 and H5, without any other additional interaction. Thus, neither phosphorylation, nor CDK5 presence affect the opposite face of the receptor, not affecting the receptor-coactivator interaction. However, both TIF2 and PGC1-α coactivators exhibited a different behavior, presenting higher interaction with PPARγ in the phosphorylation inhibited state (**Figure 3**).

Additionally, PGC1-α, which is known as PPARγ's preferred coactivator (52, 53), showed preferential binding to PPARγ wt by its unique ID (**Figure 6E**). Moreover, this coactivator shows to make additional contacts with the receptor in the phosphorylated state, as the deletion of ID1 increased the interaction between the PGC1-α and PPARγ S273D (**Figure 7D**). Possibly, this contact may be mediated by an additional and inverted LXXLL motif that exists between amino acids 210 to 214 of PGC1-α, which has been shown to interact with other NRs, such as ERRα (54) and is called L3. Despite it is well known that the main PGC1-α ID with most NRs is the ID corresponding to L2 (aas 144-149, here called ID1), our results show that when the strongest ID is inactivated, other motifs, as L3 becomes to anchor to the PPARγ, but only if the S273 is phosphorylated. Nevertheless, the existence of this phosphorylation-responsive interaction might explain the decreased interaction of PGC1-α wt with the phosphomimic mutant PPARγ S273D (**Figure 3A**). In this case, phosphorylation would increase the affinity of L3 motif for the receptor, generating a competition between L2 (or ID1) and L3 motifs, which, for structural reasons, cannot bind at the same time to the receptor, weakening the interaction that was previously made only *via* ID1-H12. This possibly occurs through the CDK5-PGC1-α competition on the PPARγ coupling site. Interestingly, the decreased PGC1-α expression in adipose tissue when such phosphorylation occurs is associated with increased insulin resistance (55, 56).

Interestingly, TIF2, which did not present high preference to bind PPARγ (**Figure 2** and **4C**), was also responsive to phosphorylation. Its role in regulating adipose tissue homeostasis, and its expression appears to be linked to increased insulin resistance in mice (57). Our results show that it binds to PPARγ canonically *via* ID3 (**Figure 6D**), however its other IDs are responsive to phosphorylation in opposite manners. According to our data, while ID1 seems to bind better when the phosphorylation is inhibited (**Figure 7A**), ID2 seems to bind better to the phosphorylated receptor (**Figure 7B**). This exchange of interaction interfaces with the receptor due to its phosphorylation state might induces exposure of different interaction surfaces to factors in the transcription activation/repression complex and may lead to different metabolic responses. This type of modular protein IDs is used by the cells as a broad device to decode and respond to the state of its protein, with different IDs, being dedicated to the selective recognition of distinct PTMs (51).

Concerning corepressors and IDs interaction profile, NCoR and SMRT presented some similar behavior. Interestingly, our results showed that there are differences in the IDs recruitment depending on the corepressor. This difference may be explained due to the different mechanisms of binding of the ID1, ID2 and ID3 to the receptor, related to the variants on IDs motifs which are LXXXIXX (V/I) IXXX (Y/F), LXXIIXXXL, and IXXIIXXXI, respectively (37, 58). Each of them has its own particularities on receptor binding. The ID2 for example, attach to PPARα by adopting an irregular three turn helix that fits tightly into a receptor groove formed by open conformation of H12. In this case, this surface can also act as a coactivator binding site (59). Both corepressors showed strongest interaction with PPARγ *via* ID2, corroborating with previous studies that demonstrate the importance of this ID to PPARγ interaction (48). On the other side, both ID1 seems to have little or no interaction with PPARγ. However, NCoR ID3 appears to be responsive to phosphorylation, as the lack of ID1 decreased the PPARγ binding in phosphorylated and no phosphorylated state, and the absence of ID3 did not respond to phosphorylation (**Figure 7**). This NCoR ID3 response to phosphorylation suggests that possible alternative contacts might be formed between this NCoR ID and the S273 region, as the S replacement for A or D amino acids might provoke particular conformational modifications in PPARγ structure. Interestingly, although the used isoform of SMRT does not have the ID3, the same responsiveness to the phosphorylation was observed, since the lack of ID1 also decreased PPARγ interaction when S273 is mutated.

Furthermore, our results revealed that the CDK5 presence also disturbs the PPARγ-coregulators interaction in different ways. Possibly the CDK5 has some coupling interface with PPARγ that overlaps the interaction interface with the coregulators, as it seems to compete with TIF2, PGC1-α, and NCoR (**Figure 8**). However, the interaction of PPARγ with SMRT is increased in the presence of CDK5, suggesting that, in this case, it is somehow coupling this corepressor, also through interaction interface intersection. These results were confirmed by *in vitro* phosphorylation assays were the complexes TIF2:PPARγ, PGC1-α:PPARγ, and NCoR:PPARγ presented increased ADP activity and SMRT:PPARγ presented the opposite profile (**Figure 9**).

This study adds details to the mechanisms of obesity induced by PPARγ phosphorylation. Our data not only confirm that the coregulators' interaction profile could change due this phosphorylation (30, 31), but also show that this PTM could lead to new interactions sites within coregulators:PPARγ and coregulators:CDK5. A better understanding of this mechanism of action opens new pathways for anti-diabetic drug development. Previous studies show that there is a range of

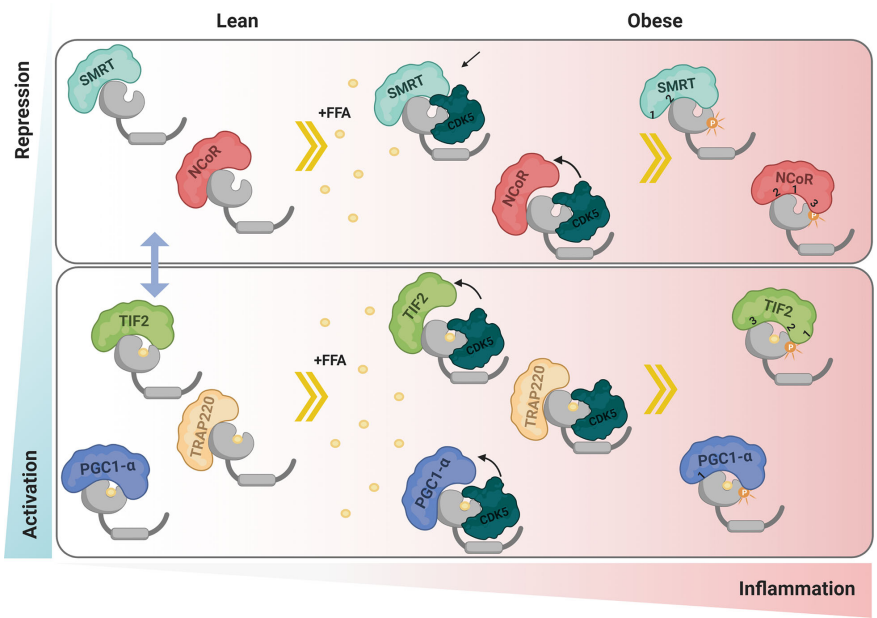

FIGURE 10 | Proposed interaction mechanism. In lean adipose tissue the mechanism of interaction with coactivators and corepressors is in equilibrium, represented by the blue arrow. Under conditions of obesity, free fatty acids and other inflammatory factors act by activating the enzyme CDK5 that phosphorylates PPARγ. The presence of CDK5 generates an imbalance in the coregulators homeostasis, increasing the interaction of PPARγ with SMRT while decreasing with NCoR, PGC1-α and TIF2. Ser273 phosphorylation performed by CDK5 also modulates the interaction with coregulators. Both corepressors canonically bind *via* ID2-H12, and respond to modification in Ser273, both in the absence and presence of phosphorylation. PGC1-α, although interacting more strongly with the receptor *via* ID1, showed to make additional contact in a region near Ser273 that is favored in the presence of the ligand. TIF2 binds to H12 *via* ID3, however ID2 seems to interact better in the absence of phosphorylation and ID1 seems to interact better in the phosphorylation condition. TRAP 220 does not make contact near Ser 273, so it was not responsive to either phosphorylation or the presence of CDK5. Red represents the intensity of inflammation in adipose tissue. Blue represents levels of PPARγ activation due to interaction with the coregulators. The numbers 1, 2, and 3 represents the IDs (Created with BioRender.com).

molecules that can bind to PPARγ preventing Ser273 phosphorylation, without cause the high activation characteristic of strong agonists (20–22, 27) and these results opened a new target possibility, the PPARγ:coregulator interaction. Inhibitors of this interaction can act either by binding to the binding groove formed by the IDs or by binding to the receptor's H12 (60). Moreover, our results showed that in addition to these interaction sites, other unusual regions may have their interaction induced by the PPARγ phosphorylation state, further opening the range of possibilities for the new molecules searching.

Based on our results, we build a panel of possible PPARγ: coregulators interactions in different phosphorylation states (**Figure 10**). In summary, we showed that the phosphorylation inhibition increases PPARγ activation through higher interaction with PGC1-α and TIF2 coactivators and decreased interaction with SMRT and NCoR corepressors. The coregulators mutation assays results provide us insights to elucidate the importance of phosphorylation for the different coregulators anchorages possibilities. In particular our results show that the PGC1-α has been shown to make additional non-ID mediated contact with PPARγ in the region near Ser273. The ID3 of TIF2 coactivator seems to be the most important for canonical binding *via* H12 and IDs 1 and 2 make some contacts in the region near Ser273, depending on the phosphorylation state. Both tested corepressors showed that ID2 is the most important for the canonical interaction with PPARγ. However, ID1 is important in cases where modification of receptor S273 occurs, regardless of the receptor phosphorylation state. Finally, we have shown that the presence of CDK5 disrupts interaction with PGC-α, TIF2, and NCoR, probably through competition for the coupling site. Meantime, the interaction with SMRT is increased in this condition. These two different profiles of interaction indicate that the presence of CDK5 imbalance the coregulators natural activity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AF designed the research and article and revised the article. MD, TT, FB, HR, FT, AO and LS performed the research. AM provided essential material, discussed results and methodology. MD and AF wrote the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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Unorthodox Transcriptional Mechanisms of Lipid-Sensing Nuclear Receptors in Macrophages: Are We Opening a New Chapter?

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Work over the past 30 years has shown that lipid-activated nuclear receptors form a bridge between metabolism and immunity integrating metabolic and inflammatory signaling in innate immune cells. Ligand-induced direct transcriptional activation and protein-protein interaction-based transrepression were identified as the most common mechanisms of liganded-nuclear receptor-mediated transcriptional regulation. However, the integration of different next-generation sequencing-based methodologies including chromatin immunoprecipitation followed by sequencing and global run-on sequencing allowed to investigate the DNA binding and ligand responsiveness of nuclear receptors at the whole-genome level. Surprisingly, these studies have raised the notion that a major portion of lipid-sensing nuclear receptor cistromes are not necessarily responsive to ligand activation. Although the biological role of the ligand insensitive portion of nuclear receptor cistromes is largely unknown, recent findings indicate that they may play roles in the organization of chromatin structure, in the regulation of transcriptional memory, and the epigenomic modification of responsiveness to other microenvironmental signals in macrophages. In this review, we will provide an overview and discuss recent advances of our understanding of lipid-activated nuclear receptor-mediated non-classical or unorthodox actions in macrophages.

Keywords: macrophage, lipid sensing nuclear receptors, ligand-insensitive role of nuclear receptor, nuclear receptor cistrome, epigenetic regulation

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INTRODUCTION

A generally accepted paradigm in endocrinology has been over the last 30 years that if not the sole, but the main function of lipid-sensing nuclear receptors is to translate microenvironmental chemical cues into distinct biological effects through tight regulation of gene transcription. In the 1980s, the cloning of the first nuclear receptors including the glucocorticoid and estrogen receptors was followed by the identification of dozens of evolutionarily related proteins and initiated the development of this concept. Although at the time the newly identified proteins showed similar domain structure containing DNA binding, ligand binding, and transactivation domains, initially

the nature of their endogenous chemical ligands was unknown. Therefore, these proteins were called ″orphan″ receptors. The approach termed ″reverse endocrinology″ (having the receptor first and looking for the ligand later) allowed the discovery of dietary lipid-derived, often lower-affinity endogenous ligands, for many of them including liver X receptors (LXRs) and peroxisome proliferator-activated receptors (PPARs) (1, 2). Since the identification of their first endogenous lipid ligands, several natural and synthetic nuclear receptor activity modifier molecules including agonists, antagonists, partial agonists, and inverse agonists have been discovered. This chemical toolbox helped us to learn more about the transcriptional basis of lipid-sensing nuclear receptor actions and the functional consequences of their ligand activation in various cell types including macrophages under different physiological and pathological conditions (3–5). Beyond the classical transcriptional effects, it has been demonstrated that nuclear receptors may have non-genomic activities. Various orphan and lipid sensing nuclear receptors can also be located in the cytoplasm and in the plasma membrane often in lipid rafts. The extranuclear localization of nuclear receptors not only influences transcription through the modulation of the receptor's availability in the nucleus but also control various biological processes interacting with other signal transduction pathways (6–9). We will not cover these activities in this overview. Importantly, until the end of the 2000s, the applied methods were not particularly suitable to thoroughly investigate the receptors in an unbiased fashion and discover potential nonclassical, but nuclear and chromatin associated functions of lipid sensing nuclear receptors. This is because the widely used approaches were ligand activation centric and biased and researchers were looking for ligand-induced changes with the goal to identify regulated genes. This was a significant bias and severely limited the scope of investigations. The spread of next-generation sequencing-based epigenomic and transcriptomic technologies has changed all this and given new impetus to nuclear receptor research resulting in comprehensive genome-wide maps and led to the identification of a ligand-independent and novel ligand-directed transcriptional regulatory roles of lipid-sensing nuclear receptors. In this review, we will summarize this voyage and our current understanding about nonclassical transcriptional regulatory actions of lipid-activated nuclear receptors including PPARs and LXRs in macrophages. We chose to focus on macrophages due to the remarkable plasticity of this cell type and the relatively large amount of genome-wide and functional analyses available (10–12).

TRANSCRIPTIONAL BASIS OF MACROPHAGE HETEROGENEITY AND PLASTICITY

Macrophages play an essential role in the maintenance of normal tissue homeostasis and the protection against different pathogen infections but also participate in different human diseases including atherosclerosis, cancer, and obesity. Phenotypic and functional features of macrophages are tightly determined by the combination of their developmental origin and tissue microenvironment (13, 14). Many tissue-resident macrophage-subsets including brain, liver, lung, and kidney macrophages are derived from fetal progenitors and produce self-renewing populations, while intestinal and dermal macrophages are continuously replenished by bone marrow-derived monocytes. Monocyte-derived macrophages are infiltrating and thus observed at sites of injury and inflammation. Both functional heterogeneity and polarization of tissue-resident and monocyte-derived macrophage subsets are precisely regulated by their complex molecular microenvironment (15, 16). Two extreme endpoints of macrophage polarization are Th1-type cytokine interferon-gamma (IFNγ) or Gram-negative bacteria-derived lipopolysaccharide (LPS)-induced classical [M(IFNγ) or M(LPS)], and Th2-type cytokines interleukine-4 (IL-4) or IL-13-promoted alternative [M(IL-4) or M(IL-13)] polarization. Classically polarized macrophages have inflammatory properties and high antibacterial activity, while alternative macrophage polarization is associated with anti-inflammatory features supporting protection against nematode infections and tissue regeneration. However, numerous transient macrophage polarization states are identified *in vitro* and *in vivo* which can be switched depending on changing microenvironmental milieu (14, 16, 17).

Macrophage identity and response to changing molecular milieu require strict regulation of their gene expression program at the transcriptional level through complex and well-organized collaboration between genomic regulatory regions and DNA-binding transcription factors (TFs) (10, 18). Gene-proximal promoters and distal regulatory elements (so-called enhancers) are associated with characteristic and partially distinct covalent post-translational histone modification patterns and contain several transcription factor-specific DNA motifs. Promoters are marked by H3K4m3, while enhancers exhibit high levels of H3K4m1 and H3K4m2. Besides, both regulatory elements are associated with H3K27Ac following their activation and H3K27m3 in a repressed state (19–21). The available enhancer repertoire is of great importance to specify the identity of the macrophage lineage and is primarily determined by the collaborative binding of general macrophage-specific lineage determining transcription factors (LDTFs) such as PU.1, AP-1, and CEBPβ. The complex interaction between these LDTFs results in chromatin opening, enhancer activation, and new loop formation between promoters and enhancers leading to the formation of macrophage-specific enhancer repertoire (11, 22). Intriguingly, recent studies have raised the possibility that additional transcription factors including GATA6, SALL1, and nuclear receptors can also act as LDTFs participating in the determination of tissue-specific enhancer sets in various tissue-resident macrophages (23–28).

The macrophage subset-specific enhancer repertoires serve as a binding platform for the signal-dependent transcription factors (SDTFs). Many microenvironmental signals including pathogen-derived molecules, cytokines, and lipids can activate SDTFs turning on signal-specific gene expression programs (29–32). Toll-like receptor (TLR) ligands such as LPS and poly(I:C) as well as

tumor necrosis factors (TNFs) activate nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and Activator protein 1 (AP-1) transcription factor complexes initiating a transcriptional program of the inflammation (33, 34). Various cytokines can activate different members of the signal transducer and activator of transcription (STAT) transcription factor family. Each member of the STAT family binds to different DNA motifs and regulates different gene sets leading to the emergence of distinct macrophage polarization states including IL-4-STAT6 signaling pathway-induced alternative and IFNγ-STAT1 axis-activated classical macrophage polarization (35). Finally, the lipid microenvironment can also directly control the gene expression at the transcriptional level by activation of lipid-sensing nuclear receptors influencing macrophage metabolism and inflammation (3–5). Recently, many *in vitro* and *in vivo* pieces of evidence indicate that different microenvironmental signals can interact with each other at the epigenomic level affecting genome-wide chromatin accessibility, cofactor binding, and enhancer activity in human and murine macrophages. These complex interactions decisively influence transcriptomic profiles of macrophages resulting in complex macrophage phenotypes under different physiological and pathological conditions (36–40). It is the context one needs to consider the role and contribution of nuclear hormone receptors.

THE GENERAL ARCHITECTURE AND REGULATORY MECHANISMS OF NUCLEAR RECEPTORS

The nuclear receptor superfamily contains various transcription factors that act as SDTF and play a crucial role in the signal translation from constantly changing lipid microenvironment to gene expression alterations. This functional complexity is based on the evolutionarily conserved protein structure. All nuclear receptors consist of N-terminal ligand-independent activation function (AF-1), DNA binding (DBD), hinge or linker, ligand binding (LBD), and ligand-dependent terminal activation (AF-2) domains. Highly conserved two zinc-finger motifs containing DBDs are responsible for the recognition and binding of specific DNA sequences known as hormone response elements. More diverse LBDs recognize receptor-specific lipid ligands and form dimerization surfaces, while AF-2 domains within LBDs create a binding surface for coactivator and corepressor complexes (41).

The nuclear receptor superfamily includes both classical endocrine receptors such as receptors for steroid hormones, thyroid hormones, and fat-soluble vitamin A or D and various orphan receptors whose ligands were initially unknown. Since their discovery, many orphan receptors become ″adopted″ by identification of their specific endogenous ligands for instance oxysterols for LXRs or short-chain fatty acids for PPARs. However, several receptors remained orphans without known endogenous ligands (42). Despite structural similarities, ligand-sensitive nuclear receptors show significant differences in their mechanisms of action. The first type of these receptors includes steroid receptors and can be found in the cytoplasm associating with heat-shock proteins in an unliganded state. Ligand activation of steroid receptors leads to the dissociation from heat-shock proteins, homo-dimerization, translocation into the nucleus, and binding their specific hormone-responsive DNA elements activating their target genes at the transcriptional level. The second type of ligand-sensing nuclear receptors including various classical hormone and dietary lipid-sensing receptors form heterodimers with RXRs and bind constitutively to DNA in the nucleus regardless of their ligand binding states, but their interaction partners and functional properties are tightly dependent on the presence of their ligands (43). In an unliganded state, these heterodimers interact with corepressor proteins such as silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (NCoR) complexes and attenuate basal mRNA expression levels of their target genes. Ligand binding induces conformation changes in the LBD leading to corepressor/coactivator exchange and consequential transcriptional activation (44). On the other hand, several liganded nuclear receptors can also inhibit the transcription activator activity of another SDTFs through transrepression. This transcriptional repressor mechanism is based on protein-protein interactions without direct sequence-specific DNA binding of nuclear receptors and associated with sumoylation and corepressor complex recruitment (4, 45).

THE CLASSICAL ROLE OF DIETARY LIPID-SENSING NUCLEAR RECEPTORS IN THE REGULATION OF MACROPHAGE METABOLISM AND INFLAMMATION

Several lipid-sensing nuclear receptors including LXRs, PPARγ, PPARδ, and their heterodimerization partners RXRα and β are expressed in macrophages and their expression levels and ligand-dependent activities are tightly regulated by various microenvironmental signals (46–48). The classical paradigm of nuclear receptor biology relies on lipid-sensing nuclear receptors to form a bridge between macrophage metabolism and inflammation (3, 43). This notion was supported by several lines of evidence: i) endogenous ligands for PPARs and LXRs are small lipid molecules; ii) macrophages are often present in a lipid-rich environment and are themselves metabolically active cells; iii) metabolic and inflammatory genes are regulated in parallel but with different mechanisms by endogenous or synthetic agonists-activated lipid-sensing nuclear receptors. A good example of the metabolic role of lipid-sensing nuclear receptors is that liganded PPARγ and LXRs tightly control cholesterol transport and storage in macrophage-derived foam cells from atherosclerotic lesions. oxLDL induces PPARγ expression and oxLDL-derived 9-HODE and 13-HODE serve as endogenous ligands for PPARγ. Liganded PPARγ enhances further oxLDL uptake through the increase of the scavenger receptor CD36 expression, the LXR, and the endogenous LXR ligand 27-hydroxicholesterol producing enzyme CYP27A1 expressions. LXR activation leads to elevated cholesterol efflux through induction of ABCA1 and ABCG1, facilitated

intracellular cholesterol trafficking by enhancement of NPC1 and 2 expressions, as well as attenuated cholesterol uptake. The latter process is mediated by liganded LXR-induced E3 ubiquitin ligase inducible degrader of the LDLR (IDOL), which triggers proteasomal degradation of LDLR and VLDLR [reviewed in (49)]. In addition to their metabolic roles, lipid sensing nuclear receptors modify the immunological features of the macrophages. On the one hand, both ligand-activated PPARγ and LXRs can repress various inflammatory signal-activated transcriptional programs in macrophages through inhibition of different SDTFs such as IFNγ-activated STAT1 or LPS-activated NFκB and AP-1 transcription factor complexes [reviewed in (4, 45)]. On the other hand, liganded LXRs also promote phagocytic capacity and survival in macrophages, while PPARγ controls the regenerative activity of muscle infiltrating macrophages following muscle injury (50–52).

Importantly, however, LXRs and PPARs act in permissive heterodimers with RXRs meaning that these heterodimers can also be activated by ligands of both RXRs and LXRs or PPARs (53). Initially, 9-cis-retinoic acid was a widely accepted endogenous RXR ligand, but it proved to be difficult to detectable under physiological conditions in vertebrates, thus raising doubts about its *in vivo* relevance. However, several pieces of evidence show that 9-cis-13,14-dihydro retinoic acid meets better the criterion of a physiological RXR ligand (54, 55). Even though the true identity of the endogenous RXR ligand(s) is one of the remaining mysteries of nuclear receptor biology, various studies demonstrated that synthetic RXR ligands can activate an RXR-specific transcriptional program in macrophages resulting in the changes of their phenotypic and functional characteristics (56, 57). It has been shown that RXR ligand activation leads to elevated VEGFα production, enhanced leukocyte migration, and altered inflammatory response and metabolism (57, 58). The majority of liganded RXR-regulated genes are overlapping with LXR or PPAR-activated gene signatures, but some experimental evidence shows that RXR can also act as a homodimer or in a heterodimer with orphan nuclear receptors such as Nur77 (57–60). These findings indicate that RXR ligand activation results in a unique transcriptional and biological responses in macrophages thus RXR is more than a "simple and silent" interaction partner for PPARs and LXRs.

NUCLEAR RECEPTOR LIGAND SENSITIVITY FROM AN EVOLUTIONARY PERSPECTIVE

If one wants to study the ligand responsiveness of nuclear receptors from a broad perspective, it is useful to take an evolutionary point of view. The ancient and conserved nuclear receptor superfamily believed to emerge in the metazoan lineage, but significant differences can be observed in the number of encoded nuclear receptors between different species. Notably, 2 nuclear receptors have been identified in the sponge *Amphimedon queenslandica*, 284 in the *Caenorhabditis elegans* (*C. elegans*), 21 in the fruit fly, 33 in the amphioxus, 47 in the rat, 49 in the mouse, and 48 in the human genome (61–65). According to the currently accepted view, nuclear receptors originate from ancestral fatty acid sensors of sponges, and the evolutionary shifts in ligand preference are the consequences of mutations altering the ligand-binding cavity (61). Furthermore, the evolution of ligand binding is not simple ligand-receptor coevolution, because the nuclear receptor ligands are not proteins but products or intermediates of various metabolic pathways such as isoprenoids, fatty acids, or fatty acid metabolites. Consequently, certain nuclear receptors may be activated by completely different ligands during an early evolution compared to the mammals (66). However, approximately half of the nuclear receptors in mammals fall into subclass lacking traditional ligands and the proportion of ligand-responsive nuclear receptors is very low in many primitive invertebrate species. For instance, one out of 284 nuclear receptors have been identified as ligand-responsive in the *C. elegans*, while only two out of 21 nuclear receptors have traditional ligands in the fruit fly (65, 67). These findings indicated that the nuclear receptor/ligand evolutionary relationship is very complex and dynamic, but ligand-independent transcriptional regulatory activities of nuclear receptors are important from primitive invertebrates to humans. Nevertheless, until recently a potential ligand insensitive action of metabolite sensing nuclear receptors was difficult to investigate.

NEXT-GENERATION SEQUENCING-BASED METHODOLOGIES AS A TRANSFORMING TOOL FOR DISCOVERY OF NEW LAYERS IN NUCLEAR RECEPTOR-MEDIATED TRANSCRIPTIONAL AND EPIGENOMIC REGULATION

In the last decade, the development and expansion of next-generation sequencing-based methodologies in transcriptomics and epigenomics contributed to the better understanding of the transcriptional basis of cell specification and cellular responses to the changing microenvironment. Among these methods, Chromatin Immunoprecipitation Sequencing (ChIP-seq) is routinely used to study the genome-wide binding of transcription factors and cofactors as well as post-translational histone modification patterns in eukaryotic cells. Assay for Transposase Accessible Chromatin Sequencing (ATAC-seq) is suitable for the identification of open chromatin regions, while Global Run-On Sequencing (GRO-seq) can detect and quantify the nascent RNA expression (68–70). Therefore, key questions such as: Where does chromatin open? Where does a particular transcription factor bind? and Where is transcription initiated? can be answered by covering the entire genome in an unbiased manner. The combination of RNA sequencing-based global transcriptome analysis with these techniques helped to identify new layers of connection between the genome-wide binding of

LDTFs and SDTFs (i.e. their cistromes), cell state-specific active enhancer landscape, and their transcriptional output. Thereby, it became possible to carry out the systematic analysis of nuclear receptor binding and function in different cellular systems. The initial ChIP-seq studies confirmed many elements of our prior knowledge about non-steroid nuclear hormone receptors including nuclear localization and DNA and chromatin binding in the unliganded state or their binding to receptor-specific hormone response elements but also resulted in some unexpected findings. For instance, a single nuclear receptor can bind numerous (10.000–25.000) sites in the genome and a not negligible part of the nuclear receptor-bound regions does not contain known hormone response elements (1). Comparing the number of nuclear receptor-bound genomic regions to ligand-activated genes, it could be observed that a large number of nuclear receptor-bound genomic sites are associated with a relatively small number (250–1.000) of ligand-responsive genes. It was also demonstrated that genome-wide nuclear receptor binding may be significantly rearranged after molecular microenvironmental changes (1, 71, 72). Besides, the combination of nuclear receptor-specific ChIP-seq with quantification of nascent RNA expression at genomic regulatory and coding regions by GRO-seq allowed the investigation of the ligand-dependent direct transcriptional regulatory role of nuclear receptors. The liganded nuclear receptor-regulated enhancers could be identified in different cell types using the following simple, correlative criteria: i) nuclear receptor binding; and ii) dynamically changing nuclear receptor ligand-induced or repressed nascent RNA expression at the given regulatory regions. These sites then can be annotated to the closest similarly regulated gene. A representative example is shown in **Figure 1** and (57). Interestingly, these studies also revealed that a portion of nuclear receptor-bound enhancers is insensitive to ligand stimulation indicating a potential ligand-independent function (73–76). Overall, these early studies suggested that cell-type and cellular state-specific nuclear receptor cistromes are tightly dependent on other LDTFs or SDTFs and lipid-sensing nuclear receptors may also act in a non-classical, ligand-independent way.

LIPID SENSING NUCLEAR RECEPTORS IN THE SPECIFICATION OF TISSUE-RESIDENT MACROPHAGE SUBSETS AND AS MODULATORS OF INFLAMMATORY RESPONSE

Several studies demonstrated that the interactions between lipid-sensing nuclear receptors and LDTFs are largely macrophage subset and microenvironment-specific. It has been described that the macrophage-specific LDTF PU.1 plays a central role in the formation of macrophage-specific cistromes for various SDTFs and is required for SDTF-directed transcriptional programs in murine BMDMs and macrophage cell line. Both the LXRα cistrome and LXR agonist GW3965-induced expression of various selected LXR target genes including *Elovl5*, *Abca1*, and *Abcg1* was diminished in the absence of PU.1. Conversely, PU.1 binding, and the active enhancer mark H3K4m1 pattern did not show any differences in LXRα/β double deficient BMDMs (**Figure 2A**) and (22). Applying combined bioinformatic and ChIP-seq approaches, we reported that PPARγ binding also depends on both PU.1 binding and quality of PPARγ-specific DR1 motif in macrophages (77). However, the systematic analysis of murine tissue-resident macrophage enhancer landscapes identified many nuclear receptor-binding motifs in the macrophage subset-specific enhancer clusters including LXRα binding motif in the Kupffer cells and splenic macrophages or PPARγ binding DNA element in the splenic and alveolar macrophages. These findings raised the possibility that these lipid-sensing nuclear receptors may also act as LDTFs in different tissue-resident macrophages (78). In recent years, this hypothesis has been confirmed by several independent studies. Sakai and colleagues described the crucial role of liver-specific molecular microenvironment including hepatocytes-derived LXR ligand desmosterol and sinusoidal endothelial cell-produced Notch ligand DLL4 and TGFβ in the initiation and maintenance of Kupffer cell identity in murine diphtheria toxin-induced Kupffer cell ablation model. DLL4 rapidly induces LXRα expression in repopulating monocytes and LXRα acts as LDTF in collaborative interactions with TGFβ and Notch signaling pathways during

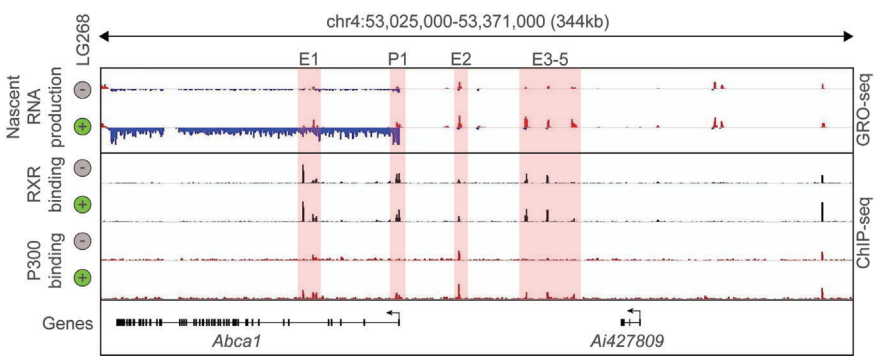

FIGURE 1 | Definition of putative RXR regulated enhancers. Genome browser view on the ABCA1 locus. GRO-seq and ChIP-seq results for RXR and P300 are shown in control (Veh, 60 min) and stimulated (LG268, 60 min) macrophages. Putative enhancers are highlighted (E1-E5 and P1).

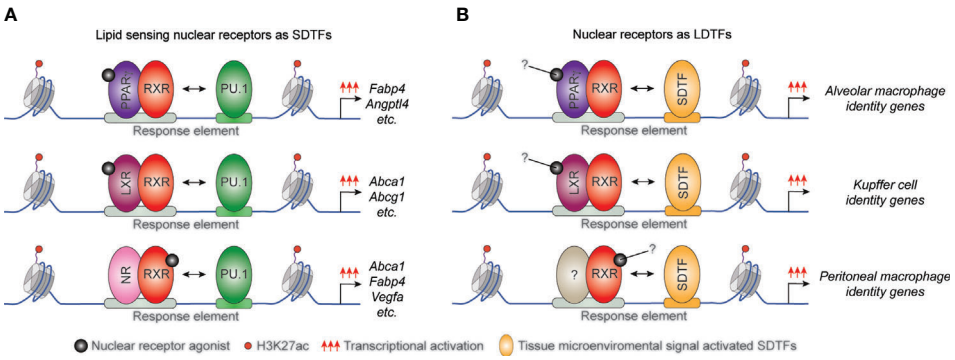

FIGURE 2 | The position of macrophage-expressed lipid sensing nuclear receptors in transcription factor hierarchy shows gene and tissue-dependency. **(A)** General macrophage lineage-specific LDTFs such as PU.1 contribute to the classical STDF action and ligand-mediated transcriptional activation capacity of lipid sensing nuclear receptors controlling macrophage metabolism. **(B)** Lipid sensing nuclear receptors also can act as LDTFs determining the tissue-specific characteristics of various tissue-resident macrophage subsets.

Kupffer cell differentiation (28). It has also been observed that diet-induced non-alcoholic steatohepatitis induces changes in the expression levels of collaborative LDTFs including downregulation of SPI-C and upregulation of ATF3 leading to altered binding and function of LXRs in Kupffer cells. The rearranged LXR cistrome contributes to disease-specific gene expression patterns and phenotype in this macrophage subtype (79). The crucial role of LXRα was identified in the differentiation of macrophages in the marginal zone of the spleen by A-Gonzales and colleagues. It was found that marginal zone macrophage specification is defective in LXRα-deficient mice resulting in abnormal responses to blood-borne antigens. The lack of marginal zone macrophages was restored in LXRα-deficient mice by myeloid-specific expression of LXRα or the adoptive transfer of wild-type monocytes (27). The lineage-determining role of PPARγ was also confirmed in alveolar macrophages in *in vivo* mouse experiments. Schneider et al. demonstrated that GM-CSF induces PPARγ expression in fetal monocytes which is responsible for the determination of the perinatal differentiation and the identity of alveolar macrophages through the regulation of several transcription factors and the alveolar macrophage differentiation and function-linked genes (26). It has been previously described that retinoic acid receptor (RAR) activation is required for the functional specialization of peritoneal macrophages through direct induction of GATA6 transcription factor expression (23). However, it has recently been reported that the RXRs themselves can also contribute to the neonatal expansion of large peritoneal macrophage pool and survival of adult large peritoneal macrophages through the regulation of chromatin accessibility and peritoneal macrophage-specific gene signature (80). In addition, Fonseca and colleagues recently showed that PPARγ is an essential collaborating factor for an AP-1 transcription factor complex in resting murine thioglycollate elicited macrophages. On the one hand, the AP1 transcription factor complex member, Jun binding was markedly reduced in PPARγ deficient macrophages at a specific enhancer set, while ATF3 and JunD bindings were not affected. On the other hand, complex protein-protein interactions were observed between PPARγ and AP-1 family members including Jun, JunD, and ATF3. Nevertheless, the functional consequences of the collaboration between PPARγ and AP-1 transcription factor complex are still not completely understood (81).

Genome-wide epigenomic approaches also allowed the testing of the proposed transrepression mode of anti-inflammatory gene regulation by nuclear hormone receptors, which effect inflammatory as well as tissue specific macrophages. The pre-genomic models of lipid sensing nuclear receptor-mediated anti-inflammatory actions were mainly based on the sumoylation-dependent transrepression of various inflammatory signals-activated TFs or TF complexes including NFkB, AP-1 and STAT1 (3, 4, 45) In agreement with the previous studies, the application of genomic approaches could confirm some elements of this regulatory mechanism including the necessity of SMRT and NCoR corepressor proteins for LXR-mediated transrepression (82). However, many questions about transrepression are still waiting for an answer at the whole-genome level. Perhaps the most important of these is the details of the overlap, extent and specificity of the interactions between liganded nuclear receptors and the inflammatory signals-activated transcription factors. In addition, recent studies identified additional mechanisms of liganded lipid sensing nuclear receptor-dependent inhibition of inflammation. Thomas and colleagues showed that liganded LXR can bind directly to inflammatory gene enhancers containing LXR binding sites independently from AP-1 transcriptional factor complex leading to reduced chromatin openness and inflammatory responsiveness (83). Interestingly, it has been also demonstrated that LXR can inhibit inflammatory gene expression through the ligand-dependent induction of Abca1-mediated cholesterol efflux and membrane lipid reorganization rather than transrepression (83, 84). Thus the transrepression mechanism is not fully validated yet and requires additional studies.

These findings indicate that lipid sensing nuclear receptors can also act as macrophage subtype-specific LDTFs (**Figure 2B**) and having a much broader impact on macrophage biology, including the inflammatory response than previously thought and raising the issue that these activities might not be all requiring ligand activation.

LIPID-SENSING NUCLEAR RECEPTOR-DIRECTED LIGAND INSENSITIVE REGULATORY MECHANISMS IN MACROPHAGES

After the initial observations indicating ligand insensitive fraction of nuclear receptor cistromes, several genome-wide studies investigated the ligand responsiveness of lipid sensing nuclear receptors in macrophages. Interestingly but not unexpectedly, our ChIP-seq and GRO-seq-based analysis demonstrated that only 13% of the identified RXR peaks (718/5206) are associated with significantly regulated nascent RNA expression following RXR agonist LG268 treatment in non-polarized murine BMDMs. In the case of the remaining part of the RXR cistrome, RXR binding is observed at transcriptionally silent (GRO-seq negative) or transcriptionally active (GRO-seq positive) but LG268 insensitive genomic regions. These findings suggest that a significant part of RXR cistrome is ligand insensitive or just responds to ligands of heterodimerization partners (57). Although the biological significance of ligand insensitive RXR cistrome is not completely understood in macrophages, our recent study demonstrated that it can play important roles in the suppression of a metastasis-promoting transcriptional program. We observed that myeloid-specific RXR deficiency leads to enhanced lung metastasis formation without influencing primary tumor growth in murine Lewis lung carcinoma (LLC) and B16-F10 melanoma tumor models. This prometastatic phenotype of RXR deficient myeloid cells is characterized by the elevated expression of prometastatic gene signature as well as increased cancer cell migration and invasion promoting capacity (**Figure 3A**). The repressive activity of RXR is based on direct DNA binding of the receptor together with silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (NCoR) corepressors and is largely insensitive to RXR ligand activation (**Figure 3B**) (85). Recently, the synthetic agonist- and antagonist-insensitive (so-called pharmacologically non-responsive) fractions of LXRα and β cistromes were also identified in non-polarized murine immortalized BMDMs further confirming those opinions that the ligand insensitive fraction of lipid sensing nuclear receptor cistromes is general rather than cell type or nuclear receptor-specific phenomenon (86).

It has been previously described that IL-4 can enhance the ligand-dependent activity of PPARγ in human and murine alternatively polarized macrophages through three different mechanisms including EGR2 transcription factor-dependent activation of its expression, induction of endogenous ligand producing mechanisms, and direct protein-protein interaction with IL-4-activated STAT6 (87–90). Nevertheless, a significant contradiction can be observed between the PPARγ-dependency of alternative macrophage polarization and PPARγ ligand-activated gene expression signature. Odegaard and colleagues demonstrated that PPARγ is necessary for proper alternative macrophage polarization. PPARγ deficiency in myeloid cells impairs alternative macrophage polarization in mice predisposing the animals to the development of diet-induced obesity, insulin resistance, and glucose intolerance (91). However, alternatively polarized human and murine macrophages have PPARγ ligand responsiveness and can produce endogenous ligands, but PPARγ ligand activation cannot induce alternative polarization-linked genes in IL-4-exposed human and murine macrophages (88). Overall, these contradictory findings raised the possibility that PPARγ controls alternative macrophage polarization in an unorthodox and potentially ligand-independent manner. To solve this mystery, we systematically investigated genome-wide RXR and PPARγ bindings and evaluated the PPARγ/RXR heterodimer-directed transcriptional events in the presence and absence of their specific ligands in alternatively polarized murine BMDMs using the combination of next-generation sequencing-based approaches including ChIP-seq, GRO-seq, and ATAC-seq. We observed that both RXR and PPARγ cistromes are expanded in the applied long- and short-term alternative macrophage polarization models following 6-day or 24-h IL-4 stimulation. Interestingly, the IL-4-expanded RXR cistrome is not associated with either IL-4-induced RXR expression or IL-4-reduced RXR binding at many genomic sites further confirming our theory about the existence of a non-chromatin associated RXR pool in the nucleus (92). The expansion of genome-wide RXR and PPARγ

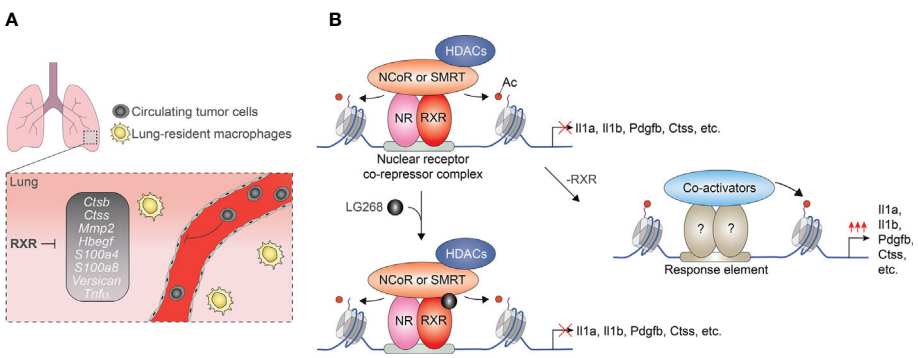

FIGURE 3 | Myeloid-cells-expressed RXRs suppress lung metastases formation in a ligand-independent manner. **(A)** RXRs repress the pro-metastatic gen set in murine lung-derived myeloid cells. **(B)** RXRs interact with SMRT and NCoR corepressor complexes and act as a direct transcriptional repressor regardless of the presence of RXR agonists.

bindings is directed by STAT6. The newly formed PPARγ/RXR co-peaks are associated with IL-4-induced chromatin accessibility, PU.1, P300, and RAD21 bindings (56, 93). Although we could identify ligand-activated and repressed PPARγ/RXR heterodimer-bound enhancers with GRO-seq and RNAPII-specific ChIP-seq methods, the majority of PPARγ cistrome were insensitive to both nuclear receptor ligands and IL-4 (56, 93). The ligand insensitive PPARγ cistrome is associated with IL-4-induced and PPARγ-dependent chromatin accessibility as well as P300 and RAD21 bindings. These genomic regulatory elements are responsible for facilitated STAT6 signaling and induction of extracellular matrix-related gene set after second IL-4 stimulation, indicating that ligand insensitive PPARγ acts as an epigenomic ratchet and provides transcriptional memory in alternatively polarized macrophages (**Figure 4**) (93). We also studied the IL-4-induced rearrangement of genome-wide RXR binding in human CD14+ monocyte-derived, differentiating macrophages. Unlike murine BMDM-based long- and short-term alternative macrophage polarization models, RXR cistrome is not expanded after very short (30 min) IL-4 stimulation, but it shows extensive overlap with IL-4-activated STAT6 cistrome in this experimental model. Examining a limited number of IL-4-activated genes and their RXR/STAT6 co-bound enhancers, we could distinguish three distinct interaction types between RXR and IL-4-STAT6 signaling pathways based on the modulatory effect of RXR agonist LG268 on basal gene expression and IL-4 responsiveness: i) basal and IL-4-dependent gene expression and enhancer activations are insensitive for liganded RXR; ii) RXR agonist activates transcription alone and acts synergistically with IL-4; iii) RXR agonist enhances IL-4-dependent transcriptional activations without influencing basal gene expression. The latter suggests a novel function of liganded RXR that it potentiates the macrophage response to other microenvironmental signals without affecting basal gene expression in a gene-specific manner (**Figure 5**) (94). Overall, these findings show that lipid-sensing nuclear receptors play a multifaceted role in macrophages through classical ligand-dependent and novel ligand-insensitive transcriptional regulatory activities.

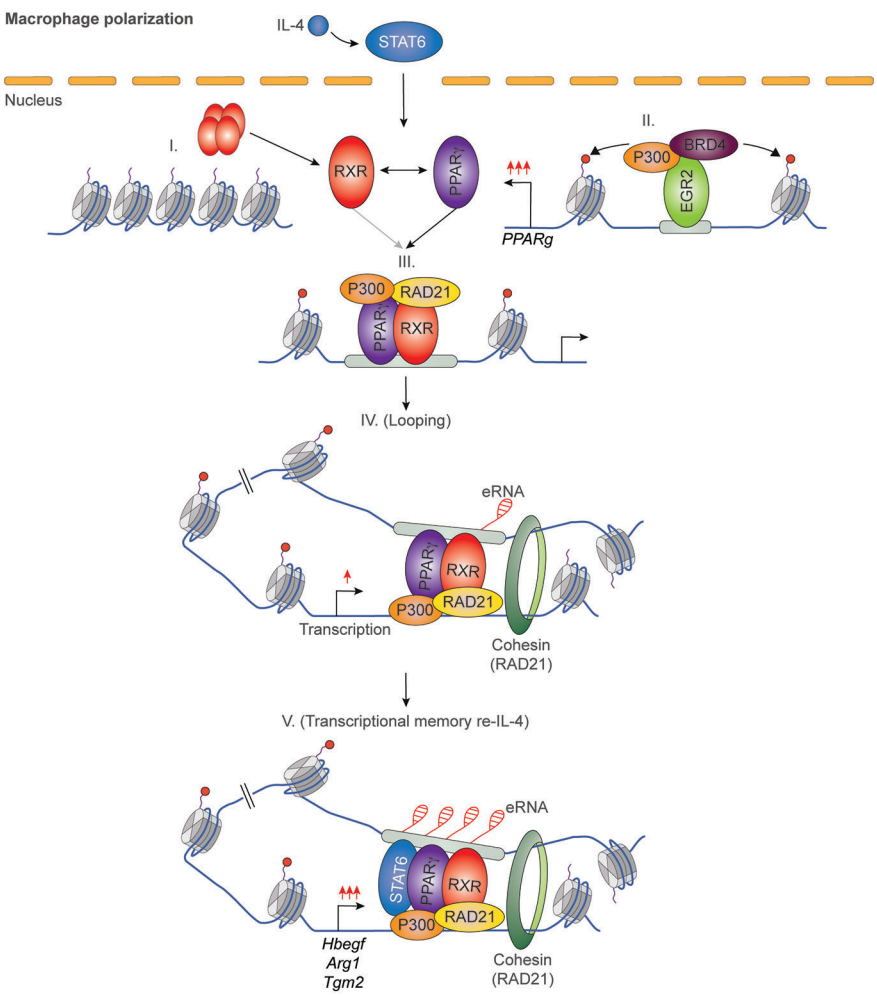

FIGURE 4 | IL-4/STAT6/EGR2 axis-induced PPARγ acts as an epigenomic ratchet in a ligand-independent manner in alternatively polarized macrophages resulting in transcriptional memory and enhanced gene-specific responsiveness to IL-4 re-stimulation.

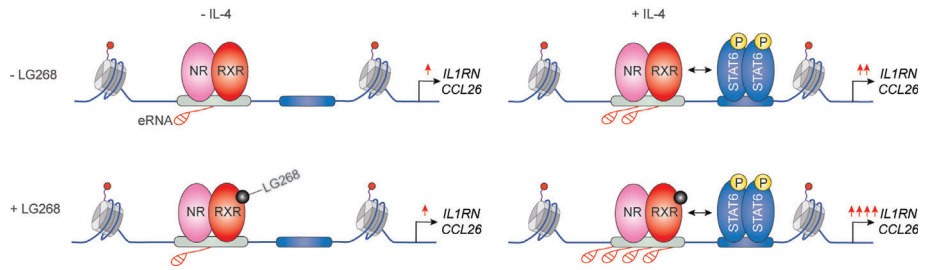

FIGURE 5 | Synthetic RXR agonist supports a gene-specific elevated IL-4 response without any effect on basal gene expression in human differentiating macrophages.

THE POTENTIAL DETERMINANTS OF LIGAND SENSITIVITY OF LIPID-SENSING NUCLEAR RECEPTORS: FROM THE FACTS TO THE THEORIES

Even though the above-mentioned studies indicate that a part of lipid-sensing nuclear receptor cistromes is ligand insensitive, the factors affecting their enhancer-specific ligand sensitivity are partially enigmatic. Our knowledge about the three-dimensional structure of lipid sensing nuclear receptors is essential to solving this enigma. In the last two decades, the structural biologists extensively studied the three-dimensional structures of lipid-sensing nuclear receptor complexes such as PPARγ:RXR and LXR : RXR heterodimers contributing to the better understanding of their interactions between various ligands, co-factors, or DNA (95–98). Initially, we thought that the ligand-dependent activation of nuclear receptors is an obvious 'on-off' switch, but the structural studies modified this theory. For example, recent findings show that different synthetic PPARγ agonists can interact with both a known ligand-binding pocket of LBD and an alternate binding surface resulting in the complex output of PPARγ activation in the presence of endogenous ligands or synthetic agonists (99). Moreover, endogenous metabolites such as serotonin derivates and butyrate also bind to non-canonical ligand-binding surfaces of PPARγ leading to its activation (100, 101). Overall, these results suggest that lipid-sensing nuclear receptors can integrate different signals from distinct signaling pathways. Therefore, the activation of the pharmacologically insensitive portion of lipid sensing nuclear receptor cistromes *via* the binding of endogenous metabolites to non-canonical ligand-binding surfaces cannot be completely excluded.

It has been also demonstrated that the nucleotide sequence of the binding motif can modulate the three-dimensional nuclear receptor structures and their interactions with DNA influencing the ligand sensitivity. Studying the PPARγ cistrome in alternatively polarized murine BMDMs, we found that the extended DR1 motif was significantly enriched at the synthetic agonist rosiglitazone-activated enhancers. In contrast, ligand insensitive regulatory regions lack this extension and harbor a shorter, more canonical RXR binding site (93). It has been shown that the identified extra 5' sequence (A-G/C-T) in DR1 can affect the DNA binding affinity of PPARγ:RXR heterodimer and is essential for the PPARγ hinge region to form an interaction with DNA. This interaction is required for the proper conformation and the ligand-binding ability of the receptor suggesting that the PPARγ:RXR heterodimer conformation is suboptimal for the binding of the ligand in the absence of DR1 extension in ligand insensitive enhancers (93, 95, 102, 103). Both pharmacologically sensitive and insensitive parts of LXR cistromes were associated with LXR-specific DNA elements similar to PPARγ, but the extension of LXR-response elements was not identified at the pharmacologically sensitive genomic sites (86). Taken together, these results indicate that the sequence of nuclear receptor binding motifs is one determinant of ligand responsiveness, but not the only one.

The lipid sensing nuclear receptor conformation and activity are also regulated in a ligand-independent manner by covalent post-translation modifications. These include acetylation, phosphorylation, O-GlcNacylation, SUMOylation, or ubiquitination at numerous modification sites influencing different features of nuclear receptors including ligand sensitivity and trans-activation capacity [reviewed in (104, 105)]. Many of them generally affect the activity of nuclear receptor signaling pathways in various cell types, but some modifications can influence the expression of a specific subset of nuclear receptor target genes. For instance, PPARγ Ser273 phosphorylation does not affect the adipogenic capacity of PPARγ but attenuates PPARγ ligand-induced activation of a specific subset of target genes promoting insulin sensitivity *via* inhibited recruitment of Thyroid hormone receptor-associated protein 3 (THRAP3) (76, 106). It has also been described that LXRα phosphorylation at Ser196 regulates its target gene selectivity in macrophages. Chemical inhibition of Ser196 phosphorylation and generation of LXRα S198A phosphorylation-deficient mutant leads to the identification of specific changes in LXR/RXR regulated gene expression. Some LXR target genes such as AIM and LPL showed significantly enhanced LXR agonist-dependent induction in LXRα S198A phosphorylation-deficient mutant but others including ABCA1 or SREBPc1 proved to be insensitive to phosphorylation. Interestingly, the S198A mutation or chemical inhibition of phosphorylation also resulted in significantly elevated basal and LXR ligand-induced CCR7 and CCL24 expression levels (107, 108). These findings indicate that the post-translational modifications of nuclear

receptors can attenuate gene-specific responsiveness to various endogenous and synthetic nuclear receptor ligands but their contribution to ligand insensitive nuclear receptor cistromes is currently unknown.

CONCLUDING REMARKS

We have attempted to illustrate above that the transcriptional regulatory role of lipid sensing nuclear receptors is much more comprehensive in macrophages than previously suspected. Although previously we and others have identified many lipid sensing nuclear receptor-activated pathways, our knowledge was limited to the regulation of macrophage metabolism and inflammation through ligand-induced direct transcriptional activation and transrepression. This was because our studies and methods were biased toward ligand-regulated events. Recent progress in epigenomic and transcriptomic methodologies has greatly increased our understanding of different aspects of nuclear receptor biology including their relationships with other LDTFs and SDTFs or their non-canonical transcriptional regulatory actions. Using these approaches, both lineage-determining and ligand insensitive activities of lipid sensing nuclear receptors were identified. These novel transcriptional regulatory mechanisms contribute to tissue-resident macrophage subtype specification, organization of chromatin structure, regulation of transcriptional memory, and modification of responsiveness to other microenvironmental signals. A systematic investigation of the molecular background of newly identified regulatory functions will be necessary for re-thinking of the importance of lipid sensing nuclear receptors in macrophage biology. However, this will take a considerable amount of time and the integration of methodologies of various disciplines including structural biology and epigenomics with genome editing technologies. Furthermore, the application of *in vivo* chemical affinity capture and massively parallel DNA sequencing (Chem-seq) is suitable method to identify the genomic sites bound by small chemical molecules including nuclear receptor activity modifier molecules (109, 110). Therefore, its combination with the nuclear receptor-specific ChIP-seq and GRO-seq methods may help to determine whether the ligand insensitive portion of nuclear receptor cistromes can bind ligand without transcriptional response or is unable to ligand binding. Finally, additional immunological approaches would need to analyze the *in vivo* functional consequences of ligand-independent actions of lipid sensing nuclear receptors in macrophages under different physiological and pathological conditions. After all, we will be able to assess whether these transcriptional regulatory mechanisms play a significant role in the development and progression of various human immunological diseases. Also, there is no reason to believe that nuclear receptors in other cell types are not behaving the same way as in macrophages. These studies requiring comprehensive and unbiased analyses should keep us as a research field occupied for the foreseeable future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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REVIEW
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Role of the Orphan Nuclear Receptor NR4A Family in T-Cell Biology

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The nuclear orphan receptors NR4A1, NR4A2, and NR4A3 are immediate early genes that are induced by various signals. They act as transcription factors and their activity is not regulated by ligand binding and are thus regulated *via* their expression levels. Their expression is transiently induced in T cells by triggering of the T cell receptor following antigen recognition during both thymic differentiation and peripheral T cell responses. In this review, we will discuss how NR4A family members impact different aspects of the life of a T cell from thymic differentiation to peripheral response against infections and cancer.

Keywords: NR4A nuclear receptor, CD4 T cell, CD8 T cell, thymus, immune response, Nur77, Nurr1, Nor1

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INTRODUCTION TO T-CELL BIOLOGY

T cells are central players of the adaptive immune response. They recognize, *via* their T cell receptor (TCR), a peptide fragment of antigen (Ag) in association with class I or II molecules of the major histocompatibility complex (MHC). The generation of a repertoire of T cells endowed with the ability to recognize almost all the possible foreign Ags is possible due to TCR gene rearrangement, a process where random juxtaposition of TCR gene segments occurs to create TCR sequence diversity. This requires that developing thymocytes undergo an education process during their differentiation. Therefore, only thymocytes expressing a useful TCR (eventually able to recognize a foreign Ag in association with self-MHC molecules) will survive (positive selection) during differentiation while those expressing an auto-reactive TCR will be physically or functionally eliminated from the repertoire (negative selection). This stringent selection process ensures that only useful (MHC restricted) and self-tolerant T cells will colonize lymphoid organs as naïve T cells. The molecular events controlling thymic T cell differentiation and selection are still not fully understood. The first part of this review will highlight how deciphering the role of NR4A family members has helped to better understand the T cell differentiation events taking place in the thymus.

The detection and engulfment of pathogens by dendritic cells (DCs) within the tissue will induce their maturation and the presentation of peptide fragments from the pathogens within MHC class I or class II molecules expressed at their surface. These DCs will then migrate to the draining lymphoid organs where they will activate Ag-specific T cells. For efficient activation and differentiation into effector T cells able to control the infection, naïve T cells require three signals: TCR stimulation, co-stimulatory signals provided by mature DCs *via* CD28-CD80/CD86 interactions, and an inflammatory milieu (cytokines produced by DCs or the environment). This

will lead to massive expansion of T cells to increase their numbers. Concomitant with T cell proliferation, differentiation will occur leading to the acquisition of effector functions crucial for the elimination of the infectious agent. After clearance of infection, most Ag-specific T cells will die by apoptosis while a few will survive and differentiate into memory T cells that will confer long-lived protection against reinfection. A different picture emerges in the context of chronic infection or cancer where the persistence of Ags and inflammation lead to a state of T cell exhaustion. In the second part of this review, we will present how the study of the role of the orphan nuclear receptor NR4A family members has provided a better understanding of the molecular events controlling peripheral T cell responses to infection and cancer.

OVERVIEW OF NR4A ORPHAN NUCLEAR RECEPTORS

The NR4A family of orphan nuclear receptors is composed of NR4A1 (Nur77), NR4A2 (Nurr1), and NR4A3 (Nor-1). They work as transcription factors in a ligand-independent manner. Like other nuclear receptors, they are composed of a central two-zinc DNA-binding domain, a N-terminal transactivation domain, and a C-terminal ligand-binding domain (LBD). The LBD lacks a classical hydrophobic binding pocket, explaining ligand-independent action. They recognize the NBRE motif (AAAAGGTCA) on DNA as monomers and they can bind as homodimers to the palindromic DNA binding motif, NurRE ($TGATATTTX_6AAATGCCCA$) (1, 2). Their functions are mostly controlled by the rapid and transient induction of their expression by a variety of extracellular signals, and thus are considered as immediate-early genes. The NR4As are involved in various cellular functions including apoptosis, survival, proliferation, angiogenesis, inflammation, DNA repair, and fatty acid metabolism (3, 4).

NR4As AND THYMIC T CELL DEVELOPMENT

Overview of T Cell Development

The thymus is organized into two distinct regions; an outer cortical area and an inner medullary area that are composed of different cell populations. During T cell selection in the thymus, thymocyte fate is largely determined by the affinity of the TCR for self-peptide presented in the context of MHC molecules (spMHC). In the cortex, the generation of the αβ-TCR through random somatic recombination processes leads to the formation of a large pool of $CD4^+CD8^+$ double-positive (DP) thymocytes that express a highly diverse TCR repertoire. DP thymocytes that receive low affinity TCR signals undergo positive selection and lineage commitment, and traffic to the thymic medulla where maturation to the $CD4^+$ or $CD8^+$ single positive (SP) lineage is completed (5, 6). Upon receipt of a high affinity TCR signal, self-reactive thymocytes undergo negative selection which includes apoptosis induction (clonal deletion) or functional inactivation (anergy). Alternatively, thymocytes that receive strong TCR signals can undergo agonist selection and be diverted into nonconventional lineages such as T regulatory cells (Treg), invariant natural killer T cells (iNKT), or $CD8\alpha\alpha^+$ intestinal intraepithelial lymphocytes (IEL) (7). In the thymic cortex, developing thymocytes encounter self-peptides derived from ubiquitously expressed proteins (6). It is necessary to remove T cells expressing autoreactive TCR directed against all self-proteins, including the ones whose expression is tissue-restricted. Therefore, in the thymic medulla and at the single-positive (SP) stage of thymocyte differentiation, thymocytes will encounter a different repertoire of self-peptides which includes those derived from proteins expressed in a tissue-restricted manner and driven by the promiscuous transcriptional activities of Aire and Fezf2 (8, 9).

TCR signals received by nascent thymocytes lead to transcriptional changes that regulate positive and negative selection (10). Among the set of genes consistently associated with clonal deletion is NR4A1 (10, 11). The NR4A family has long been investigated for their putative role in thymocyte selection. NR4A1 and NR4A3, but not NR4A2, are expressed in thymocytes undergoing selection (12), but NR4A1 is the most extensively studied of the three NR4As and will receive the most attention in this section of the review. An initial connection between the NR4A family of proteins and thymocyte selection developed when NR4A1 induction was demonstrated in apoptotic immature thymocytes and T cell hybridomas (13, 14). Subsequent studies on the role of the NR4A family in thymocyte development utilized many different approaches and model systems which will be explored in the following sections. Additionally, while there is clear redundancy within the family, it is becoming apparent that phenotypic and functional changes in thymocyte development can be observed when the expression of individual family members is manipulated. This is further emphasized by emerging evidence suggesting that the induction of individual NR4A family members is differentially regulated downstream of TCR signaling (15). Added complexity stems from the fact that the function of NR4A family members during thymocyte development has been reported to be dependent on transactivation (16–19) or extra-nuclear activities (20–23) (**Figure 1**). Finally, this section will focus on the role of the NR4A family in αβ-thymocyte selection, since to our knowledge the NR4As have not been reported to regulate the development of any other thymic lineages.

Cortical Negative Selection to Ubiquitously Expressed Self-Antigen

The thymic cortex is the compartment in which nascent thymocytes that express an αβ-TCR first encounter spMHC, and where both positive and negative selection are known to occur (6, 7). DP thymocytes are selected based on the affinity of the TCR for "ubiquitous" self-Ag (UbsA) presented in the context of MHC on the surface of cortical thymic epithelial

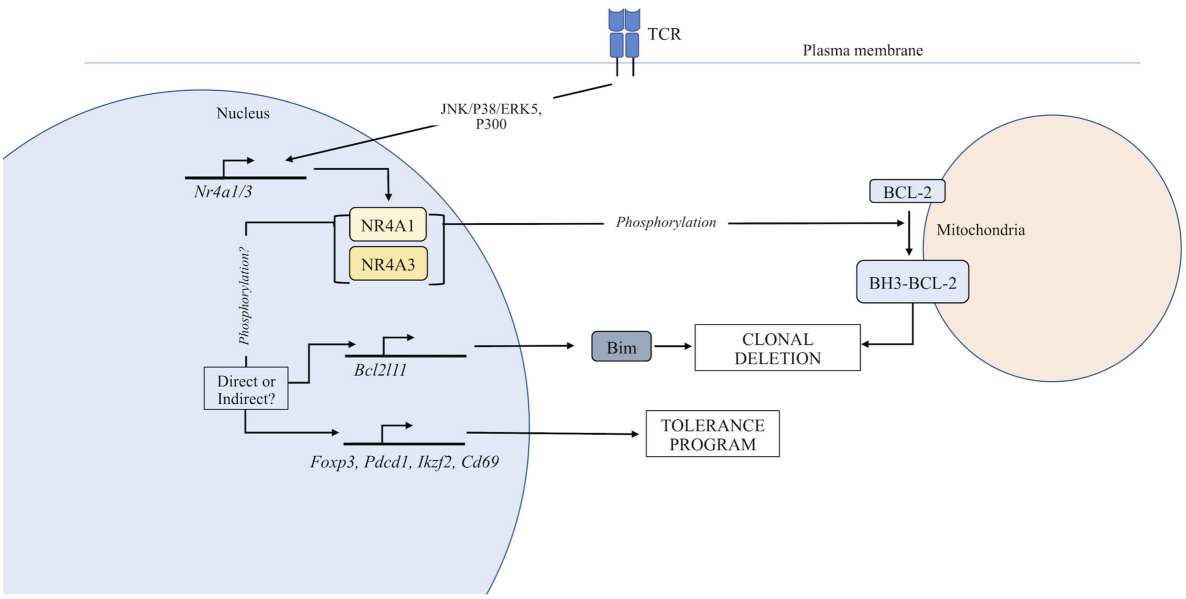

FIGURE 1 | Function of the NR4A family in thymocyte development. TCR stimulation induces expression of the NR4A family. In the nucleus, NR4As can regulate the expression of genes that control T cell tolerance. Additionally, NR4A family members can be exported from the nucleus where they directly regulate apoptosis through an interaction with Bcl-2.

cells (cTEC) and DCs (6). NR4A1, NR4A3 (24), and NR4A2 (25) share variable sequence homology in the N-terminal transactivation domain and C-terminal "ligand binding domain," but close similarity in their DNA-binding domains. Original studies implicating the NR4As in thymocyte clonal deletion demonstrated that a NR4A1 dominant negative mutant that lacked the transactivation domain or antisense NR4A1 RNA inhibited TCR-induced cell death in T cell hybridomas (13, 14, 26, 27). However, it was proposed early on that T cell hybridomas more closely model the responses of mature T cells than developing thymocytes (28, 29). *In vivo* models of negative selection to ubiquitous antigen were subsequently employed. Dominant negative NR4A1 expressed in HY TCR transgenic mice, wherein thymocytes recognize the ubiquitous male-specific HY antigen in the context of H-2D^b (30, 31), resulted in a partial rescue of DP and CD8SP populations from clonal deletion; however, tolerance to HY male antigen was maintained. Similar results were observed for models that involved injection of exogenous antigen to induce thymic clonal deletion such as in F5 TCR transgenic mice (specific for influenza nucleocapsid peptide) (27, 30, 32). More contemporary studies investigating the transcriptional regulation of NR4As provided additional support for the NR4A family in regulating negative selection and tolerance. In pre-selection DP thymocytes, histone deacetylase 7 (HDAC7) in complex with MEF2D represses NR4A1 and NR4A3 expression (33, 34). Following TCR signaling, HDAC7 is phosphorylated and exported from the nucleus, discontinuing its repressive activity (33–36). Transgenic mice harboring an HDAC7 mutant putatively incapable of being phosphorylated downstream of the TCR and exported from the nucleus (S155/318/448A) showed impaired induction of NR4A1 and NR4A3, impaired negative selection, and lethal autoimmunity (37). While this may indicate the importance of NR4A1/NR4A3 in clonal deletion, the cause of autoimmunity in this model is unclear, especially since these HDAC7 mutants demonstrated a generalized suppressive impact on the negative selection transcriptional program and impaired generation of Tregs (37).

As a result of the similarity among the NR4As in their DNA-binding domains, dominant negative mutants of individual NR4As have the potential to inhibit the transactivation activities of other NR4A family members (12). Therefore, studies employing the NR4A1 dominant negative mutant have provided support for the hypothesis that NR4A receptors drive thymocyte clonal deletion *via* their transcriptional activity (38–40). In harmony with this assertion, overexpression of full-length NR4A1 or NR4A3 (but not NR4A2) induced thymocyte apoptosis *in vivo*, while that of the NR4A1 dominant negative did not (12, 27, 30, 41) but this assumes that the dominant negative NR4A1 mutant only impairs the transcriptional activity of NR4A1. Transcriptional targets of NR4A1 thought to potentially mediate its pro-apoptotic effect were FasL, TRAIL, and Nur77 downstream gene 1 and 2 (NDG1/2) (40). While none of these targets were shown to be required for the thymocyte apoptosis induced by a full-length NR4A1 transgene, we caution that thymic phenotypes in NR4A transgenic mice may not accurately represent endogenous NR4A function during thymocyte selection since NR4A

transgenes were active during the DN stage (e.g., Lck proximal promoter), not the DP stage where negative selection first occurs (15, 39, 42, 43).

Conversely, NR4A1/3 have been proposed to induce apoptosis by their nuclear export and conversion of anti-apoptotic Bcl-2 to a pro-apoptotic form *via* exposure of its Bcl-2 homology domain 3 (BH3) at the mitochondria in a variety of cell types including thymocytes (20–23). Bcl-2/BH3 conversion has been shown to occur *in vivo* in the HY and F5 TCR transgenic UbsA selection models specifically within DP, but not SP thymocytes, implying this NR4A activity is unique to UbsA-mediated negative selection (21). A study that used a Bcl-2 transgene wherein the key amino acid residues critical for the pro-apoptotic function of the Bcl-2 BH3 domain were mutated demonstrated that expression of the BH3-mutant Bcl-2 enhanced rescue of high affinity and specific Vβ TCR-expressing clones experiencing negative selection to endogenous superantigen compared to wildtype Bcl-2. This indicated that pro-apoptotic conversion of Bcl-2 may be a mode of influence for NR4A1/NR4A3 on clonal deletion. However, subsequent work using the HY^{cd4} TCR transgenic model, which is specifically designed for physiological timing of expression of the αβ-TCR in contrast to traditional TCR transgenics (44), failed to observe exposure of the Bcl-2 BH3 domain during negative selection (18).

As a better approach to study the function of NR4A1 in thymocyte development, a NR4A1 knock-out (KO) mouse was generated. NR4A1 deficiency did not impair clonal deletion in the HY TCR and AND TCR transgenic models of negative selection to UbsA (28). The dispensability of NR4A1 for UbsA-mediated clonal deletion was further reinforced by a study using the physiological HY^{cd4} transgenic TCR model (18). Due to putative redundancy in the transcriptional activities of the NR4As mentioned above, the lack of phenotype in NR4A1-deficient mice has long been thought to be due to compensation by the remaining intact NR4A family member NR4A3 (12), and has received support from the study of the NR4A family in Treg development (45) (see below). However, even though NR4A1 deficiency did not impair clonal deletion, it altered the expression of proteins induced during negative selection to UbsA such as PD-1, Helios, and CD69 demonstrating a NR4A1 transcriptional footprint on UbsA-mediated negative selection (18, 40, 46).

Against complete functional redundancy of NR4A1/3 is the differential regulation of NR4A family member expression. NR4A1 is induced in thymocytes receiving both positive and negative selection signals, though to a greater degree for the latter (10, 42), while NR4A3 expression appears to be induced only by high affinity signals (15). This supports the notion that the different NR4A family members can differentially contribute to thymocyte selection events. NFAT has been proposed necessary for the induction of NR4A3 (but not NR4A1 expression) in $CD4^+$ and $CD8^+$ peripheral T cells, especially in the context of exhaustion (15, 47, 48). Meanwhile, ERK signaling is required for optimal NR4A1 and NR4A3 induction in peripheral T cells (based on chemical inhibitor experiments) (15). Most recently, a specific pathway has been proposed for NR4A1 regulation in thymocytes, wherein ASK1-JNK/P38 MAP kinases promote the induction of NR4A1, while Fas apoptotic inhibitory molecule (FAIM) and Akt inhibit this cascade (49). Some additional proposed positive regulators of NR4A1 and NR4A3 induction are P300, MEF2D, and ERK5 (2, 50).

Negative Selection to Tissue-Restricted Self-Antigen Expressed in the Medulla

Following positive selection in the thymic cortex, developing thymocytes traffic to the medulla *via* upregulation of the CC-chemokine receptor 7 (CCR7) coordinated with maturation to the SP stage (5). Within this compartment, negative selection is induced by a new "tissue-restricted" self-antigen **(TRsA)** repertoire regulated by the transcription factors AIRE and Fezf2 and mediated by a distinct complement of antigen presenting cells (6, 9, 51). Investigation of *in vivo* negative selection to TRsA revealed that NR4A1 contributes to clonal deletion in a manner not fully compensated for by NR4A3. Using the MHC II-restricted OT-II RIP-mOVA model, Fassett et al. showed that deficiency in NR4A1 impaired clonal deletion of CD4SP transgenic thymocytes (46). In this model, the OT-II transgenic TCR recognizes a peptide derived from the chicken protein, ovalbumin (OVA) (52) and OVA is expressed in both pancreatic beta cells and thymic medulla owing to its control by the rat insulin promoter (53). Additionally, Fassett et al. showed that combined deficiency in NR4A1 and Bim, a key inducer of thymocyte apoptosis (54), did not further impair clonal deletion compared to NR4A1-deficiency alone. From a mechanistic perspective, they found a reduction in mRNA levels of *Bcl2l11* (coding for Bim) in the absence of NR4A1, suggesting that NR4A1-mediated induction of Bim was at least one way NR4A1 regulated clonal deletion (46). This contrasts with a study using the OT-I TCR RIP-mOVA transgenic model (55), in which Bim does not require NR4A1 for its transcriptional induction (19). In this model, deficiency in NR4A1 only modestly impaired clonal deletion as evidenced by a small increase in mature OT-I thymocytes. Single deficiency in either NR4A1 or Bim did not result in broken tolerance, however, combined deficiency resulted in broken self-tolerance signified by the development of diabetes. Since the number of mature OT-I thymocytes and T cells was similar in Bim-deficient and NR4A1/Bim doubly deficient situations, the break in tolerance suggests NR4A1 regulates tolerance through altering the functional capabilities of the thymocytes or T cells (19). A role for NR4A3 and redundancy between NR4A1 and NR4A3 has not been investigated in a medullary antigen-specific model system.

How do NR4A family members regulate medullary negative selection? In addition to regulating the expression of Bim as seen in the OT-II Rip-mOVA model, nuclear export of NR4A1 following "death signals" (e.g., etoposide, calcium ionophore, phorbol ester, TCR signal) has been observed and appears to occur following the phosphorylation of a serine residue (S354 in mouse) in the DNA binding domain (20, 23, 38, 56, 57). The localization of highly phosphorylated NR4A1 to the cytosolic fraction has also specifically been associated with SP, but not DP

thymocytes stimulated *in vitro* with TCR- and CD28-specific plate-bound antibodies (38). While S354 of NR4A1 has been proposed as a target residue for Akt and the ERK1/2-RSK pathway (23, 57, 58), there is debate as to whether phosphorylation at this site mediates nuclear export or retention (23, 38, 59); however, it appears to vary across model systems and cell types (23, 59–61). In thymocytes, protein kinase C (not AKT, JNK, ERK1/2 or p38) is thought to phosphorylate both NR4A1 and NR4A3, leading to mitochondrial translocation (59), yet both MAPK and PI3K inhibitors have been shown to inhibit this phenomenon specifically in SP thymocytes in a separate study (38). Nuclear export would thus be consistent with a role for NR4A1 in directly mediating TRsA-induced clonal deletion in the OT-II model (46), if NR4As indeed drive clonal deletion by translocation to mitochondria. However, deficiency in Bim alone impaired clonal deletion in this model (62), suggesting that in this case NR4A1 is not sufficient to drive clonal deletion independently of Bim, lending further support for NR4A transcriptional activity as the major modulator of thymocyte fate. In both thymocytes and T cell hybridomas, the MEK5-ERK5 pathway has been proposed to regulate thymocyte apoptosis by both inducing NR4A1 expression downstream of the TCR signal (50, 63), and phosphorylating NR4A1 leading to enhanced NR4A1 transcriptional activity (64). In further support of the concept of diverging functions of the NR4As across the stages of thymocyte development (and in UbsA- and TRsA-mediated negative selection), Akt is thought more active in DP than SP thymocytes (38, 65), and thought to direct the ubiquitination and degradation of NR4A1 in both T cell hybridomas and thymocytes (66). However, the relationship between NR4A post-translational modification and function, whether it be mitochondrial translocation or transactivation (or perhaps a combination of both), requires further investigation using *in vivo* model systems paying close attention to discrete T cell subsets. Overall, no consistent dependence on induced pro-apoptotic genes has been observed across studies. As a result, an emerging view is that the NR4As may influence selection outcomes by transcriptionally modulating the T cell tolerance program (18, 46, 67–70). Supporting evidence includes an increased susceptibility in NR4A1 deficient mice for experimental autoimmune encephalomyelitis (EAE; 2D2 transgenic TCR model), allergic contact dermatitis, collagen-induced arthritis (69), and diabetes (19). However, it is unclear whether this increased autoimmunity is due to changes in thymocyte development or peripheral T cell function. Studies employing conditional NR4A1 knock-out models will be necessary to resolve this question.

Positive Selection/Lineage Commitment

Despite induction of NR4A1 during positive selection (10, 42), neither it nor the other NR4As have been shown to be required for this process. As was discussed in the preceding section, there are differences observed between MHC I- and MHC II-restricted models of negative selection, which suggest the NR4As may play diverging roles in the selection of $CD8^+$ and $CD4^+$ T cell lineages. This view is reinforced by the observation that both CD4SP thymocytes and mature $CD4^+$ T cells from a polyclonal repertoire express elevated basal levels of a Nur77-GFP reporter compared to $CD8^+$ lineages, perhaps owing to the proposed enhanced signal delivered by the CD4 coreceptor compared to that of CD8 (42, 71, 72). In polyclonal thymocytes and peripheral T cells, NR4A1 has been shown to negatively regulate the transcriptional activation of *Runx3*, a critical operator of $CD8^+$ T cell lineage commitment, which may alter the relative stability of selection into the $CD8^+/CD4^+$ lineages (43, 73). However, RNA-sequencing analysis of NR4A triple-KO CAR-transduced $CD8^+$ T cells revealed no effect on *Runx3* expression, perhaps suggesting diverging influences by individual NR4As (67) or differences in thymocytes versus mature T cells. Nevertheless, deficiency in both NR4A1 and Bim led to enhanced efficiency of positive selection in female mice bearing the MHC I-restricted HY^{cd4} transgene (18) and NR4A1 deficiency alone resulted in increased positive selection in the OT-II TCR transgenic model (46). However, NR4A1 deficiency did not enhance positive selection in the OT-I transgenic model (19). Overall, the contribution of NR4A1 to positive selection requires further examination and remains unclear, though its influence may vary with selection circumstances including lineage commitment and the intrinsic self-reactivity of transgenic TCR models investigated.

Alternate Thymocyte Fates

It is clear the NR4As do not always function as proapoptotic mediators. Indeed, the NR4As have been implicated in non-apoptotic processes following strong TCR signaling such as the development of non-conventional $CD4^+$ fates (Treg and anergic $CD4^+$ T cell) (19, 45, 46, 74, 75). Treg are a distinct lineage of T cells that are selected in the thymus and are critical for tolerance. Lineage specification and function of Treg is controlled by expression of the transcription factor Foxp3. Each NR4A member has been implicated in variably promoting the activation of *Foxp3* transcription and other genes associated with the Treg signature (e.g., *Ikzf4* and *Il2ra*) (17, 19, 46, 75). As such, triple NR4A deficiency resulted in the loss of Tregs in thymic and peripheral compartments, systemic multiorgan autoimmunity, and a skewing of the mature $CD4^+$ T cell repertoire toward an activated phenotype ($CD44^{hi}$ $CD62L^{lo}$) (45). Combined NR4A1 and NR4A3 deficiency nearly recapitulated the phenotype of the triple knock out, thus NR4A2 may not play as key a role in Treg development and homeostasis. It should be mentioned, however, that mice lacking all three NR4As displayed generalized thymic abnormalities, which may connote defects independent of the Treg compartment or could result from excessive inflammation. A more recent study has provided evidence for a positive feedback loop between the NR4A family members and Foxp3 which involves reciprocal promoter binding and transactivation, and functions to reinforce Treg development from the $CD25^+$ $Foxp3^-$ $CD4^+$ precursor stage (45, 74, 75). Despite its putative ability to transactivate *Foxp3*, deficiency in NR4A1 has been shown to enhance selection efficiency of the natural Treg (nTreg) lineage, suggesting that NR4A contributions to agonist selection extend beyond the direct transactivation of lineage-associated genes (19, 46). In both a polyclonal and OT-II transgenic context,

deficiency in NR4A1 alone resulted in enhanced selection of CD4SP thymocytes into the Foxp3$^+$ Treg lineage and the early CD25$^+$ Foxp3$^-$ CD4$^+$ Treg precursor subset (18, 46) in a cell-intrinsic manner, the former of which displayed a normal transcriptional footprint and suppressive activity (46). These findings are intriguing as they suggest that in the absence of NR4A1 other NR4A family members are sufficient to drive Foxp3 expression, and that in addition to promoting Foxp3 expression, NR4A inhibits selection into the Treg lineage. Future studies are required to determine how NR4A1 negatively regulates Treg selection.

An additional outcome of high affinity TCR signaling is the generation of anergic phenotype FR4^{hi} CD73^{hi} CD4$^+$ T cells, which are also thought a precursor to Foxp3$^+$ Tregs (76–78), and which demonstrates enhanced thymic development in a NR4A1-deficient context (18). While this may be attributable to the cell-extrinsic generation of anergic phenotype CD4$^+$ T cells by Foxp3$^+$ Tregs, these anergic phenotype CD4$^+$ T cells may also feed into the Foxp3$^+$ Treg population as precursors when NR4A1 is deficient (18, 78). How NR4A1 impacts the relationship between Treg and anergic phenotype CD4$^+$ T cells requires further investigation. Finally, NR4A1 transgenic overexpression has most recently been associated with driving developing iNKT cell apoptosis and an "exhausted" phenotype in a cell-intrinsic and extrinsic manner (79). Continued study into the role of the NR4As in iNKT development and function may prove critical to understanding iNKT-driven autoimmune responses.

Future Perspectives

Given the reported differences in NR4A family member function across different MHC-restricted models and systems modeling UbA- and TRA-mediated negative selection, it appears probable that the NR4As perform multiple specific roles—both nuclear and extranuclear—within discrete lineages and stages of T cell development. Differences observed between MHC I- and MHC II-restricted TCR models could be due to the differential selection contexts determined in part by the type of antigen presenting cell (APC). This would include antigen processing and presentation efficiency and associated co-stimulatory molecules (6). In future studies, the importance of heeding differences in model systems is thus apparent. In this regard, the non-physiological timing of typical TCR transgenes may introduce additional difficulty in interpreting the roles of the NR4As (44, 79, 80). In addition, the classically utilized NR4A1 "knock-out" results in the translation of the N-terminal 117 amino acids of NR4A1, which is not present with Cre-Lox removal of the NR4A1 translational start codon. This truncated NR4A1 is not inert, but is in fact associated with liver immune infiltration, loss of splenic architecture, and altered hematopoietic stem cell homeostasis (81). The N-terminal region of NR4A1 has been shown to inhibit the MDM-2 induced degradation of HIF-1α, which regulates HSC mobilization (82, 83). Whether this feature of the germline NR4A1 KO influences thymocyte development is unclear, but suggests additional studies using the conditional NR4A1 KO be considered.

NR4As AND PERIPHERAL CD8$^+$ T CELL RESPONSES

It was rapidly realized that NR4A expression was not only induced in thymocytes following TCR stimulation but also in peripheral mature T cells. Their possible role in T cell response was first suggested by the identification of a correlation between their expression and the ability of T cells to differentiate into memory T cells (84, 85). Recent studies have revealed an important role for NR4As during acute and chronic CD8$^+$ T cell responses (**Table 1**).

CD8$^+$ T cells are potent cells of the adaptive immune system able to eradicate intracellular infections, control chronic infections, and eliminate tumors. The success of a primary immune response to acute infection requires proper control of cell fate to generate a large number of short-lived effector cells (SLECs; CD127^{lo}KLRG1^{hi}) that will control the pathogen and memory precursor effector cells (MPECs; CD127^{hi}KLRG1^{lo}) that differentiate into long-lived memory CD8$^+$ T cells to confer long-term protection. In chronic infection or cancer, where antigen and inflammation persist, T cell exhaustion is associated with an expression of inhibitory receptors

TABLE 1 | Role of NR4A family members in CD8 T cells.

Expression	***Nr4a1***	***Nr4a2***	***Nr4a3***	**References**
Rapid and transient induction by *in vitro* TCR stimulation	✓	✓	✓	(42, 85–90)
Rapid and transient induction during *in vivo* acute response	✓	✓	✓	(42, 87, 89, 91)
Constitutive expression by resident memory T cells	✓	✓	✓	(92–95)
High expression by exhausted T cells	✓	✓	✓	(47, 48, 67, 96–99)
Function	**NR4A1**	**NR4A2**	**NR4A3**	**References**
T cell proliferation	↓	N.D.	No effect	(68, 88, 89)
Cytokine production	↓	N.D.	↓	(67, 68, 88, 89)
SLEC differentiation	↓ or no effect	N.D.	↑	(68, 88, 89)
MPEC differentiation	↑ or no effect	N.D.	↓	(66, 88, 89)
Central memory T cell generation	No effect	N.D.	↓	(89, 100)
Resident memory T cell generation	↑	↑	↑	(94, 95, 100)
T cell exhaustion	↑	↑	↑	(48, 67, 88, 89)
Molecular mechanism	**NR4A1**	**NR4A2**	**NR4A3**	**References**
Competition for bZIP transcription binding activity	✓	N.D.	✓	(67, 89)

(PD1, Tim3, 2B4, Lag3, etc.) and a progressive loss of T cell functions (101). During chronic response, exhausted $CD8^+$ T cells can be divided into stem-like, transitory, and terminally differentiated subsets (102–104). The restoration of T cell functions following checkpoint blockade (e.g., anti-PD1 or anti-PD-L1) has been reported to act on the stem-like subsets.

Expression of NR4As During Acute $CD8^+$ T Cell Responses

Studies showing that *Nr4a* gene transcription was induced in thymocytes by TCR signaling raised the possibility that this could similarly occur in mature $CD8^+$ T cells. Using the NR4A1-GFP reporter mouse model, Moran et al. have shown that only TCR signaling, not inflammatory signals, can rapidly induce *Nr4a1* transcription both *in vitro* and *in vivo* and that the level of GFP expression is proportional to the strength of TCR signaling (42). This reporter mouse model is now widely used to measure the *in vivo* timing and strength of TCR signaling in thymocytes and peripheral T cells as GFP^+ cells are those that have recently received a TCR signal. The induction of the other NR4A family members during $CD8^+$ T cell responses was first supported by the rapid and transient transcription of *Nr4a1*, *Nr4a2*, and *Nr4a3* during the immune response to *Listeria monocytogenes* infection (91) with a peak of expression at 12h post T cell activation and a return to baseline levels at 48h. This is consistent with induction by TCR signaling and suggests an early role for NR4As in $CD8^+$ T cell response. Later on, it was shown using single cell RNA sequencing (scRNAseq) of *in vitro* antigen-stimulated $CD8^+$ T cells that *Nr4a* genes transcription is weak in unstimulated $CD8^+$ T cells, high at 1 and 3h post-TCR stimulation and already lower at 6h (86). Furthermore, the level of *Nr4a1* mRNA was proportional to the strength of TCR signaling (86). Finally, it was reported that NR4A1 expression is also an accurate and specific marker to identify human T cells that have recently been activated *via* their TCRs thus validating the use of *Nr4a1* induction as specific marker of recent TCR signaling (90).

The fact that *Nr4a3* is also induced following TCR stimulation has led to the development of *Nr4a3*-Tocky reporter mouse. Instead of GFP, Tocky reporter protein possesses time-dependent decay fluorescence shifting its emission from blue to red. This property allows for the observation of transient versus persistent TCR activation both *in vitro* and *in vivo* (87).

Although all these studies identified NR4As as early immediate genes induced by TCR signaling, the identification of the role of this induction in peripheral immune responses has only been recently uncovered.

Contribution of NR4A Family Members During Acute $CD8^+$ T Cell Response

Transcriptomic studies suggesting that NR4As may have an important role in the early stages of the $CD8^+$ T cell response (91) were later supported by the analysis of the dynamics of chromatin accessibility following T cell activation (48, 105). Indeed, the NBRE motif was enriched in chromatin regions that are highly accessible following acute $CD8^+$ T cell stimulation (48), as early as 2h post *in vitro* TCR activation (105). This enrichment was maintained up to 24h post-activation but was less apparent in *in vivo* effector $CD8^+$ T cells (at day 7 post-infection) and memory $CD8^+$ T cells (105). Altogether, this dynamically regulated chromatin accessibility from naïve to recently activated cells suggested a role for NR4A transcription factors during early $CD8^+$ T cell activation, which was then revealed by different groups.

In a first study, $Nr4a1^{-/-}$ mice showed better $CD8^+$ T cell proliferation following *in vitro* anti-CD3 or antigen stimulation. Similar enhancement of $CD8^+$ T cell proliferation was observed *in vivo* after adoptive transfer of $Nr4a1^{-/-}$ $CD8^+$ T cells into wild-type recipient followed by antigen administration or into lymphopenic MHC class I-deficient hosts (68). Furthermore, *ex vivo* production of IFN-γ by $CD8^+$ T cells is increased in absence of NR4A1 (68). Using full body knock-out $Nr4a1^{-/-}$ mice, the authors also showed increased Ag-specific $CD8^+$ T cell expansion, SLEC generation, and granzyme B production following infection with *Listeria monocytogenes* (68). Unfortunately, cytokine production or the generation of $CD8^+$ T cell memory were not evaluated in this setting. A more recent study using adoptive transfer of $Nr4a1^{-/-}$ TCR transgenic $CD8^+$ T cells followed by acute LCMV infection showed that NR4A1 deficiency increased $CD8^+$ T cell expansion and function but did not affect MPEC/SLEC differentiation, although T-bet expression, a transcription factor important for SLEC generation, was increased (88). The discrepancy on the effect of NR4A1 on MPEC/SLEC differentiation between the two studies might be due to the use of a full body knock-out *versus* T cell specific deletion (68, 88). We recently demonstrated that NR4A3 also influences $CD8^+$ T cell differentiation during acute response to *Listeria* and LCMV infection and vaccination (89). NR4A3 ablation in $CD8^+$ T cells did not affect Ag-specific T cell expansion but did influence MPEC/SLEC differentiation and cytokine production. Indeed, $Nr4a3^{-/-}$ $CD8^+$ T cells differentiated less into SLECs and more into MPECs, As a consequence, more central memory $CD8^+$ T cells were generated in the absence of NR4A3 (89). Similar to $Nr4a1^{-/-}$ $CD8^+$ T cells, NR4A3 deletion enhanced cytokine production (IL-2, IFN-γ, and TNF-α) by Ag-specific $CD8^+$ T cells. Therefore, NR4A1 and NR4A3 have a similar impact on cytokine production but not on MPEC/SLEC differentiation, suggesting that these have redundant and non-redundant functions. Further studies should reveal if NR4A2 induction contributes to $CD8^+$ T cell response.

While *Nr4a* are immediate early response genes that are rapidly induced following TCR activation, they are also transcribed by resident memory $CD8^+$ T cells (Trm), a subset of memory T cells that establish permanent residency at the site of infection. The transcription of *Nr4a* genes is part of the gene signature characterizing $CD8^+$ Trm cells (92, 93, 95) and a study aiming at identifying transcriptional factors involved in $CD8^+$ Trm cell differentiation identified NR4As among possible important regulators (95). Indeed, in a pooled shRNA screen, $CD8^+$ T cells containing shRNAs against *Nr4a1*, *Nr4a2*, and *Nr4a3* were less present in the Trm pool (95).

Boddupalli et al. formally demonstrated the importance of NR4A1 in Trm cells. In the context of influenza infection, NR4A1 deficiency in $CD8^+$ T cells decreased the number of Trm cells in the liver, Peyer patches, and intestinal epithelial lymphocytes (IELs) without any effect on lungs or bone marrow $CD8^+$ Trm cells (100). Other subsets of memory $CD8^+$ T cells, effector memory (Tem) and central memory (Tcm), which do not transcribe *Nr4a1*, were not affected by NR4A1 deficiency, demonstrating a specific requirement for *Nr4a1* in Trm biology (100). This is in opposition to the role NR4A3 plays in the development of memory T cells, as we have observed enhanced $CD8^+$ Tcm cell generation in the absence of NR4A3 (89). Interestingly, recent studies evaluating the heterogeneity of Trm cells following LCMV infection by scRNA-Seq have revealed that *Nr4a1*, *Nr4a2*, and *Nr4a3* are particularly enriched in the highly functional $CD28^+$ subset of $CD8^+$ Trm cells and that knockdown of *Nr4a2* specifically decreased the proportion of these $CD28^+$ Trm cells (94). Further studies should be done to determine which signals mediate the expression of NR4A family members in $CD8^+$ Trm cells and how they play a role in Trm cell differentiation.

In summary, during an acute immune response, the expression of NR4As is rapidly induced and this contributes to $CD8^+$ T cell response (**Figure 2A** and **Table 1**). At the effector

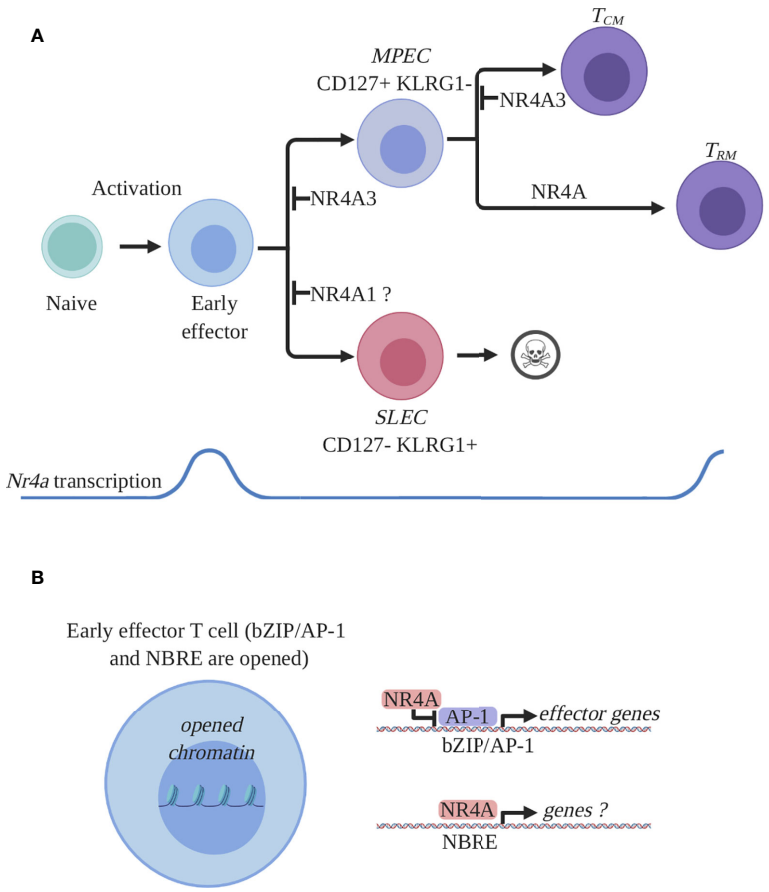

FIGURE 2 | NR4A family members expression and function in $CD8^+$ T cells during an acute immune response. **(A)** Expression and role of NR4A members in $CD8^+$ T cell response. Antigen recognition by naïve $CD8^+$ T cells will induce a transcriptional program responsible for activation, proliferation, and differentiation and proliferation. Among the activation-induced genes are all the *Nr4a* transcription factors which are rapidly and transiently induced at the early effector stage. Early effector $CD8^+$ T cells will further differentiate into effectors endowed with the ability to control the infection. Two main subpopulations of effectors are generated: short lived effector cells (SLEC) and memory precursor effector cells (MPEC). SLECs will die by apoptosis following pathogen clearance while MPECs will survive and differentiate into memory T cells. At the effector stage, NR4A1 was shown to either inhibit or have no effect on SLEC differentiation while NR4A3 was shown to diminish MPEC differentiation. At the memory stage, the *Nr4a* transcription was shown to be enriched in a particular subset of memory $CD8^+$ T cells, the resident memory $CD8^+$ T cells (Trm). All the NR4A family members participate in the differentiation of $CD8^+$ Trm cells while only NR4A3 was shown to influence central memory CD8 T cell (Tcm) differentiation. **(B)** Proposed molecular mechanism by which NR4A influences effector $CD8^+$ T cell differentiation. $CD8^+$ T cell activation will lead to the opening of the chromatin allowing for the transcriptional activity of different transcription factors involved in $CD8^+$ T cell response. Among these transcription factors are bZIPs and NR4As which bind bZIP or NBRE DNA-binding motifs. Thus, bZIP TFs will occupy their recognition motifs on DNA and will drive the transcription of the effector- and differentiation-related genes. NR4A will influence $CD8^+$ T cell transcriptional response by competing with bZIPs for DNA occupancy and by directly regulating genes containing NBRE motifs. The identity of the genes directly regulated by NR4A are still unknown. This figure was created with Biorender.com.

stage both NR4A1 and NR4A3 reduce cytokine production, while only NR4A3 promotes SLEC differentiation. At the memory stage, *Nr4a* genes are selectively transcribed by $CD8^+$ Trm cells with the three members possibly contributing to Trm cell differentiation while NR4A3 represses $CD8^+$ Tcm cell generation. As not all NR4As were properly studied at each differentiation steps of $CD8^+$ T cells, a full understanding of their respective role during acute $CD8^+$ T cell response await further studies.

Contribution of NR4A Family Members During Chronic $CD8^+$ T Cell Response

One of the first studies that predicted involvement of NR4As in $CD8^+$ T cell exhaustion was based on a model system called CA-RIT-NFAT where the NFAT protein was made constitutively active, but could not interact with the AP-1 transcription factor complex. This NFAT construct induces an exhausted/dysfunctional transcriptional program in $CD8^+$ T cells (96). In this model, transcription of both *Nr4a2* and *Nr4a3* was upregulated. A subsequent study also pointed toward a role for NR4As in $CD8^+$ T cell exhaustion during chronic LCMV infection. Using ATAC-seq, it showed that the NBRE motif was highly enriched in opened chromatin regions associated with $CD8^+$ T cell exhaustion (47). Similar observations were made in exhausted $CD8^+$ tumor-infiltrating T lymphocytes (TILs) where the NBRE motif was enriched in open chromatin regions of Ag-specific $CD8^+$ TILs when compared to bystander $CD8^+$ TILs (48). The transcription of *Nr4a2* and *Nr4a3* was upregulated in $CD8^+$ TILs in an autochthonous melanoma mouse model (97). Evidence for a similar involvement of NR4A in human exhausted $CD8^+$ T cells came from a study of TILs in colorectal cancer where *Nr4a1* transcription and NBRE motif in transcriptionally active hypomethylated DNA regions were enriched in Ag-specific $CD8^+$ TILs ($CD39^+CD103^+$) compared to bystander TILs ($CD39^-CD103^+$ or $CD39^-CD103^-$) (98).

The functional importance of NR4As in $CD8^+$ T cell exhaustion during cancer was recently described (67, 88). Chen et al. demonstrated in mouse melanoma that there was substantial enrichment of NBRE motifs in the open chromatin regions of exhausted TILs and that the expression of NR4As is highly enriched in severely exhausted ($PD\text{-}1^{hi}$ $Tim\text{-}3^{hi}$) compared to exhausted ($PD\text{-}1^{hi}$ $Tim\text{-}3^{lo}$) $CD8^+$ T cells (67). Similarly, the analysis of human melanoma TILs scRNA-Seq data highlighted a correlation of *Nr4a1*, *Nr4a2*, and *Nr4a3* transcription with inhibitory receptor expression (*Pdcd1* and *Havcr2*) (67). In addition to regulating the function of endogenous TIL, NR4As also regulated the function of chimeric antigen receptor (CAR) T cells. Adoptive T cell therapy (ACT) of B16 melanomas with CAR-T cells deficient for all three NR4A family members dramatically improved tumor control and survival over ACT with wild-type CAR-T cells (67). CAR TILs lacking NR4A1/2/3 expressed lower levels of the inhibitory receptors PD-1 and Tim-3 and produce more cytokines (TNF-α, IFN-γ) than their wild-type counterpart. In this setting, individual NR4A deletion did not confer any therapeutic effect suggesting redundant functions of the different NR4A members in $CD8^+$ T cell exhaustion (67).

A similar role for NR4A1 in $CD8^+$ T cell exhaustion was simultaneously reported by the group of Dong (88). In their study, the authors hypothesized a role for NR4A1 in $CD8^+$ T cell exhaustion based on the observation that *Nr4a1* transcription is abolished and that the NBRE motif is lost in open chromatin regions in $CD8^+$ TILs following anti-PD1 treatment, a treatment that reinvigorate exhausted T cells. They showed that ACT with *Nr4a1*$^{-/-}$ $CD8^+$ T cells provide better tumor control than ACT with wild-type $CD8^+$ T cells (88). This was associated with an increase in the number of CD8 TILs, reduction of PD-1 and Tim-3 expression, enhanced cytokine production (TNF-α and IFN-γ) as well as increased degranulation by $CD8^+$ TILs (88). Similar observations were made using chronic infection with LCMV clone 13 further supporting an essential role for NR4A1 in programming $CD8^+$ T cell exhaustion (88). This contrasts with the above CAR T cell model where the deletion of all three NR4A family members was required for therapeutic efficacy (67) and might result from more severe exhaustion in the CAR T cell system, which in turn requires the complete loss of NR4A activity.

Another recent study further supports the central role of NR4A family members in $CD8^+$ T cell exhaustion. It was shown that NR4A and TOX transcription factors act downstream of NFAT to induce the transcriptional program of exhaustion. Furthermore, a positive feedback loop where both TOX and NR4A positively regulate each other is at play during $CD8^+$ T cell exhaustion (99).

The role of NR4As in $CD8^+$ T cell exhaustion (**Table 1**) suggests that manipulating their expression or developing drugs that modulate their activity represents a very promising strategy to prevent exhaustion during cancer immunotherapy treatment. Before doing so, it will be essential to determine whether NR4As act similarly in human T cells and, if so, to consider if inhibitors targeting these molecules have the undesired side-effect of enhancing autoimmunity.

MOLECULAR MECHANISMS BY WHICH NR4As AFFECT ACUTE AND CHRONIC $CD8^+$ T CELL RESPONSE

Acute $CD8^+$ T Cell Response

As discussed above, there is experimental evidence suggesting a role for NR4A1 and NR4A3 in the $CD8^+$ T cell response to acute infection (68, 88, 89) with both members affecting the production of cytokines. Furthermore, NR4A1 and NR4A3 deficiency seems to have opposite effects on SLEC/MPEC differentiation and the three members were reported to have an impact on the memory generation (89, 94, 100). Very few studies have addressed the molecular events control by NR4As during $CD8^+$ T cell response.

The group of Hedrick has reported that NR4A1 directly binds to the *Irf4* promoter, an event that leads to the inhibition of *Irf4* transcription (68). This transcriptional repression could be

mediated *via* the demonstrated ability of NR4As in other settings to recruit the corepressor complex CoREST (43, 106) but a formal demonstration in $CD8^+$ T cells is still lacking. In the absence of NR4A1, elevated *Irf4* transcription could explain the increased T cell expansion, cytokine production, and SLEC differentiation (107–111). Whether the phenotype of NR4A1-deficient T cells is solely the consequence of change in IRF4 expression levels needs further investigation. Both NR4A1 and NR4A3 deficiency led to better cytokine production, however, using RNA-seq we did not observe an increase in *Irf4* transcription by *Nr4a3*$^{-/-}$ $CD8^+$ T cells (89), suggesting that other mechanisms are important in $CD8^+$ T cells. To gain insight into the mechanism of NR4A3 action during acute $CD8^+$ T cell response, we have used RNA-seq and ATAC-seq to identify the genes that are regulated by NR4A3. As NR4A3 is expressed very early following T cell activation, we performed these analyses at relatively early time points (12h after *in vitro* stimulation for ATAC-seq and *in vivo* day 3 post-infection for RNA-seq). The transcripts that are differentially expressed between *Nr4a3*$^{+/+}$ and *Nr4a3*$^{-/-}$ $CD8^+$ T cells were associated with the signature of memory T cells. Furthermore, as early as day 3 post-infection, the expression of the transcription factors controlling MPEC differentiation (*Eomes*, *Tcf7*, *Id3*, *Bcl6*, *Bach2*, and *Zeb1*) is increased in absence of NR4A3 while the transcription of transcription factors involved in SLEC differentiation is reduced (*Tbx21*, *Prdm1*, *Id2, Rbpj*, and *Zeb2*), which explains why more MPECs and memory T cells are generated without NR4A3 (89). Further studies are needed to determine whether NR4A3 directly regulates the expression of the transcription factors controlling MPEC/SLEC differentiation as ATAC-seq analysis did not reveal differences in chromatin accessibility at the genes encoding these transcription factors, except for *Bach2* and *Rbpj* (89). Within the differentially accessible regions (DARs) that are less open in *Nr4a3*$^{-/-}$ $CD8^+$ T cells, there was an expected enrichment for the NBRE motif, which suggests that several genes within these regions are direct targets of NR4A3. However, with the current knowledge, the list of genes within these regions did not help to explain how NR4A3 affects $CD8^+$ T cell differentiation and function (89). Intriguingly, most of the DARs are more opened in absence of NR4A3 and within these regions there is an enrichment for the DNA binding motifs for the transcription factors of the bZIP family, which includes Fos and Jun (89). It is therefore possible that NR4A3 prevents the activity of bZIP transcription factors during $CD8^+$ T cell response. As members of the bZIP transcription factor family such as AP-1 (Fos/Jun) and Bach2 are known to regulate cytokine production, these observations provide a mechanism for how NR4A3 influences T cell functions. However, whether the same mechanism contributes to enhance MPEC and central emory $CD8^+$ T cell differentiation requires further investigation and it is unclear how NR4A3 prevents the accessibility to DNA of bZIP transcription factors. In $CD4^+$ T cells, Liu et al. (discussed below) propose that NR4A1 directly compete with the binding of bZIP transcription factors to DNA (88). Combined with our ATAC-seq data in *Nr4a3*$^{-/-}$ CD8+ T cells, this suggests a common molecular mechanism used by different NR4A family members to influence gene transcription (**Figure 2B**). However, it remains unclear why NR4A1 and NR4A3 have different effects on MPEC/SLEC differentiation. Furthermore, how NR4A family members regulate $CD8^+$ Trm differentiation at the molecular level is still unknown, but of critical importance.

Chronic $CD8^+$ T Cell Response

NR4A family members are important players in the induction of $CD8^+$ T cell exhaustion (67, 88, 89). Transcriptomic analysis reveals that better tumor control by NR4A triple-deficient $CD8^+$ T cells is associated with the induction of the effector T cells gene signature while those for exhausted and memory T cells were down-regulated (67). ATAC-seq further showed that a large fraction (36%) of DARs with lower accessibility in NR4A triple-deficient TILs contains the NBRE motif. A smaller fraction (11%) of DARs with lower accessibility contains a NFAT binding motif without an adjacent AP-1 site, a molecular pattern that is found within chromatin regions associated with exhaustion (47, 67). Interestingly, the DARs less accessible in NR4A triple-deficient TILs are very similar to those more open in $CD8^+$ T cells expressing the exhaustion-inducing engineered form of NFAT that cannot interact with AP-1 (CA-RIT-NFAT) (47, 48, 96). Overall this suggests that NR4As directly contribute to the regulation of genes involved in $CD8^+$ T cell exhaustion and explains why their deletion reduces T cell exhaustion. On the other hand, DARs that are more accessible in absence of NR4A members are enriched in the DNA binding motifs of bZIP (71%) and Rel/NFKB (25%) transcription factors (67). As these transcription factors have been reported to control T cell activation and effector functions, these molecular changes are probably responsible for the enhanced functionality that is observed in TILs deficient for NR4A family members (**Figure 3**). It is interesting to note that a similar impact on accessibility of chromatin regions containing bZIP transcription factor binding motifs was observed in $CD8^+$ T cells lacking NR4A3 expression during response to acute *Listeria* infection and in $CD4^+$ T cells lacking NR4A1 (88, 89). Limiting access of bZIP transcription factors to DNA is therefore a general mechanism of action of NR4As. Further studies should reveal whether each NR4A family member affects different sets of genes and/or influences different members of the bZIP transcription family.

NR4As AND PERIPHERAL $CD4^+$ T CELL RESPONSES

$CD4^+$ T cells act as central orchestrator of the adaptive immune response to pathogens and cancer. These cells help a variety of other immune cells, such as B cells, macrophages, and $CD8^+$ T cells, to mount an immune response that is adapted to the type of infectious agents. Following Ag recognition of peptide fragments on MHC class II molecules on DCs, naïve $CD4^+$ T cells will proliferate and differentiate into effector cells. Depending on the inflammatory context induced by the infecting pathogen, naïve $CD4^+$ T cells can differentiate into several types of effectors: Th1, Th2, Th17, Tfh, or Treg. Briefly, Th1 cells are characterized by their ability to secrete

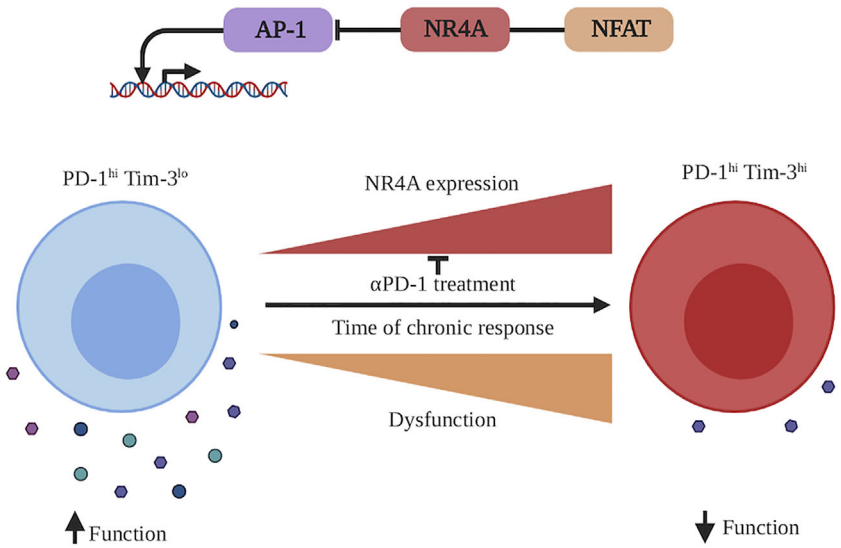

FIGURE 3 | NR4A involvement in CD8+ T cell exhaustion during a chronic immune response. During a chronic immune response, antigen persistence as well as inflammation milieu will induce $CD8^+$ T cell exhaustion. This state is characterized by the acquisition of the expression of different inhibitory receptors (as PD-1, Tim-3 etc.), which dampens $CD8^+$ T cell function to protect the organism against the chronically activated $CD8^+$ T cells. $CD8^+$ T cell exhaustion is accompanied by an increased transcription of *Nr4a1, Nr4a2*, *and Nr4a3*. At the molecular level, NR4A family members cooperate with NFAT and potentially other transcription factors to decrease the activity of AP-1 (bZIP family transcription factor) and increase the dysfunctional/exhaustion state of the CD8+ T cells. An effective reinvigorating therapy to reverse $CD8^+$ T cell dysfunction is treatment with anti-PD-1 antibodies, which decreases the *Nr4a* transcription. This figure was created with Biorender.com.

IFN-γ, which will help to induce anti-microbial activity of macrophages and enhance $CD8^+$ T cell response, Th2 production of IL-4 will help B cells to undergo class-switch to IgE, Th17 production of IL-17 will help recruit neutrophils, Tfh (T follicular helper) cells will help B cell responses and Treg cells will suppress the response of auto-reactive T cells. As for $CD8^+$ T cells, after pathogen clearance most of the effectors will die while a few will further differentiate into long-lived $CD4^+$ memory T cells.

In naïve CD4 T cells, NR4A1 is expressed at low level as a consequence of homeostatic/tonic TCR signaling (39). *Nr4a* transcription is also rapidly induced in $CD4^+$ T cells following engagement of the TCR by Ag. *In vitro*, this occurs within 1h of stimulation, peaks after 3–4h and return to basal level at 12h (38, 69, 112). The studies that have revealed some of the role of NR4A family members in $CD4^+$ T-cell biology will now be discussed (**Table 2**).

TABLE 2 | Role of NR4A family members in CD4 T cells.

Expression	***Nr4a1***	***Nr4a2***	***Nr4a3***	**References**
Rapid and transient induction by *in vitro* TCR stimulation	✓	✓	✓	(38, 69, 74, 87, 88, 90, 112)
Th1	Low	Low	Low	(113)
Th2	Low	Low	Low	(113)
Th17	Low	Low	Low	(113)
Tfh	✓ or low	✓ or low	Low	(112, 113)
T reg	✓	✓	✓	(113)
Dysfunctional/exhausted or tolerant CD4 T cells	✓or low	✓or low	✓	(88, 96, 97)
Function	**NR4A1**	**NR4A2**	**NR4A3**	**References**
T cell proliferation	↓	N.D.	N.D.	(69)
Cytokine production	↓	↑	N.D.	(69, 88, 114)
Th1 polarization	↓	↓ or no effect	N.D.	(69, 74, 88, 115)
Th2 polarization	No effect	N.D.	N.D.	(88)
Th17 polarization	↓	↓ or ↑	N.D.	(69, 74, 88, 115)
iTreg differentiation	No effect	↑	N.D.	(74, 88)
Treg identity	↑	↑	↑	(113)
Tfh development and function *in vivo*	No effect in TKO	No effect in TKO	No effect in TKO	(112)
T cell tolerance	↑	N.D.	N.D.	(88)
Molecular mechanism	**NR4A1**	**NR4A2**	**NR4A3**	**References**
Competition for bZIP transcription binding activity	✓	N.D.	N.D.	(88)

The transcription of *Nr4a2* is highly enriched in peripheral blood T cells of multiple sclerosis (MS) patients and in T cells during experimental autoimmune encephalomyelitis (EAE), a mouse model of MS (114, 116). The overexpression of NR4A2 in primary mouse T cells increased the production of IFN-γ and IL-17, the main cytokines involved in MS/EAE pathogenesis. Conversely, its suppression decreased IFN-γ and IL-17 production and the induction of EAE was reduced following the adoptive transfer of encephalitogenic $CD4^+$ T cells in which NR4A2 expression was reduced using siRNA when compared to control $CD4^+$ T cells (114). Importantly, siRNA-mediated knockdown of NR4A2 in $CD4^+$ T cells from MS patients also led to reduced IFN-γ and IL-17 production (114). A luciferase promoter assay suggests that NR4A2 acts directly on the transcription of *Ifng* and *Il17* genes (114). A follow-up study by the same group, showed *Nr4a2* transcription was selectively higher in IL-17- or IL-17/IFN-γ-producing $CD4^+$ T cells when compared to IFN-γ-producing $CD4^+$ T cells during EAE and experimental autoimmune uveitis (EAU) (115). This increase in NR4A2 expression by autoimmune T cells was not observed in the STZ model of autoimmune diabetes, which is mediated by Th1 cells, suggesting that the enhanced expression of NR4A2 is associated with autoimmune diseases where IL-17 plays a pathogenic role (115). To understand the role of NR4A2 induction in IL-17 production by $CD4^+$ T cells the authors used siRNA knockdown of *Nr4a2* and showed that NR4A2 decreases *in vitro* Th17 differentiation but not Th1 differentiation. The effect on Th17 differentiation was not due to decreased expression of RORγt, the master transcription factor controlling Th17 differentiation. Instead, NR4A2 was necessary for the production of IL-21, which then upregulates the expression of the IL-23 receptor, necessary to enhance and stabilize the Th17 phenotype. Furthermore, the addition of IL-21 rescued Th17 differentiation by *Nr4a2* knockdown $CD4^+$ T cells (115). Injection of mice, early or late during EAE, with siRNA directed against *Nr4a2* was able to significantly reduce EAE clinical scores with a concomitant decrease of IL-17, but not IFN-γ, production by $CD4^+$ T cells that have infiltrated the central nervous system (115). Therefore, NR4A2 expression in $CD4^+$ T cells promotes Th17 differentiation and targeting its expression represents a promising strategy to treat MS patients. It is intriguing that NR4A2 promotes cytokine production by $CD4^+$ T cells since NR4A1 and NR4A3 were shown to dampen cytokine production by $CD8^+$ T cells. As the three family members recognize the same motifs on DNA, further studies are required to determine whether this is the consequence of a different function of NR4A2 or a cell type specific effect.

Although NR4A expression is transiently induced following $CD4^+$ T cell activation, it was reported that Tfh cells transcribe *Nr4a1* and *Nr4a2* (112). This is probably the consequence of the continuous TCR stimulation of Tfh cells by antigen-presenting cognate B cells within the germinal centers. However, the deletion of the three family members in $CD4^+$ T cells did not affect Tfh differentiation and function (112).

The role of the NR4A family members in $CD4^+$ T cell response was recently broadened by the identification of the involvement of NR4A1 in $CD4^+$ T cell activation, metabolism, tolerance, and autoimmunity (69, 88). Liebmann et al. demonstrated that NR4A1 deletion in $CD4^+$ T cells enhances T cell proliferation and cytokine production both *in vitro* and *in vivo* (69). The deletion of *Nr4a1* in the 2D2 TCR transgenic mouse model of EAE led to accelerated and more severe disease with an increase in IFN-γ and IL-17 secreting $CD4^+$ T cells within the central nervous system. The authors further confirmed that it was the lack of NR4A1 in T cells that was involved using adoptive T cell transfer experiments (69). They also showed a general role for NR4A1 in different T cell mediated inflammatory diseases such as allergic contact dermatis and collagen-induced arthritis (69). Increased proliferation in absence of NR4A1 was not the consequence of reduced apoptosis but correlated with an increase in cell cycle entry. As entry into the cell cycle is regulated by metabolism, the authors evaluated whether NR4A1 deficiency impacted T cell metabolism. In absence of NR4A1, activated $CD4^+$ T cells showed increased respiration, glycolysis, and glycolytic activity. As a consequence, the pharmacological inhibition of respiration or glycolysis had much less effect on proliferation of $Nr4a1^{-/-}$ than $Nr4a1^{+/+}$ $CD4^+$ T cells (69). In agreement with a role for NR4A1 in regulating T cell metabolism was the regulation of several genes involved in T cell metabolism such as electron transport genes and genes controlling glucose metabolism. Intriguingly, the analysis of motifs within the promoters of metabolic genes that are differentially expressed between $Nr4a1^{-/-}$ and $Nr4a1^{+/+}$ activated $CD4^+$ T cells did not reveal NR4A1 as a possible upstream regulator but predicted a role for the nuclear receptors ERRα, ERRγ, ERRβ, NR2F1, and NR0B1. Furthermore, NR4A1 was shown to bind to the *Esrra* gene, encoding for ERRα. The authors demonstrated that pharmacological inhibition of ERRα or shRNA knockdown of *Esrra* partially reversed the phenotype (cytokine production and metabolism) of NR4A1-deficient $CD4^+$ T cells and reduced EAE disease severity of $Nr4a1^{-/-}$ mice (69). This highlights the key role of NR4A1 transcriptional induction of *Esrra*. Further studies should reveal which other mechanisms contribute to the $CD4^+$ T cell phenotype in absence of NR4A1 and whether other family members regulate T cell metabolism.

A pivotal role of NR4A1 in $CD4^+$ T cell dysfunction was recently described by the group of Dong (88). The authors observed a specific upregulation of NR4A1 in $CD4^+$ tolerant T cells. The overexpression of NR4A1 in $CD4^+$ T cells strongly suppressed the expression of genes associated with effector functions while inducing the expression of anergy-related genes following TCR stimulation. Under Th polarizing culture conditions, overexpression of NR4A1 impaired Th1 and Th17 differentiation without affecting Treg and Th2 generation. On the other hand, the inactivation of *Nr4a1* in $CD4^+$ T cells enhanced IL-2 and IFN-γ production. This suggests that NR4A1 is overexpressed in $CD4^+$ tolerant T cells precisely to induce tolerance. This was tested *in vivo* using an oral tolerance model where *Nr4a1* deletion increased IL-2 and IFN-γ production and prevented the establishment of $CD4^+$ T cell tolerance. Further supporting the role for NR4A1 in repressing

effector functions of $CD4^+$ T cells, the adoptive transfer of naïve *Nr4a1*$^{-/-}$ $CD4^+$ T cells into RAG-deficient mice induced more severe colitis than wild-type $CD4^+$ T cells, with an increase in IFN-γ and IL-17 producing T cells in the colon (88). The comparison of the transcriptome of NR4A1 overexpressing $CD4^+$ T cells and $CD4^+$ tolerant T cells revealed a common gene signature containing a core cluster of genes controlling T cell activation or dysfunction. A ChIP-seq experiment revealed that approximately 70% of the $CD4^+$ tolerance T cells genes were direct targets of NR4A1. Further analysis of the ChIP-seq data unexpectedly identified AP-1 consensus sequences and canonical NBRE motifs at sites where NR4A1 bound. This suggests that NR4A1 might be able to compete with AP-1 (bZIP) family members for overlapping sites on DNA and would explain the fact that most of the NR4A1 target genes that were downregulated by NR4A1 overexpression had reduced c-Jun binding. Inhibition of AP-1 activity by NR4A1 was demonstrated using luciferase reporter and EMSA assays suggesting that after being recruited to AP-1 binding sites, NR4A1 can repress effector gene expression. In addition, for the genes bound by NR4A1 and whose transcription is increased by NR4A1, these largely co-localized with H3K27 acetylation marks and include genes involved in tolerance. In summary, NR4A1 modulates the transcriptional program of $CD4^+$ T cells by directly upregulating the expression of target genes containing NBRE motifs while downregulating the expression of AP-1 target genes (88). Interestingly, the repression of AP-1 target genes by NR4As is important in $CD8^+$ T cells (67, 89) indicating that this is general mechanism by which they repress effector functions in T cells. Whether NR4A members solely influences the activity of AP-1 requires future investigation as other transcription factor binding motifs, such as NFKB, were identified in open chromatin regions of NR4A-deficient $CD8^+$ T cells (67, 89). In agreement with this possibility is an older study performed in the Jurkat T cell lines showing that NR4A1 can compete with NFKB for binding to DNA (117).

NR4A-family members do not only have a role in the development of regulatory $CD4^+$ T cells, as discussed above, but they are also required to maintain a pool of fully functional Tregs. NR4A1, NR4A2 and NR4A3 protein and transcript levels in peripheral Treg largely exceed those measured in other mature T cell subsets (42, 113). To evaluate the importance of NR4As in more mature Treg cells, specific deletion of *Nr4a1* and *Nr4a2* in this subset was obtained using a *Foxp3* Cre/Lox system (113). This was combined to a germline *Nr4a3* deletion to generate *Nr4a* triple knockout (TKO) in Foxp3 expressing cells. While generated efficiently in the thymus, Treg cells from these mice have a competitive disadvantage in the periphery (113). In addition, TKO Treg cells have decreased Foxp3 expression, lose their suppressive functions and gain Tfh and Th2 gene expression programs (113). This is because, as demonstrated by NR4A1 ChIP-seq, NR4As directly bind and maintain active chromatin marks on Treg-associated genes *Foxp3, Il2ra*, and *Ikzf4*. In addition, specifically in Tregs, NR4A1 directly binds and represses the *Il4* and *Il21* loci. A luciferase reporter assay also demonstrated that NR4A2 suppresses *Il4* promoter activity. Therefore, in mature $CD4^+$ Treg cells, NR4As serve to maintain regulatory identity while suppressing Th2 and Tfh programs. The different NR4A family members vary in their capacity to induce Foxp3 expression (74) and the fact that the effects on Treg function was, at least partially, reproduced in single *Nr4a2* deficient mice suggests that perhaps NR4A2 could be the main driver of Treg identity in mature CD4 T cells (74). However this is in slight contradiction with the fact that autoimmunity and reduced lifespan was observed in *Nr4a1*/*Nr4a3* but not in *Nr4a2*/*Nr4a3* double deficient mice (113). Finally, conditional acute deletion of *Nr4a1* and/or *Nr4a2* in ERT2-Cre mice resulted in the loss of Treg-associated transcriptional targets and inhibited the *in vitro* differentiation of inducible Treg cells (74, 113). It is thus unlikely that the effects observed in TKO mice generated with the Foxp3-Cre system are solely the consequence of poorly matured $Foxp3^+$ thymocytes (74). The dependence of Treg cells on NR4A expression makes it a possible target for therapy. For example, in the tumor microenvironment, regulatory T cells are detrimental and are associated with poor prognosis (118–120). Treg-specific *Nr4a1* and *Nr4a2* deficient mice have increased tumor resistance and pharmacological treatments that inhibit the expression of these NR4As in tumoral Treg cells result in improved $CD8^+$ T cell functions and tumor control (121).

The early induction of NR4A in $CD4^+$ T cells by TCR signaling influences proliferation, metabolism, function, and differentiation of conventional $CD4^+$ T cells. Interestingly, the deletion of NR4A1 or NR4A2 seems to have different impact on T cell functions where NR4A1 suppress effector gene expression while NR4A2 positively contributes to the expression of cytokine genes and Th17 polarization. As a consequence suppressing NR4A1 expression promotes autoimmunity while deletion of NR4A2 protects from Th17-mediated autoimmune diseases. Furthermore, NR4A1 contributes to the induction of the tolerance program in $CD4^+$ T cells. Therefore, targeting of a specific family member will offer unique therapeutic opportunity to either enhance or inhibit $CD4^+$ T cell response. Furthermore, NR4As play important role in the maintenance of Treg cell identity. Future studies are needed to evaluate whether NR4A3 contributes to $CD4^+$ T cell response and whether any member influences memory $CD4^+$ T cell development. Altogether, this underlies the importance of teasing apart the unique role of each of NR4A family member in $CD4^+$ T cells as this information will be key for being specifically able to appropriately target CD4-mediated autoimmune/inflammatory diseases, to enhance anti-tumor response and to therapeutically induce tolerance.

CONCLUDING REMARKS

In T-cell biology, NR4A1, NR4A2, and NR4A3 have long been considered as functionally redundant. While this certainly appears to be true to some extent, there is also significant evidence that shows that, as a function of the model, cell type, or the measured output, these molecules have some unique roles. Given the important therapeutic roles NR4As could play in

autoimmunity, vaccination, or cancer, it is exciting to think that there is still significant work left to understand their common and distinctive molecular modes of action.

AUTHOR CONTRIBUTIONS

LO, JM, SB, TB, and NL wrote the manuscript and prepared the figures. TB and NL edited the manuscript. LO and JM contributed equally to the work. All authors contributed to the article and approved the submitted version.

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Chenodeoxycholic Acid Modulates Bile Acid Synthesis Independent of Fibroblast Growth Factor 19 in Primary Human Hepatocytes

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Bile acids (BAs) are detergents essential for intestinal absorption of lipids. Disruption of BA homeostasis can lead to severe liver damage. BA metabolism is therefore under strict regulation by sophisticated feedback mechanisms. The hormone-like protein Fibroblast growth factor 19 (FGF19) is essential for maintaining BA homeostasis by down regulating BA synthesis. Here, the impact of both FGF19 and chenodeoxycholic acid (CDCA) on primary human hepatocytes was investigated and a possible autocrine/paracrine function of FGF19 in regulation of BA synthesis evaluated. Primary human hepatocytes were treated with CDCA, recombinant FGF19 or conditioned medium containing endogenously produced FGF19. RNA sequencing revealed that treatment with CDCA causes deregulation of transcripts involved in BA metabolism, whereas treatment with FGF19 had minor effects. CDCA increased FGF19 mRNA expression within 1 h. We detected secretion of the resulting FGF19 protein into medium, mimicking *in vivo* observations. Furthermore, medium enriched with endogenously produced FGF19 reduced BA synthesis by down regulating CYP7A1 gene expression. However, following knockdown of FGF19, CDCA still independently decreased BA synthesis, presumably through the regulatory protein small heterodimer partner (SHP). In summary, we show that in primary human hepatocytes CDCA regulates BA synthesis in an FGF19-independent manner.

Keywords: bile acid metabolism, cholesterol 7-alpha hydroxylase, liver, transcription, nuclear receptors, RNA sequencing

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; CYP7A1, cholesterol 7-alpha hydroxylase; ERK, extracellular signal-regulated kinase; FGF19, fibroblast growth factor 19; FGFR4, fibroblast growth factor receptor 4; FXR, Farnesoid X receptor; SHP, small heterodimer partner.

INTRODUCTION

Bile acids (BAs), synthesized from cholesterol by the liver, enter the enterohepatic circulation to function as detergents in the intestine for absorption of dietary lipids. This efficient system ensures that the majority of BA returns from the intestine to the liver and only 1–5% of the total pool need to be replaced by newly synthesized BAs on a daily basis (1–3). BA homeostasis is essential, and disturbance can cause severe complications such as malabsorption of nutrients and excess BAs may cause cell injury that in turn leads to liver fibrosis and cirrhosis. Synthesis, transport and circulation of BAs are therefore under rigorous control (1–5). BAs act as signaling molecules to the nuclear receptor Farnesoid X receptor (FXR) in both intestine and liver and FXR in turn induces regulatory pathways (5–7). In recent years, the FXR induced hormone-like protein Fibroblast growth factor 19 (FGF19) has been of particular interest in respect to BA regulation. Upon activation by re-absorbed BAs, intestinal FXR induces FGF19 expression. FGF19 is released to the portal blood stream and when reaching the liver it signals to suppress the rate-limiting enzyme in BA synthesis, cholesterol 7-alpha hydroxylase (CYP7A1) (8–10). FGF19 expression is low or absent in healthy liver and this is mirrored in cultures of primary human hepatocytes. The liver starts expressing FGF19 and circulating levels increase under pathophysiological conditions for example when bile flow from the liver is restricted. Furthermore, primary human hepatocytes express FGF19 when treated with chenodeoxycholic acid (CDCA), the primary BA with highest affinity for FXR in humans (3, 8, 9, 11, 12). We have previously established circulating levels of FGF19 under physiological conditions and demonstrated that FGF19, unlike BAs, do not display a gradient over the liver (13). With this study we aimed to gain a better understanding of how FGF19 affects primary human hepatocytes and in particular how it affects BA synthesis. We evaluated the impact of CDCA and recombinant FGF19, within the physiological range of concentrations, on primary human hepatocytes in respect to BA synthesis. The concentrations used were kept around the established postprandial levels in the portal circulation of FGF19 (approximately 400 pg/ml) and CDCA (approximately 10 μM) (10, 13, 14). We further investigated the effect of conditioned medium with endogenously produced FGF19 on primary human hepatocytes. A possible autocrine/paracrine function of FGF19 was evaluated by knockdown of FGF19 and inhibition of bile acid synthesis by CDCA was assessed. Differential expression in primary human hepatocytes by RNA sequencing following treatment with CDCA, recombinant FGF19 or endogenously produced FGF19 was investigated. In short, we demonstrated that although CDCA rapidly induced FGF19 in primary human hepatocytes, and conditioned medium suppressed CYP7A1, CDCA still efficiently downregulated CYP7A1 following FGF19 knockdown. Thus, CDCA regulate BA synthesis independently of FGF19.

MATERIALS AND METHODS

Isolation of Primary Human Hepatocytes

Primary human hepatocytes were isolated from patients undergoing liver resection, from extirpated livers or from donor livers rejected for transplantation. Information about the livers in each experiment is summarized in **Table 1**. The isolation procedure followed a three-step perfusion technique developed by Berry and Friend (15) and optimized for primary human hepatocytes by Strom et al. (16). Cells were cultured on matrigel derived from Engelbreth-Holm-Swarm sarcoma (Sigma-Aldrich, St. Louis, MO) and in William's E medium (Invitrogen, Waltham, MA) supplemented with 20 mM HEPES (Lonza, Basel, Switzerland), 2 mM glutamine (Sigma-Aldrich, St. Louis, MO), 10 nM insulin, 100 nM dexamethasone, 0.01 M gentamicin (Lonza, Basel, Switzerland) and 55 nM amphotericin B, for five days at 37°C in 5% CO2.

Treatment of Primary Human Hepatocytes

Cells were kept in culture for a total of 5 days prior to harvesting and reagents were added at concentrations and time points for the different experiments as follow.

For the dose-response experiment, cells were treated with CDCA (Sigma-Aldrich, St. Louis, MO) or recombinant FGF19 (R&D systems, Minneapolis, MN) for 24 h (n = 13) or 6 h (n = 10) before harvesting, at concentrations stated.

For the time course experiment (n = 3), 10 μM CDCA was added to cultures for 10 min and up to 6 h prior to harvesting.

For the endogenous experiment (n = 10), cells were treated with 40 μM CDCA (induction medium) or regular medium without CDCA (control medium) for 6 h. After washing the cells several times with fresh medium, new medium was added and cells were kept for an additional 18 h (conditioned/control medium). The conditioned medium with all its secreted compounds was then transferred to naïve cells and treated for 24 h with 100%, 50% or 10% of conditioned or control medium (see **Figure 5A** for a layout).

A summary of livers included in the respective experiments can be found in **Table 1**.

siRNA Gene Silencing of FGF19

Knockdown of FGF19 was performed using Silencer select siRNA and transfected by Lipofectamine RNAiMAX (ThermoFisher/Life Technologies, Carlsbad, CA). Lipofectamine/siRNA solution was prepared in Opti-MEM (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Cells were transfected with 100 pmol siRNA (FGF19, assay ID s19355) or non-targeting negative control siRNA (cat# 4390843) for 18 h prior to co-treatment with 10 μM CDCA for 6 h (n = 3), CDCA was added directly to the existing medium.

RNA Preparation and Quantification by qPCR

RNA was extracted using TRIzol (Invitrogen, Waltham, MA) according to manufacturer's instructions. Quantification of mRNA was performed in triplicates with TaqMan assays on an ABI Step-One Plus instrument (Applied Biosystems, Waltham, MA). Relative mRNA expression was calculated from Ct-values against the housekeeping genes Cyclophillin A and GAPDH. TaqMan probes were purchased from Applied Biosystems (Waltham, MA): Cyclophillin A—Hs99999904_m1; GAPDH—

TABLE 1 | Demographics.

Experiment	N	Analysis	Donor ID	Gender (F/M)	Age (year) Median (min-max), Mean (±SEM)	Cell viability % Median (min-max), Mean (±SEM)	Diagnosis
FGF19/ CDCA 24 h	13	qPCR (n = 13) ELISA (n = 13) BA analysis (n = 5)[#]	13,38[#],183[#], 188,189,192, 194,195,198, 359,414[#],425[#], 432[#]	8/5	47.0 (0–77) 49.1 (±7.4)	77.0 (62–94) 76.6 (±2.4)	Donor (n = 5), CRC (n = 3), CCC (n = 2), Cholangitis (n = 1), Hyperoxaluria (n = 1), Neuroendocrine tumor (n = 1)
FGF19/ CDCA 6 h	10	qPCR (n = 10) ELISA (n = 10) BA analysis (n = 5)[#] RNA sequencing (n = 3)[†]	16[†],38[#],207, 210[#],224[†],226[†], 359,414[#],425[#], 432[#]	3/7	46.0 (0–76) 39.4 (±10.6)	80.0 (71–94) 81.9 (±2.5)	Donor (n = 3), CRC (n = 1), CCC (n = 1), HCC (n = 2) Hyperoxaluria (n = 1), Alagille syndrome (n = 1), MSUD (n = 1)
Endogenous FGF19	10	qPCR (n = 10) ELISA (n = 10) BA analysis (n = 5)[#] RNA sequencing (n = 3)[†]	16[†],38[#],39[†], 219,375[#†],414[#], 425[#],432[#],444, 445	3/7	32.0 (0–76) 38.3 (±9.4)	80.0 (66–86) 76.9 (±2.0)	Donor (n = 5), CRC (n = 2), CCC (n = 1), MSUD (n = 1), Unknown (n = 1)
Time course	3	qPCR (n = 3) ELISA (N = 3)	414,425,432	1/2	73.0 (27–76) 58.7 (±15.9)	80.0 (71–86) 79.0 (±4.4)	Donor (n = 2), CCC (n = 1)
siRNA	3	qPCR (n = 3) ELISA (n = 3)	456,458,461	2/1	57 (12–76) 48.8 (±19.9)	77.0 (72–87) 78.7 (±4.4)	Donor (n = 1), CRC (n = 2)

[#]*Donors used for BA analysis.*
[†]*Donors used for RNA sequencing analysis.*
CRC, colorectal metastasis; HCC, hepatocellular carcinoma; CCC, cholangiocellular carcinoma; MSUD, maple syrup urine disease.
Characteristics and number of livers used to isolate cells for the respective experiments.

Hs02786624_g1; FGF19—Hs00192780_m1; CYP7A1—Hs00167982.

Quantification of FGF19 by ELISA

FGF19 concentration in cell supernatants was determined using ELISA (Human FGF19 Quantikine ELISA kit, R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Samples were analyzed in technical triplicates.

Extraction and Quantification of Bile Acids by GC-MS

Levels of cholic acid (CA) in cell supernatants was analyzed by extraction from 1 ml supernatant as first described by Björkhem and Falk (17). In short, cell supernatant was mixed with 2,500 ng deuterium-labeled internal standard (D_2-CDCA and D_4-CA) and 1 M potassium hydroxide and hydrolyzed overnight at 120°C. BAs were extracted by basic ether extraction followed by acidic ether extraction, methylated with trimethylsilyl diazomethane and converted into derivates with hexamethyldisilazane–trimethylchlorosilane–pyridine. All reagents were purchased from Sigma-Aldrich (St. Louis, MO). BAs were separated and quantified against a standard curve with GC-MS (6890 Network GC system/5973 Network mass selective detector, Agilent Technologies, Santa Clara, CA), using the MassHunter Workstation software, version B.04.00/ Build4.0.225.0 (Agilent Technologies, Santa Clara, CA).

RNA Sequencing

RNA sequencing was carried out on RNA from hepatocytes treated with either 10 μM CDCA or 1000 pg/ml recombinant FGF19 for 6 h and compared to non-treated control cells (n = 3), and on hepatocytes treated with 100% conditioned or control medium (n = 3). After depleting ribosomal RNA by using Ribo-Zero Gold (Illumina, San Diego, CA), RNA sequencing libraries were prepared using the Illumina TruSeq Stranded RNA Library Prep Kit v2 (dual index) according to the manufacturer's instruction. The quality of every cDNA library was determined on an Agilent Bioanalyzer instrument according to the manufacturer's protocol. Library concentrations were quantified with the KAPA-SYBR FAST qPCR kit, and referenced to the provided standards (Roche, Basel, Switzerland). The sequencing run was performed with the

NextSeq 500/550 High Output v2 kit (Illumina, San Diego, CA) for 150 cycles, paired end, on a NextSeq 500 instrument (Illumina, San Diego, CA). All raw data (fastq files) are deposited in ArrayExpress under accession number: E-MTAB-8627.

Quality Control and Processing of RNA Sequencing Data

Next generation sequencing read quality was assessed with FastQC (v.0.11.5). Adaptor sequences were trimmed, and low-quality reads removed using Trimmomatic (v.0.32). Sequencing reads aligning (Bowtie2, v.2.2.9) to annotated ribosomal RNA genes were discarded. High-quality and ribosomal RNA depleted sequencing reads were aligned to the genome using TopHAT2 (v.2.0.3). Using sorted bam files (Samtools v.1.5), the number of aligned reads was counted (HTSeq-count v.0.7.2, **Table S1**). After normalization (TMM: trimmed mean of M-values, **Table S2**), a differential gene expression analysis (edgeR v. 3.3.3 in R v. 3.4.3) was performed. Significant differentially expressed genes were distinguished by a false discovery rate (FDR) under 0.05. Gene ontology analysis was performed in R (v. 3.4.3) with clusterProfiler (v. 3.6.0) and org.Hs.eg.db (v. 3.5.0). Additionally, the following dependent package versions were installed: DOSE (v. 3.4.0), AnnotationDbi (v. 1.40.0), IRanges (v. 2.12.0), S4Vectors (v. 0.16.0), BiocGenerics (v. 0.24.0), and Biobase (v. 2.38.0). Scripts for the analysis are deposited on Github (https://github.com/jonasns/FGF19).

Statistics

Non-parametric tests were used, and graphs are presented as median with interquartile range (IQR). Wilcoxon matched-pairs signed rank test were used to assess differences between two groups and the Friedman test was used to evaluate differences between three or more groups. Dunn's multiple comparison test was used *post-hoc* when an overall significant difference was found with the Friedman test. Differences were considered significant when $p < 0.05$.

RESULTS

Treatment With CDCA, But Not Recombinant FGF19, Reduces BA Synthesis in Primary Human Hepatocytes

To explain physiological alterations in BA synthesis, we investigated the underlying molecular responses stimulated upon FGF19 and CDCA treatment in primary human hepatocytes. We measured CYP7A1 gene expression levels and CA concentrations secreted into the cell medium from primary human hepatocytes that were treated with various concentrations of recombinant FGF19 (400–1,200 pg/ml) or CDCA (3–20 μM) for 6 h or 24 h (**Figure 1**). No significant changes in CYP7A1 gene expression levels were found upon treatment of hepatocytes with various concentrations of recombinant FGF19 at any time point when compared to untreated controls. In contrast, CYP7A1 gene expression levels were significantly lower in cultures treated with CDCA at concentrations above 3 μM for both time points compared to controls (**Figures 1A, B**). In parallel, we determined that median levels of CA in the cell supernatant was approximately two-fold lower in hepatocytes cultures after 24 h treatment with 15 μM and 20 μM of CDCA (median CA level 1.2 μM and 1.2 μM, respectively) compared to controls (median CA level 2.2 μM). No difference in CA levels in cell supernatant was observed in cultures treated with CDCA for 6 h. There was no difference in CA levels in cultures treated with recombinant FGF19 irrespective of time or concentration (**Figures 1C, D**). Our results showed that CDCA affected BA synthesis by reducing gene expression levels of the rate-limiting enzyme CYP7A1 and formation of CA in a concentration-dependent manner. No changes were observed in CYP7A1 gene expression or CA levels in cultures treated with recombinant FGF19.

CDCA Treatment Increased FGF19 Gene Expression in Primary Human Hepatocytes and FGF19 Enriched Cell Medium Subsequently Suppressed CYP7A1 Gene Expression

Given that FGF19 has been described as a main regulator of BA synthesis, the insignificant response of primary hepatocytes to recombinant FGF19 exposure was unexpected. We reasoned that recombinant versus endogenously produced FGF19 can evoke different responses. Previous reports describe low gene expression of FGF19 in untreated primary human hepatocytes, however BAs can induce FGF19 *in vitro* in primary human hepatocytes (9). We first studied CDCA induced FGF19 production by primary human hepatocytes in a dose- and time-dependent manner (**Figure 2**). Our results showed that FGF19 gene expression increased proportionally with increasing concentrations of CDCA (3–20 μM) both 6 or 24 h after treatment when compared to untreated control (**Figure 2A**). In accordance, we detected increasing FGF19 protein secretion with increasing concentrations of CDCA at both time points (**Figure 2B**). These results confirmed that FGF19 gene expression is induced in primary human hepatocytes by CDCA. To resolve temporal dynamics in FGF19 protein secretion, we treated primary human hepatocytes with 10 μM CDCA and quantified FGF19 gene expression and protein levels. We determined that FGF19 gene expression is induced between 1 to 1.5 h after CDCA-treatment and remained constant afterwards. FGF19 protein secretion into the medium commenced with a 2 h delay (between 3 to 3.5 h after CDCA-treatment) (**Figure 2C**).

Since CDCA rapidly induced FGF19, we further investigated whether FGF19 endogenously produced by primary human hepatocytes in response to CDCA can downregulate BA synthesis. To assess the effect of endogenously produced FGF19 on CYP7A1 gene expression and CA formation, we treated cells with various concentrations of FGF19 produced by primary human hepatocytes (conditioned medium) for 24 h (**Figure 3A**). We quantified FGF19 protein levels in the conditioned medium to be on average 310 pg/ml, compared to 25 pg/ml in the control medium (**Figure 3B**). We next determined that CYP7A1 gene expression levels remained unchanged when treated with 10% or 50% conditioned medium but was significantly lower in primary

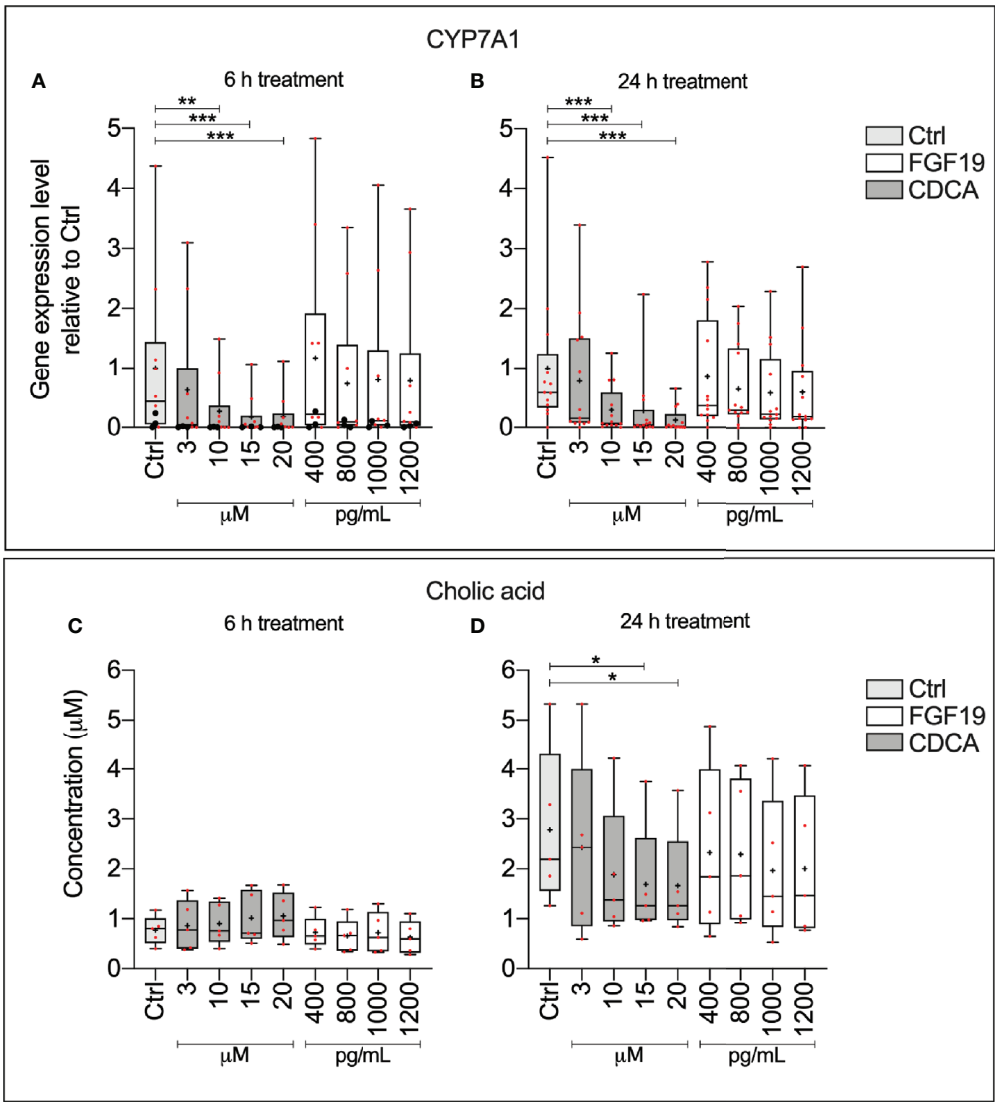

FIGURE 1 | Chenodeoxycholic acid (CDCA), but not recombinant FGF19, downregulated bile acids (BA) synthesis in primary human hepatocytes. **(A, B)** CYP7A1 mRNA expression (24 h, n = 13; 6 h, n = 10) and **(C, D)** cholic acid (CA) concentration (24 h, n = 5; 6 h, n = 5) in cell medium following treatment with FGF19 or CDCA at various concentrations for 24 h or 6 h. Data is presented as box-plot showing interquartile range (IQR) (box) and min-max (bars) with median marked with a line, red dots are the individual values, black dots in **(A)** represents RNA from donors that were also used for RNA sequencing. The plus sign represents mean value. Friedman test was used to assess differences between control and treatments and Dunn's multiple comparison test was used *post hoc*. *p < 0.05, **p < 0.01, ***p < 0.001.

human hepatocytes treated with 100% conditioned medium compared to control cultures (**Figure 3C**). Furthermore, we measured CA synthesis and found that the levels of CA between control and conditioned medium did not differ at either concentration (100%; 0.78 µM and 0.70 µM, 50%; 0.97 µM and 0.56 µM, 10%; 1.8 µM and 1.1 µM in control and conditioned medium respectively, **Figure 3D**).

Altogether, CDCA rapidly induced FGF19 in primary human hepatocytes and FGF19 enriched medium downregulated CYP7A1 gene expression. However, as the endogenously produced FGF19 was not purified and the conditioned medium contained traces of CDCA (up to 2 µM, data not shown), further validation of whether it is actually FGF19 that suppresses BA synthesis is needed.

Treatment of Primary Human Hepatocytes With CDCA Lead to Deregulation of Genes Involved in Metabolic Pathways

To discern the molecular roles of CDCA and FGF19 on hepatic gene expression, we performed a global analysis of gene expression (**Figure 4**). A principal component analysis (PC) of the experiments performed on primary human hepatocytes treated with 10 µM CDCA or 1,000 pg/ml recombinant FGF19

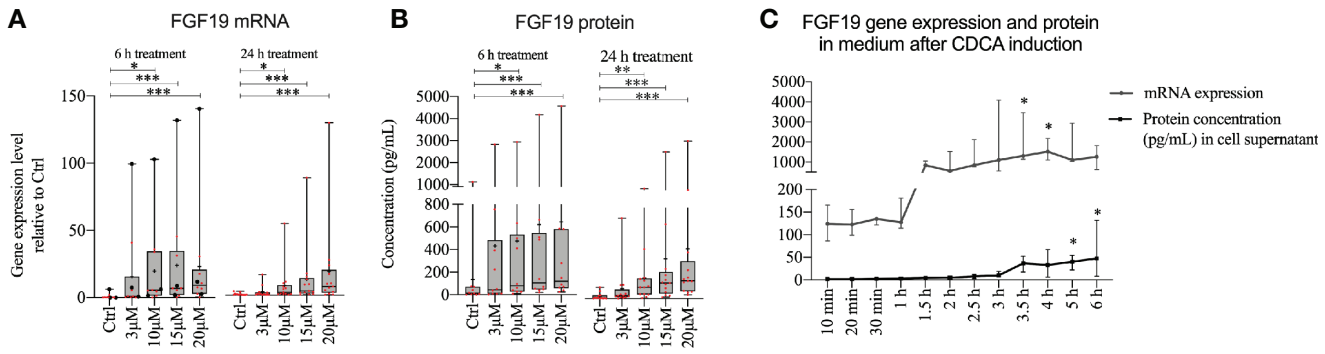

FIGURE 2 | FGF19 was rapidly induced and secreted in a dose-dependent manner following chenodeoxycholic acid (CDCA) treatment. **(A)** FGF19 mRNA expression and **(B)** protein levels in cell medium after CDCA treatment. **(C)** Time course of FGF19 mRNA expression (relative to Ctrl) and secreted protein after treatment with a single dose of CDCA. Data in **(A, B)** is presented as box-plot showing interquartile range (IQR) (box) and min-max (bars) with median marked with a line, marked with red dots are the individual values and black dots in **(A)** represents RNA from donors that were also used for RNA sequencing. (24 h, n = 13; 6h, n = 10). The plus sign represents mean value. The time course is presented as median with IQR. Friedman test was used to assess differences between control and treatments and Dunn's multiple comparison test was used *post-hoc*. *p < 0.05, **p < 0.01, ***p < 0.001.

showed that 88% (PC 1 and 2) of the variation was explained by inter-individual differences given the clustering by donors (**Figure 4A**), while 11% (PCA 3 and 4) of the variation was the consequence of treating primary human hepatocytes with CDCA or recombinant FGF19 (**Figure 4B**). We carried out a differential gene expression analysis and compared each treatment group with untreated controls. Our analysis showed that the vast majority of annotated genes remained unchanged (or highly variable between donors) in primary human hepatocytes but 2.4% and 1.9% of the annotated genes were significantly deregulated (fold-change > ±2, FDR<0.05) upon CDCA and FGF19 treatment, respectively, when compared to untreated controls (**Figure 4C**). We grouped the genes by gene categories, which revealed that both protein coding and noncoding genes were affected upon treatment (**Figure 4D**). CDCA treatment altered the expression of 627 genes (**Figure 4C**,

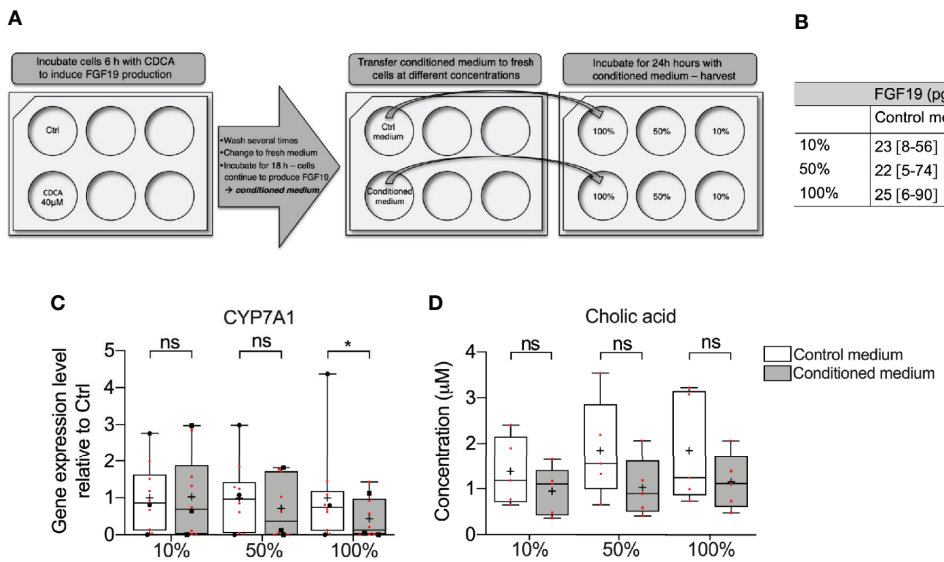

FIGURE 3 | Medium containing FGF19 produced by primary human hepatocytes downregulated CYP7A1 mRNA expression. **(A)** Layout of the experiment showing how chenodeoxycholic acid (CDCA) was used to induce FGF19 synthesis in primary human hepatocytes. Endogenously produced FGF19 was then applied to naïve hepatocytes for 24 h. **(B)** Levels of FGF19 in control and conditioned medium. **(C)** CYP7A1 mRNA expression and **(D)** cholic acid (CA) concentration in control and conditioned medium. Data is presented as box-plot showing interquartile range (IQR) (box) and min-max (bars) with median marked with a line, marked with red dots are the individual values, black dots in **(C)** represents RNA from donors that were also used for RNA sequencing. (n = 10). The plus sign represents mean value. Wilcoxon matched-pairs signed rank test was used to assess differences between control and conditioned medium. *p < 0.05, ns: non significant.

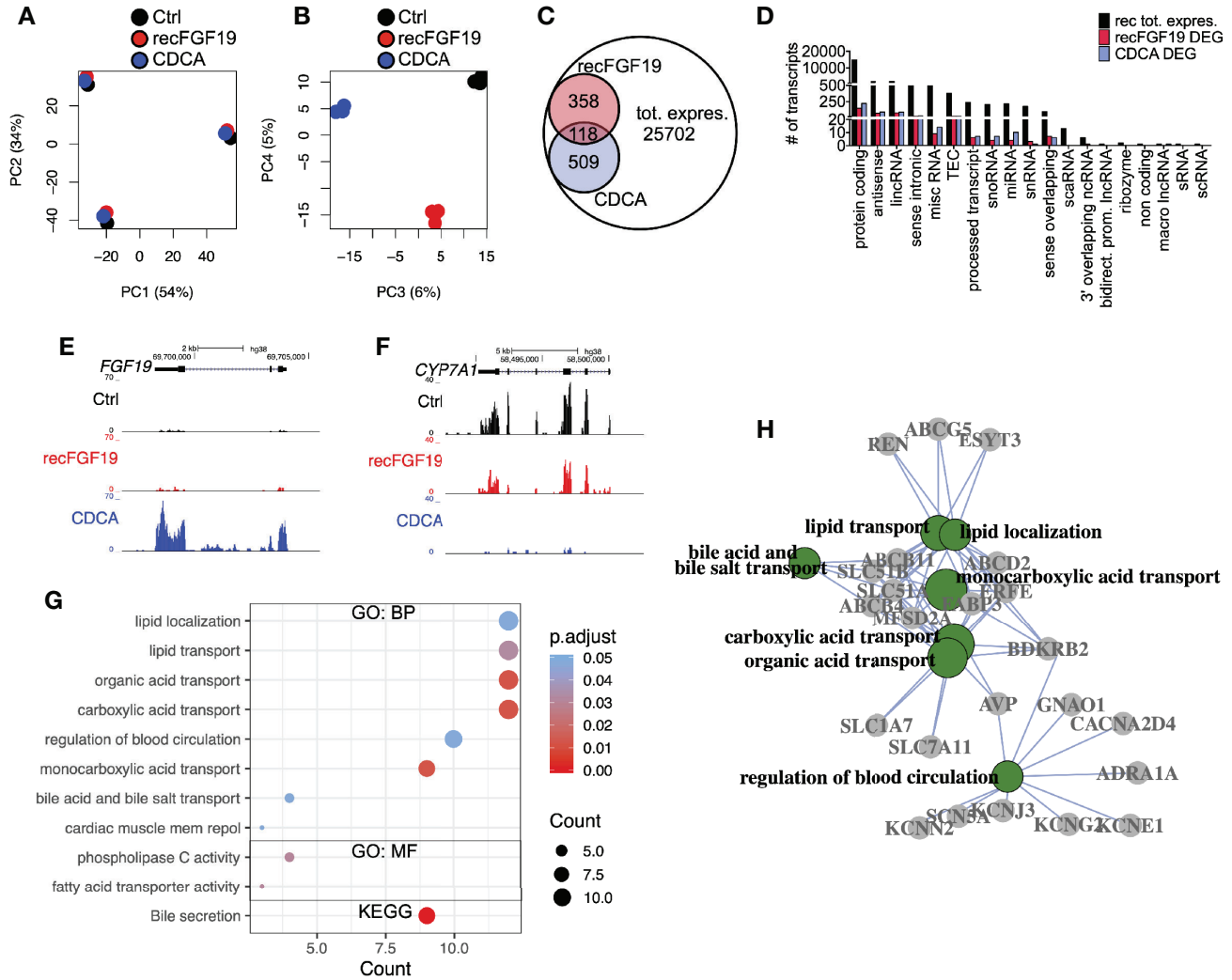

FIGURE 4 | Differentially expressed genes in primary human hepatocytes upon treatment with chenodeoxycholic acid (CDCA) or recombinant FGF19. Total RNA from primary human hepatocytes treated with recombinant FGF19, CDCA, or vehicle control was sequenced (n = 3). **(A, B)** Principal component analysis (PCA) showed that 88% of the variation in the samples could be explained by donor differences (PC1-2), while 11% of the variation could be explained by the treatment (PC3-4). **(C, D)** A limited number of transcripts were differentially expressed upon treatment. Venn diagram **(C)** and bar plot **(D)** displaying all expressed transcripts (black) and differentially expressed ones after recombinant FGF19 treatment (red) or CDCA treatment (blue). **(E, F)** Representative UCSC Genome Browser tracks of normalized FGF19 and CYP7A1 expression after recombinant FGF19 or CDCA treatment. The tracks from top to bottom show the scale in the human genome, the location in the human genome, the gene including exons (black boxes) and introns (arrows), and the RNA-seq signal from each treatment. **(G)** Unique chenodeoxycholic acid (CDCA)-DE transcripts were used for gene ontology (GO) biological processes (BP), molecular function (MF), and KEGG pathway analysis. Displayed are all significant ontologies/pathways in each ontology. The size of the bubble indicates number of genes in each category and the color represents the significance. **(H)** GO-term interaction network of the 7 most significant GO-BP terms in **(G)**. GO terms are in green circles, and gene names are in grey circles. Unique DE transcripts after recombinant FGF19 gave no significant enrichment in GO or KEGG pathway-related terms.

Table S3). Among them, we confirmed increased FGF19 (22.7-fold) and decreased CYP7A1 (26.4-fold) gene expression levels (**Figures 4E, F**). In addition, previously described genes involved in BA metabolism (1, 3), such as NR0B2 (encoding the regulatory protein small heterodimer partner [SHP]) was up-regulated (2.7-fold) and the BA transporters SLC51A (OSTα), SLC51B (OSTβ), ABCB11 (BSEP) and ABCB4 (MDR3) were up-regulated (3.1, 17.9, 6.2, and 2.3-fold, respectively) (**Table S3**).

In general, our Gene Ontology (GO) term and KEGG pathway analysis showed that CDCA treatment affected genes involved in lipid and BA transport and secretion (**Figure 4G**, **Table S4**). Categorizing the GO terms showed that many deregulated genes are interconnected (**Figure 4H**). In contrast, a total of 476 transcripts were found differentially expressed upon treatment with recombinant FGF19 when compared to control (**Figures 4C, D**). However, CYP7A1 was not among the differentially expressed genes (**Table S3**). Furthermore, GO term

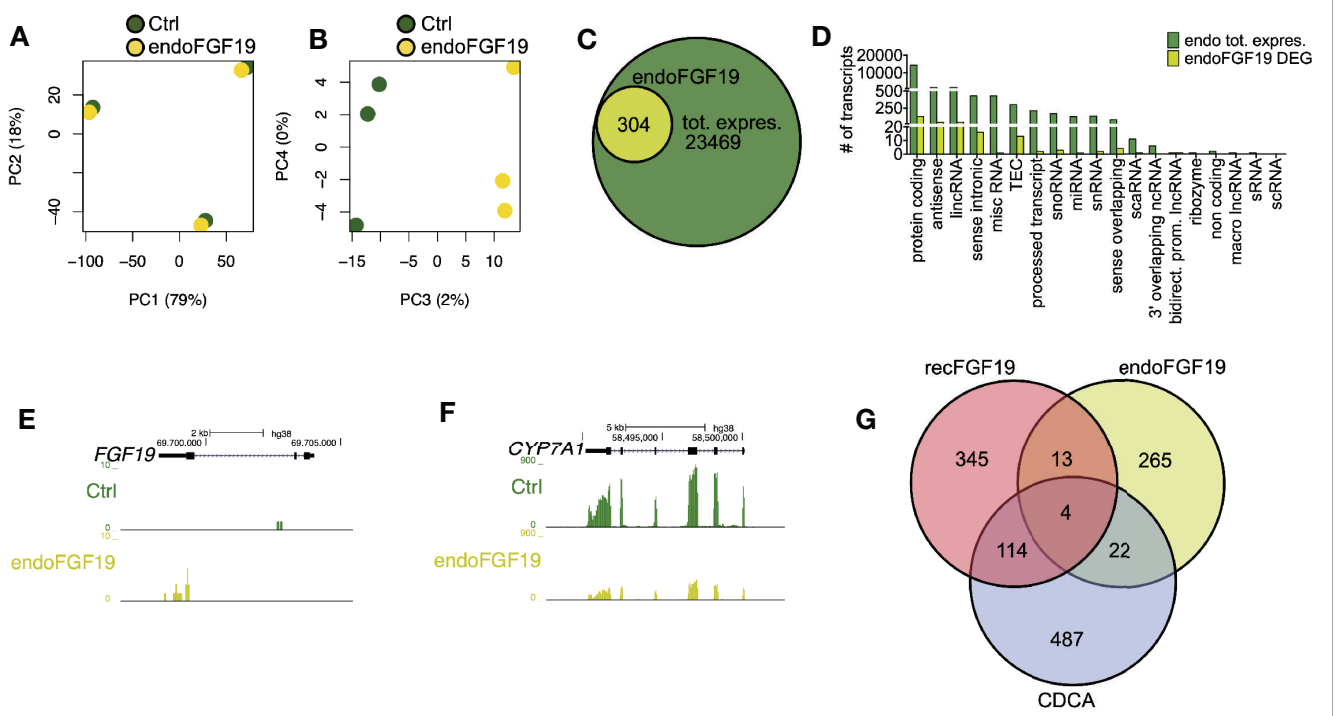

FIGURE 5 | Differentially expressed genes in primary human hepatocytes upon treatment with endogenously produced FGF19. Primary human hepatocytes were treated with endogenously produced FGF19 or vehicle control and total RNA was sequenced (n = 3). **(A, B)** Principal component analysis (PCA) showed that 97% of the variation in the samples could be explained by donor differences (PC1-2), while only 2% of the variation could be explained by the treatment (PC3-4). **(C, D)** A limited number of transcripts were differentially expressed upon treatment. Venn diagram **(C)** and bar plot **(D)** displaying all expressed transcripts (green) and differentially expressed transcripts after endogenously produced FGF19 treatment (yellow). The differentially expressed transcripts were not significantly enriched for any gene ontologies (GOs) or KEGG pathways. **(E, F)** Representative UCSC Genome Browser tracks of normalized FGF19 and CYP7A1 expression after endogenous FGF19 treatment. The tracks from top to bottom show the scale in the human genome, the location in the human genome, the gene including exons (black boxes) and introns (arrows), and the RNA-seq signal from the treatment. **(G)** Venn diagram displaying the overlap between differentially expressed transcripts after chenodeoxycholic acid (CDCA) (blue), recombinant FGF19 (red), or endogenously produced FGF19 (yellow) treatment compared to their respective vehicle controls. All three treatments resulted in mainly unique expressed transcripts, with little overlap among the treatments.

and pathway analyses did not reveal any pathway that was significantly regulated.

Since recombinant FGF19 had minor effects, we tested the effects of endogenously produced FGF19 on gene expression (**Figure 5**). Similar to the previous treatments, our PCA analysis of cells treated with endogenously produced FGF19 revealed that 97% of the variation in the samples was explained by differences between donors and only 2% by treatment (**Figures 5A, B**). A total of 304 transcripts were differentially expressed when compared to control (**Figures 5C, D**, **Table S3**). As expected, gene expression of CYP7A1 was reduced (6.1-fold) whereas FGF19 and SLC51B – genes normally associated with BA activation of FXR, increased 5.0 and 2.9-fold, respectively (**Figures 5E, F**, **Table S3**). Although these genes were differentially expressed both GO term and KEGG pathway analysis did not give any significant results.

In summary, a number of transcripts were differentially expressed upon the respective treatment and there was little overlap between treatments (**Figure 5G**). Genes involved in BA metabolism were differentially expressed upon treatment with CDCA or conditioned medium containing endogenously produced FGF19 but not with recombinant FGF19.

CYP7A1 Gene Expression Was Downregulated in Presence of CDCA Irrespective of siRNA-Mediated Knockdown of FGF19

The rapid increase of FGF19 by physiological levels of CDCA encouraged us to further validate the contribution of an autocrine pathway for FGF19 regulation of BA synthesis. We therefore performed a knockdown experiment to reduce FGF19 gene expression levels upon CDCA treatment. Following FGF19 siRNA knockdown, we exposed primary human hepatocytes to 10 μM CDCA for 6 h. In accordance to our previous results (**Figure 2**), CDCA induced FGF19 gene expression (**Figure 6A**) and protein secretion into the medium (**Figure 6B**) in primary human hepatocytes transfected with siRNA controls. In contrast, FGF19 gene expression and protein secretion was efficiently reduced upon transfection with siRNA against FGF19 and could not be increased when treated with CDCA (**Figures 6A, B**). In accordance, CYP7A1 gene expression was reduced 43-fold in primary human hepatocytes treated with CDCA when compared to untreated controls irrespective of siRNA-mediated depletion of FGF19 (**Figure 6C**). CA biosynthesis remained unchanged in

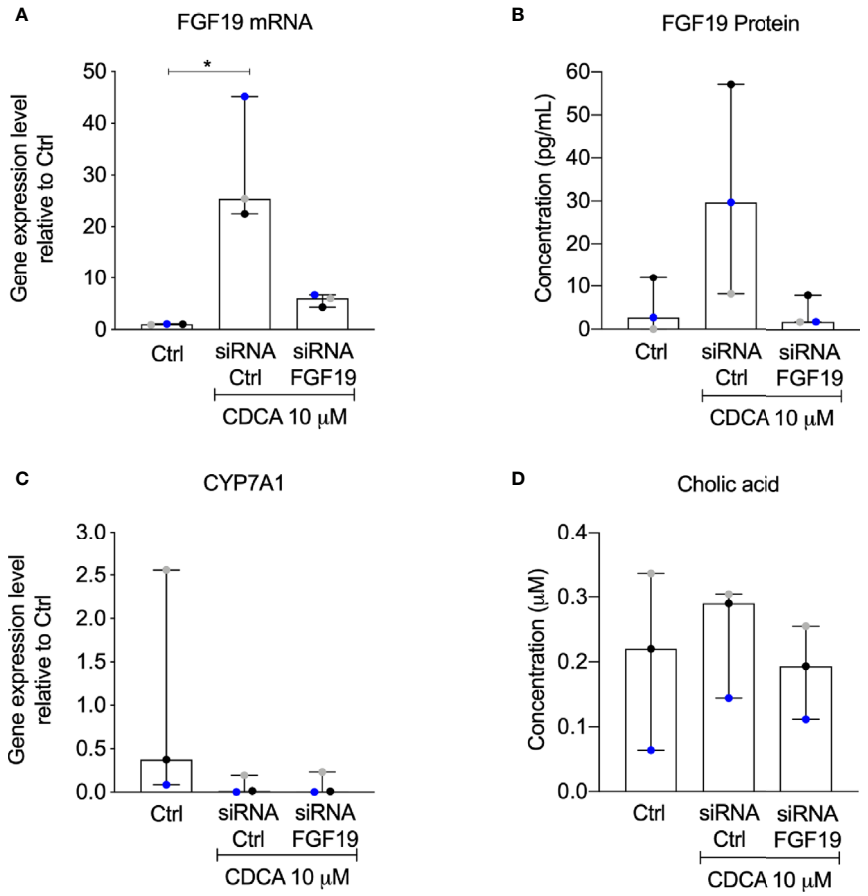

FIGURE 6 | Knockdown of FGF19 did not alter downregulation of CYP7A1 mRNA expression by chenodeoxycholic acid (CDCA). Primary human hepatocytes were treated with siRNA targeting FGF19 or non-targeting control. **(A)** FGF19 mRNA expression, **(B)** protein levels in medium in control cultures, after induction by 10 μM CDCA. **(C)** CYP7A1 mRNA expression, **(D)** cholic acid (CA) concentration in cell medium. Data is presented as median with interquartile range (IQR). Friedman test was used to assess differences between control and treatments and Dunn's multiple comparison test was used *post-hoc*. Each colored dot represents one individual case (n = 3). *p < 0.05.

response to 6 h of CDCA treatment and FGF19 depletion (**Figure 6D**). Our results showed that although CDCA induces FGF19 gene expression and protein secretion in primary human hepatocytes, regulation of BA synthesis in this setting remains intact.

DISCUSSION

BA homeostasis is regulated by a complex system controlled by BA activated feedback systems. BAs activate nuclear receptors that subsequently induce pathways involving transcription factors and other regulatory proteins to regulate transport, circulation and biosynthesis (1–5). There are different pathways for feedback regulation of BA synthesis in humans, mediated by the BA activated nuclear receptor FXR. The major hepatic pathway involves the orphan nuclear receptor SHP, which function as a transcriptional co-repressor to inhibit CYP7A1 expression (6, 18–21). FGF19 acts as an endocrine molecule, reaching the liver following secretion from intestine, to suppress CYP7A1 by binding and signaling through the fibroblast growth factor receptor 4 (FGFR4)/β-Klotho complex (8, 9, 22). Both of these FXR targets are of importance to maintain BA homeostasis, but as they are both upregulated in response to BAs at the last stages of the enterohepatic circulation, the impact of each of them on CYP7A1 expression and subsequently BA synthesis is difficult to distinguish. This is further complicated by distinct species differences in BA metabolism and conclusions originating from animal models can therefore not be directly translated to humans, which warrants studies on human systems (23–26).

To investigate the hepatic response to FGF19 and CDCA we treated primary human hepatocytes with recombinant FGF19 or CDCA at different concentrations within the physiological range (10, 13, 14). While CDCA downregulated CYP7A1 mRNA expression in a dose-dependent manner, FGF19-treated cells showed no change. RNA sequencing analysis revealed that only a minor subset of transcripts were differentially expressed by recombinant FGF19 treatment. Pathway analyses did not

confirm that these were involved in BA metabolism nor was any other pathway significantly affected by recombinant FGF19. Pathway analyses of differentially expressed transcripts from CDCA treated cells, on the other hand, showed genes involved in BA metabolism in agreement with other studies (1, 3). This surprisingly low response by the hepatocytes to recombinant FGF19 has been demonstrated in previous studies where supraphysiologic concentrations of at least 10 times more FGF19 than what is found circulating in humans has been used to see an effect in both animals and cell culture systems (9, 26, 27). The recombinant protein may have properties other than the endogenously produced and/or the biological activity may be different. A study by Kong and Guo (28) suggested that Fgf15, the mouse ortholog of FGF19, is prone to form inclusion bodies when expressed in *Escherichia coli*. They concluded that these aggregates can cause problems with re-folding of the protein *in vitro* subsequent to isolation and purification, which would then render it less biological active. This might be a contributing factor to the lack of effect from recombinant FGF19 and should be important to keep in mind for studies utilizing this synthetic form of FGF19. It should be noted that mRNA expression of the FGF19 receptor, FGFR4, and the co-factor necessary for stabilizing the interaction between FGF19 and its receptor, β-Klotho, were both stably expressed and not affected by treatment (**Supplementary Figure 1**). We designed an experiment to evaluate if the response to endogenous FGF19, produced by the primary human hepatocytes in response to CDCA, differ from recombinant FGF19 and downregulated BA synthesis. Interestingly, we found a dose-dependent decrease of CYP7A1 mRNA expression following treatment with medium enriched with endogenously produced FGF19. However, RNA sequencing analysis of cells treated with conditioned medium also revealed that non-classical FGF19-regulated genes, FGF19 and SLC51B that are both direct targets of CDCA-activated FXR (1, 27), were also among the differentially expressed genes. Upregulation of these genes could thus be explained by trace amounts of CDCA remaining in the conditioned cell medium. Indeed, BA analysis revealed CDCA levels of up to 2 μM in the conditioned medium (data not shown). Further validation of purified endogenously produced FGF19 and how it affects bile acid synthesis is thus needed.

FGF19 mRNA and protein expression in primary human hepatocytes was upregulated by CDCA in a dose-dependent manner. Interestingly, this occurred rapidly after treatment and with a dose of CDCA within the physiological range (14). FGF19 mRNA expression started increasing after 1 h followed by protein secretion after about 2 h. The rapid induction of FGF19 and the release into the cell medium is in agreement with the time frame of the postprandial peak of FGF19 observed in plasma that follows the peak of BAs. This suggests that FGF19 is potentially upregulated in liver also under physiological conditions, although it is unclear if FGF19 produced *in vivo* under normal circumstances would be enough to affect BAs synthesis. Therefore, to further evaluate the impact of hepatocyte produced FGF19 on BA synthesis, we conducted a knockdown experiment of FGF19. Following knockdown of FGF19 and after CDCA induction we found that FGF19 levels did not differ from the control cultures. Knockdown of FGF19 gene expression in primary human hepatocytes had no significant effect on CYP7A1 expression, which was still successfully downregulated 43-fold by CDCA, presumably *via* the previous described FXR-SHP-pathway (6). Song et al. (9) showed that when using an antibody against FGF19 or silencing FGFR4 in primary cell cultures in combination with the synthetic FXR agonist GW4064 the expression of CYP7A1 is increased, compared to cultures that were only treated with GW4064. However, from the data presented in Song et al. CYP7A1 is still downregulated in cultures treated with GW4064 regardless of antibody/siFGFR4 treatment when compared to cultures treated with DMSO vehicle control, which would be in support of the data in our study (9). We speculate that the upregulation of hepatic FGF19 is not crucial for maintaining BA homeostasis under normal conditions but that this may become of importance under conditions (*e.g.* cholestasis) when the liver experience excess levels of BAs and when the liver is not receiving FGF19 from the intestine (11, 12, 29). A possible explanation for the rapid response of FGF19 to CDCA has been suggested to be in the stabilization of SHP (30). SHP is rapidly degraded *via* the ubiquitin-proteasomal pathway. Studies have shown that FGF19 increase phosphorylation of extracellular signal-regulated kinase (ERK), which in turn dramatically decrease ubiquitination and proteasomal degradation of SHP. Inhibition of ERK on the other hand result in a dramatic reduction of SHP, suggesting that FGF19-mediated activation of ERK could be of importance for SHP stability and a possible autocrine or paracrine function of FGF19 in regulation of BA homeostasis (9, 30).

In summary, we have shown that levels of CDCA compatible with postprandial levels in portal blood downregulated bile acid synthesis and rapidly upregulated FGF19 expression in primary human hepatocytes. Downregulation of BA synthesis by CDCA is independent of endogenously produced FGF19.

DATA AVAILABILITY STATEMENT

The RNA-seq dataset generated during the current study is available in the ArrayExpress repository, under accession number: E-MTAB-8627 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-8627).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Swedish ethics review authority in Stockholm (Dnr:2017/269-31). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study design by EE, HJ, CK, and JNS. Sample and data collection by HJ, JNS, and CJ. Writing manuscript by EE, HJ, CJ, CK, JNS. Data analysis by EE, HJ, CJ, CK, JNS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.554922/full#supplementary-material

Supplementary Figure 1 | FGFR4 and βKlotho was not affected by FGF19 or CDCA treatment. **(A, B)** FGFR4 mRNA expression and **(C, D)** βKlotho mRNA expression following treatment with FGF19 or CDCA at various concentrations 6 h (n = 10) or 24 h (n = 13). Data is presented as box-plot showing IQR (box) and min-max (bars) with median marked with a line, red dots are the individual values. The plus sign represents mean value. Friedman test was used to assess differences between control and treatments (all non significant).

Supplementary Table 1 | htseq counts Chr1-22.

Supplementary Table 2 | TMM values.

Supplementary Table 3 | DEG analysis results.

Supplementary Table 4 | GO pathway analysis.

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REVIEW
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Nuclear Receptors in the Control of the NLRP3 Inflammasome Pathway

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The innate immune system is the first line of defense specialized in the clearing of invaders whether foreign elements like microbes or self-elements that accumulate abnormally including cellular debris. Inflammasomes are master regulators of the innate immune system, especially in macrophages, and are key sensors involved in maintaining cellular health in response to cytolytic pathogens or stress signals. Inflammasomes are cytoplasmic complexes typically composed of a sensor molecule such as NOD-Like Receptors (NLRs), an adaptor protein including ASC and an effector protein such as caspase 1. Upon stimulation, inflammasome complex components associate to promote the cleavage of the pro-caspase 1 into active caspase-1 and the subsequent activation of pro-inflammatory cytokines including IL-18 and IL-1β. Deficiency or overactivation of such important sensors leads to critical diseases including Alzheimer diseases, chronic inflammatory diseases, cancers, acute liver diseases, and cardiometabolic diseases. Inflammasomes are tightly controlled by a two-step activation regulatory process consisting in a priming step, which activates the transcription of inflammasome components, and an activation step which leads to the inflammasome complex formation and the subsequent cleavage of pro-IL1 cytokines. Apart from the NF-κB pathway, nuclear receptors have recently been proposed as additional regulators of this pathway. This review will discuss the role of nuclear receptors in the control of the NLRP3 inflammasome and the putative beneficial effect of new modulators of inflammasomes in the treatment of inflammatory diseases including colitis, fulminant hepatitis, cardiac ischemia–reperfusion and brain diseases.

Keywords: nuclear receptors, inflammasome, inflammatory disease, circadian rhythm, NLRP3, therapeutic strategy, inflammation and innate immunity

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INTRODUCTION: THE INNATE IMMUNE SYSTEM

Any living organism has to adapt to a specific environment and share common resources with others. To this purpose, organisms may collaborate in a reciprocal relationship from which each one of them benefits for its own survival. On the other hand, organisms may also be subject to threats from pathogenic offenders or from the environment itself, against which they have to defend themselves. The immune system is fundamental to anticipate and to preserve organisms from these threats. For that purpose, a specific system has been developed to allow the detection of two major classes of molecular signals, the pathogen-associated-molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (1). PAMP and DAMP classification appears to be based on

their biological sources rather than their chemical structures (1). PAMPs derive from pathogens including microbes and their products, while DAMPs originate from environmental disturbances such as the abnormal accumulation of endogenous compounds and cellular or subcellular damage. A common sensor system, defined as pattern-recognition receptors (PRRs), is able to detect both PAMPs and DAMPs. PRRs which are encoded by innate immune cells such as resident macrophages thus serve as sentinels of environmental changes including the presence of microbes and sterile tissue injury. In addition to PRRs, DAMPs are also detected by non-PRR receptors including receptor for advanced glycation end products (RAGEs), triggering receptors expressed in myeloid cells (TREMs), G-protein-coupled receptors (GPCRs), and ion channel (2). This allows the innate immune system to integrate various deleterious environmental changes to deliver the appropriate response according to nature of the threat (1).

PRRs can be distinguished based on their cellular location and the chemical nature of their ligand. Five main classes have been described: membrane-bound Toll-Like Receptors (TLRs) and C-Lectin Receptors (CLRs), cytoplasmic NOD-like receptors (NLR) and Retinoid acid-inducible gene I (RIG-1)-like receptors (RLRs), and multiple intracellular DNA sensors (CDSs) including cyclic GMP-AMP synthase (cGAS) and absent in melanoma 2 (AIM2) (2). Although TLRs were known to be activated by bacterial wall components such as LPS or proteoglycans, DAMPs including nucleic acids released from damaged cells are able to activate TLR3, TLR7, and TLR9 for instance, while intracellular proteins and extracellular matrix components released after tissue damage are able to induce a TLR2 or a TLR4-dependent signaling cascade (2). In addition, CLRs, usually known to be activated by fungi, are also able to detect lectin-derived compounds such as dendritic cell natural killer lectin group receptor 1 (DNGR1), macrophage-inducible C-type lectin (MINCLE), and Dectin-1 (2). RLRs are able to detect non-self RNA from microbial origin but also inappropriately masked self 5′ppp-RNA such as RNA generated during the unfolded protein response (2). CDSs are able to detect cytoplasmic (cGAS and AIM2) and damaged DNA in the nucleus (AIM2 only) (2). Finally, NLRs, which recognize bacterial compounds such as flagellin, are also able to detect crystals, ATP, amyloid fibers, glucose, or mitochondrial DNA. Therefore, PRRs and non-PPRs are able to sense extracellular and intracellular DAMPs, thus allowing a thorough surveillance of potential threats. Importantly, extracellular signals are considered as low-threat and resolvable problems, while cytosolic signals represent high-threat encounters that may induce pyroptosis, known as an interleukin (IL)-1β and IL-18-triggered cell death program induced by cytosolic PRRs only, mainly inflammasomes (1). When activated by DAMPs, PRRs and non-PRRs then trigger a so-called sterile inflammation, *i.e.* not induced by microbes. Therefore, a sustained activation of these receptors leads to inflammatory diseases including ischemia–reperfusion injury, colitis, systemic lupus erythematous, gout, neurodegenerative diseases, diabetes, atherosclerosis, hepatitis, rheumatoid arthritis, cancer, lung diseases, and gut diseases (2).

Inflammation is characterized by the production of a plethora of secreted immunomodulatory signaling molecules such as histamine, cytokines, chemokines, and lipid derivatives (1). The IL-1 cytokine family is a major cytokine family that includes IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ. Except for IL-1α, IL-1 cytokines are produced as inactive pro-cytokines and require maturation to biologically active forms by enzymatic cleavage. For instance, pro-IL-1β and pro-IL-18, the most studied IL-1 family members, are processed by the proteolytic activity of Caspase 1, the predominant IL-1 processing protease. Caspase 1 activity is tightly controlled by cytosolic PRR-constituted inflammasome complex. Inflammasomes form the main class of cytosolic PPRs that are activated by diverse exogenous signals including anthrax lethal toxin (NLRP1), bacterial flagellin (NLRC4), double stranded DNA (AIM2), toxin-induced modifications of Rho-GTPase (Pyrin). Unlike other inflammasomes, the nucleotide-binding domain (NOD)-, Leucine-rich repeat (LRR)- and pyrin domain containing protein 3 (NLRP3) inflammasome is not only activated by microbial and environmental molecules but also by several metabolic products including ATP, cholesterol crystals and β amyloid fibers. In this regard, NLRP3 is unique because it is able to sense a wide range of threats. NLRP3 is therefore a central PAMPs and DAMPs sensor whose erratic activation leads to numerous NLRP3-driven diseases.

THE NLRP3 INFLAMMASOME

The NLRP3 inflammasome was first identified in the cryopyrin-associated periodic syndrome (CAPS) and was later recognized to be involved in many other inflammatory/metabolic diseases including gout, atherosclerosis, type 2 diabetes, non-alcoholic fatty liver diseases (NAFLD), colitis, and neurodegenerative diseases such as Alzheimer and Parkinson diseases. The NLRP3 inflammasome is not only expressed by leucocytes (macrophages, dendritic cells, neutrophils) but also by hepatocytes, neurons, endothelial cells, cardiomyocytes, and pancreatic beta cells (3).

Structure

The NLRP3 inflammasome is a supramolecular organizing center (SMOC) which consists of a sensor (NLRP3), an adaptor (Apoptosis-associated speck-like protein containing a Caspase recruitment domain (ASC) encoded by *PYCARD*), and an effector (Caspase 1) (4). NLPR3 contains an amino terminal pyrin domain (PYD) involved in protein–protein interaction, a central oligomerization domain (NOD, nucleotide-binding and oligomerization domain, NACHT) with an ATPase activity involved in the self-association and function of NLRP3 and a carboxy terminal leucin-rich repeat (LRR) domain inducing the autoinhibition of NLRP3 by folding back onto the NACHT domain (4). Apart from an N_{ter} PYD domain, ASC also includes a C_{ter} caspase recruitment domain (CARD) that plays a role of adaptor platform for the pro-Caspase 1 protein through a CARD–CARD domain interaction. Caspase 1 structure also

includes a central catalytic domain (p20) and a C_{ter} small catalytic subunit (p10) (4).

Function

Upon stimulation, NLRP3 oligomerizes through homotypic interactions between NACHT domains of two NLRP3 proteins and the subsequent recruitment of ASC through PYD-PYD interactions (**Figure 1**). Then, helical ASC filaments nucleate and associate to form macromolecular ASC specks (5–7) (**Figure 1**). Finally, assembled ASC recruits pro-caspase 1 in a CARD-dependent manner that enables the proximity-driven self-cleavage of pro-caspase 1 to generate p33 (comprising the CARD and the p20 domains) and p10, which remains bound to ASC and becomes proteolytically active (**Figure 1**). Further processing then triggers the release of the p20 and p20–p10 complex from ASC. The p20–p10 complex is unstable in the cells, thus terminating its protease activity (8). Beyond the classical representation of NLRP3 inflammasome assembly, it has recently been demonstrated that the NIMA-related kinase 7 (NEK7) oligomerizes with the LRR domain of NLRP3 into a complex by bridging the gaps between adjacent NLRP3 subunits to mediate NLRP3 oligomerization that is essential for ASC speck formation and caspase 1 activation (9, 10) (**Figure 1**). Strikingly, NEK7 is specific to NLRP3 and does not interact with other inflammasomes such as NLRC4 (11). Regulation of NEK7-NLRP3 assembly is induced by ATP-driven potassium efflux (12) but also in a K^+-efflux independent manner (13) and by reactive oxygen species (ROS) production (9). Activated-Caspase 1 is then able to process pro-IL-1β and pro-IL-18 into mature and functional IL-1β and IL-18 (**Figure 1**).

In addition to the regulation of pro-inflammatory cytokine maturation, the NLRP3 inflammasome is also involved in the control of pyroptosis, defined as a rapid and inflammatory form of programmed cell death. Pyroptosis actually results from the cleavage of Gasdermin D (GSDMD) by inflammatory caspases including caspases 1, 4, 5, or 11 (14–16) (**Figure 1**). GSDMD possesses an N_{ter} cell death domain ($GSDMD^{NTerm}$), a central short region, which links to a C_{ter} auto-inhibition domain. Caspase 1 cleaves pro-GSDMD, thereby removing the auto-inhibition domain, thus alleviating the inhibition on the cell death domain (**Figure 1**). $GSDMD^{NTerm}$ then binds to phosphatidylinositol phosphate and phosphatidylserine in the inner leaflet of the cell membrane, oligomerizes, and inserts into the plasma membrane, thus forming a pore of 16 symmetrical protomers that kill the cell (17).

Activation of the NLRP3 Inflammasome

The activation of the NLRP3 inflammasome, as most inflammasomes, is tightly controlled by a two-step process (4) (**Figure 1**). A priming step is required to increase gene and

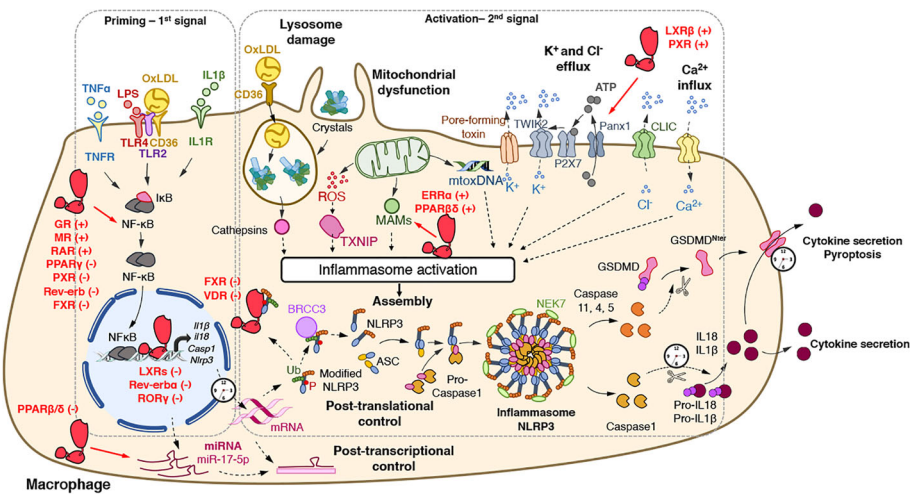

FIGURE 1 | Regulatory activities of nuclear receptors on the NLRP3 inflammasome priming and activation steps. The priming (first step) of the NLRP3 inflammasome requires the binding and activation of PRRs (TLRs,…) by PAMPs such as LPS, cytokines or ox-LDL, resulting in the transcription of the NLRP3 inflammasome components. Its activation (second step) is the result of recognition of PAMPs (such as the bacterial pore-forming toxin nigericin) or DAMPs which are released by damaged or dying cells (such as ATP) following injury or metabolic imbalance (such as mtROS), or accumulate in tissues (such as crystals). These lead to lysosomal damage, mitochondrial damages (exposition of cardiolipin, mtDNA) which ultimately modify ion (K^+, Ca^{2+}) fluxes. Upon this two-step process, the NLRP3 inflammasome assembles, caspase 1 is activated, Gasdermin-D and pro-IL-1β and pro-IL-18 are cleaved, leading to mature cytokines secretion and cell death by pyroptosis. The activity of nuclear receptors on each step is indicated when appropriate. ASC, apoptosis-associated speck-like protein containing a CARD domain; ATP, adenosine triphosphate; BRCC3, Lys-63-specific deubiquitinase BRCC36; casp, caspase; CLIC, chloride intracellular channels; DAMPs, damage-associated molecular patterns; GSDMD, gasdermin-D; IL, interleukin; IL1R, interleukin-1 receptor; LPS, lipopolysaccharide; MAM, Mitochondria-associated ER membranes; mtoxDNA, mitochondrial oxidized DNA; NFκB, nuclear factor-kappa B; NLRP3, nucleotide-binding, LRR and PYD domains-containing protein 3; Ox-LDL, oxidized low-density lipoproteins; P, Phosphate; PAMPs, pathogen-associated molecular patterns; Panx1, Pannexin-1; PRRs, Pattern Recognition Receptors; ROS, reactive oxygen species; P2X7, purinergic receptor P2X7; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TWIK2, two-pore domain weak inwardly rectifying K^+ channel; TXNIP, Thioredoxin-interacting protein Ub, ubiquitin. (+): activates; (–): inhibits.

protein expression of its components in order to sense stimuli and become activated. Once the cytoplasmic levels of NLRP3 mRNA reach an activating threshold, inflammasome assembly can be triggered by a secondary signal. This activation step initiates the NLRP3 SMOC assembly that promotes Caspase 1 autocatalytic activity and its subsequent maturation.

Priming the NLRP3 Inflammasome

The priming step has two main purposes: the transcriptional induction of the inflammasome complex components NLRP3, Caspase 1, IL-1β, and IL-18 and the induction of post-translational modifications of NLRP3 (**Figure 1**). The first one can be induced through the recognition of various PAMPs and DAMPs by PRRs such as TLRs and NLRs including NOD1 and NOD2 or cytokine receptors (*e.g.* TNFR) whose activation promotes nuclear factor κB (NF-κB) transcriptional factor activation and the subsequent induction of *Nlrp3* and *Il1β* gene transcription (**Figure 1**).

In addition to classical TLR ligands, disruption of metabolic homeostasis has also been involved in the NLRP3 inflammasome priming. For instance, NLRP3 mediates trained immunity following western diet feeding (18), suggesting that a lipid-rich diet potentializes the NLRP3-mediated response to pro-inflammatory stimuli. Accordingly, oxidized LDL (oxLDL), but also islet amyloid polypeptide (IAPP) and Alzheimer Disease beta amyloid peptides ($A\beta_{1-42}$), induce *Nlrp3* and *Il1β* gene expression, and thus the priming of this pathway in a CD36-TLR2-TLR4 heterotrimer-dependent manner in bone marrow-derived macrophages (BMDM) (19, 20) (**Figure 1**). Finally, cholesterol crystal-induced release of Neutrophils Extracellular Traps (NETs) from neutrophils is also able to prime NLRP3 in macrophages through the activation of several TLRs (21).

In addition to transcriptional regulation, the stability of mRNA of inflammasome components such as NLRP3, Casp1, and Casp8 can be controlled at the post-transcriptional level by miRNA [see (22) for review]. For instance, miR-223-3p negatively regulates the NLRP3 inflammasome by targeting the 3′-untranslated region (UTR)-binding sites of NLRP3 mRNA in myeloid cells (23). In addition, miRNAs can also target the mRNA of upstream regulators of the NLRP3 inflammasome including TXNIP, TRAF6, and SOD2 (11). As an example, miR-17-5p decreases TXNIP mRNA stability and NLRP3 activation in insulin producing cells and in the brain, thus inhibiting NLRP3 pathway activation (24, 25) (**Figure 1**). Accordingly, altered expression of several miRNAs is associated with the development of numerous NLRP3-driven diseases such as rheumatoid arthritis (26), multiple sclerosis (27), and systemic lupus erythematosus (28, 29).

While this transcriptional priming allows the production of NLRP3 pathway components, additional mechanisms are necessary to maintain NLRP3 in an inactive but poised configuration to rapidly respond to an activation signal. The second function of priming is then the induction of rapid transcription-independent mechanisms that regulate NLRP3 stability in order to rapidly progress from this poised state to an active one. Such non-transcriptional mechanisms are mainly classical post-translational modifications including ubiquitination, phosphorylation and SUMOylation (**Figure 1**) [see for review (30)]. For instance, ubiquitination of NLRP3 by FBXL12, TRIM1, ARIH2 or the dopamine-induced E3 ligase MARCH7 promotes the proteasomal degradation of NLRP3 in resting macrophages (30), whereas deubiquitylation of NLRP3 LRR domain on K63 by BRCC3 triggers ASC oligomerization and inflammasome activation (31, 32) (**Figure 1**).

Activation

The NLRP3 inflammasome is unique as it can assemble in response to a wide range of stimuli with various chemical properties. These include exogenous molecules of various origins such as environmental particulates (silica crystals) or pathogens. In addition, many endogenous molecules that abnormally accumulate are able to activate the NLRP3 inflammasome. This abnormal accumulation usually reflects tissue damage or metabolic dysfunction, which are thus sensed by NLRP3. For instance, under physiological conditions, LDLs normally circulate in blood. When LDL level abnormally increases in the context of dyslipidemia and when the vascular endothelium is damaged, LDLs infiltrate into the vascular wall, are eventually oxidized and trigger macrophage recruitment as seen in atherogenesis. CD36-mediated uptake of oxLDLs by macrophages contributes to the formation of intracellular cholesterol crystals and leads to the subsequent activation of NLRP3 (19) (**Figure 1**). Likewise, while normal extracellular ATP levels are harmless, tissue damage or cell death increases extracellular ATP levels acting as NLRP3-activating DAMPs. NLRP3 activation is often due to cellular stress resulting in lysosomal destabilization, ion flux imbalance, and redox potential alteration.

Lysosomal Damage

Crystals (cholesterol, urea, hydroxyapatite crystals) or fibrillar protein aggregates (β-amyloid, IAPP) can be phagocytosed by immune cells and then traffic toward lysosomes. These crystals often lead to lysosomal damage resulting in the release of proteases such as cathepsins (**Figure 1**). Although lysosomal disruption appears as a critical step for NLRP3 activation (33), downstream mechanisms between lysosome alteration and activation of the NLRP3 inflammasome still need to be unequivocally identified. Lysosome-released cathepsin B was considered for long as an essential trigger of NLRP3 activation (33). Nevertheless, the use of a broad spectrum of cathepsin inhibitors and individual knock-out experiments of several cathepsins confirmed that NLRP3 activation however, relies on several cathepsins that may exert redundant activities (34, 35). Importantly, Leu-Leu-OMe-induced lysosomal damage enhances K^+ and Ca^{2+} efflux that may account for lysosomal damage-controlled NLRP3 activation (36).

Ion Fluxes

Ion fluxes are important regulators of NLRP3 inflammasome activation. Changes in ion homeostasis such as increased intracellular Ca^{2+} levels as well as decreased intracellular K^+ and Cl^- levels also appear to play a pivotal role in NLRP3 activation (**Figure 1**). Lower extracellular concentrations of K^+

compared to intracellular K^+ concentrations are sufficient to induce K^+ efflux and to promote NLRP3 activation while high levels of extracellular K^+ prevent its activation in THP1 cells and BMDM (37, 38). In addition, nigericin, a K^+ ionophore, as well as the ATP-mediated activation of P2X purinoceptor 7 (P2rx7), a ligand-gated ion channel, promotes K^+ efflux-dependent IL-1β maturation (39–41) (**Figure 1**). Interestingly, P2rx7 does not directly control K^+ efflux, but instead, promotes Ca^{2+} and Na^{2+} influx after ATP stimulation and coordinates with the K^+ channel two-pore domain weak inwardly rectifying K^+ channel (TWIK2), which mediates K^+ efflux (42) (**Figure 1**).

Interestingly, K^+ efflux must be associated with Ca^{2+} influx to promote mitochondrial-mediated ROS production (43), where Ca^{2+} influx appears critical for NLRP3 activation (44, 45) (**Figure 1**). At the molecular level, CHOP, a transcription factor activated during ER stress, promotes Ca^{2+} release from the ER, thus stimulating the calcium-sensing receptor (CASR) and promoting NLRP3 assembly (44). K^+ efflux also controls Ca^{2+} release from the ER demonstrating the interconnection between the different activating signals (43, 44).

In addition to K^+ efflux and Ca^{2+} influx, Cl^- flux has also been demonstrated to activate NLRP3 (**Figure 1**). Indeed, while low extracellular Cl^- enhances ATP-induced IL-1β secretion, high extracellular Cl^- concentration or Cl^- channel blockers inhibit NLRP3 activation (46, 47). Two recent reports demonstrated that chloride intracellular channels (CLICs), especially CLIC1 and CLIC4 mediate NLRP3 activation by promoting Cl^- efflux downstream nigericin-induced K^+ efflux and mitochondrial ROS production, which promotes CLIC translocation to the plasma membrane (46, 47) (**Figure 1**). Interestingly, K^+ seems to drive NLRP3 oligomerization, probably in a NEK7-dependent manner (12, 48), while Cl^- efflux is prone to induce ASC polymerization (48). Finally, although ion fluxes were shown to control NLRP3 assembly and activation, the link between ion fluxes and the inflammasome activation remains to be identified.

ROS Production and Mitochondrial Dysfunction

Since ROS scavengers attenuate NLRP3 activation, the generation of ROS was considered a common cellular response critical for NLRP3 activation (49). Although the source of NLRP3-activating ROS was controversial, the inhibition of the lysosomal NADPH oxidase did not alter NLRP3 activation in mouse and human cells, thus suggesting an alternative source of NLRP3-activating ROS, likely the mitochondria (33, 50, 51). After stimulation with various NLRP3 activators, mitochondrial ROS (mtROS) altogether with Ca^{2+}, contribute to the rapid release of mtDNA into the cytosol (52) where it is eventually oxidized (53). Oxidized mtDNA then specifically interacts with NLRP3 and activates the inflammasome (53) (**Figure 1**). In addition, mtROS promotes Thioredoxin-interacting protein (TXNIP)-NLRP3 interaction involved in NLRP3 expression (54) (**Figure 1**).

Notably, NLRP3 is mainly localized at the membrane surface of ER in unstimulated cells (49). However, in the presence of MSU, nigericin or alum, mtROS production leads to the rapid relocation of NLRP3 and cardiolipin at the mitochondria outer membrane and promotes K^+ efflux (49). Then, the ASC adaptor accumulates at Mitochondria-associated ER membranes (MAMs) where the NLRP3-ACS complex is formed (49). In addition, NLRP3 may also interact with mitochondrial antiviral-signaling protein (MAVS), which is another mitochondrial outer MAM (55–57). In this context, mitofusin 2 can also be found in the outer mitochondrial membrane, the ER and MAM. Mitofusin 2 plays an important role in NLRP3 activation during RNA viral infections since it interacts with MAVS to support the relocation of NLRP3 to the mitochondria (58) (**Figure 1**).

Alternative Inflammasome Activation and Non-Canonical NLRP3 Activation

In addition to the classical/canonical NLRP3 inflammasome activation, an alternative NLRP3 activation process has been identified in which LPS alone is sufficient to induce inflammasome activation without the involvement of another second activator (59). This signaling pathway relies on a cascade involving TLR4, TIR domain-containing adapter molecule 1 (TRIF), RIPK1, FADD and caspase 8 that finally promotes NLRP3 activation. Interestingly, in addition to LPS, the pro-atherogenic apolipoprotein ApoC3 is able to trigger TLR2 and TLR4 heterodimerization and promotes the alternative activation of NLRP3 (60), thus mirroring the effect of oxLDL in the canonical activation of NLRP3. Strikingly, the alternative inflammasome activation is characterized by its independency on K^+ efflux and the absence of pyroptosome formation and pyroptosis. Then, this pathway is likely involved in the control of cytokine secretion without affecting cell viability.

In addition to caspase 1, cytosolic gram negative bacteria-derived LPS may also be sensed independently of TLR4 signaling by human caspases 4 and 5, and mouse caspase 11, to induce the non-canonical NLRP3 inflammasome (61, 62). In this pathway, Caspase-4/5/11 promote pyroptosis by processing pro-GSDMD and pannexin-1, a protein channel that releases ATP from the cell. This extracellular ATP then activates P2xr7 to promote K^+ efflux and NLRP3 activation (63, 64).

NUCLEAR RECEPTORS

In addition to the above-described regulators, priming and activation processes are also controlled by nuclear receptors (NRs), a subclass of transcription factors. Although numerous studies have reported this alternative activation pathway, such regulatory processes are rarely mentioned. We provide here the first review of the literature describing how these lipid-regulated receptors control both priming and activation processes in the context of different NLRP3-driven diseases. We will also describe in which pathophysiological contexts this regulation has been reported and how the pharmacological modulation of these NRs prevents the progression of NLRP3-driven diseases. Finally, we will discuss also the role of NLRP3 in NR regulation.

Nuclear Receptors: Generalities

Discovered in the mid-80s, NRs represent a superfamily of structurally conserved ligand-dependent transcription factors that regulate gene expression (65–67). The nuclear receptor

superfamily can be sub-divided into four classes based on their ligand- and DNA-binding properties and on the nature of their partner (68). NRs usually work as homo- or heterodimers, which bind to a specific response element composed of two AGGTCA half-sites separated by one to four nucleotides in the promoter of target genes (**Figure 2**). These half-sites are organized either as a palindromic sequence or a direct repeat. The first class, mostly classical steroid hormone receptors, is probably the best characterized and consists of nuclear hormone receptors such as Androgen Receptor (AR), Glucocorticoid receptor (GR), Estrogen receptors (ERs: ERα, ERβ), Mineralocorticoid Receptor (MR), Progesterone Receptor (PR). These NRs work as homodimers and are recruited to a palindromic arrangement of core recognition motifs. The second class consists of so-called adopted receptors that were initially identified as orphan receptors meaning without known ligands, but subsequent studies characterized naturally occurring ligands and determined their physiological roles. Its members encompass eicosanoid and fatty acid receptors Peroxisome Proliferator-Activated Receptors (PPARs: PPARα, PPARβ/δ, PPARγ), the oxysterol receptors Liver X Receptors (LXRs: LXRα and LXRβ), Thyroid hormone Receptors (TRs: TRα, TRβ), the Retinoic Acid Receptors (RARs: RARα, RARβ, RARγ), the Vitamin D3 Receptors (VDR), the xenobiotic receptor Pregnane X Peceptor (PXR). NRs from class II heterodimerize with one of the Retinoid X Receptors (RXRs: RXRα, RXRβ or RXRγ) and are recruited to a response element organized in two-half sites in tandem repeat (69–71). The third NR class is composed of adopted receptors such as RXRs, the heme receptors Rev-erb (Rev-erbα and Rev-erbβ), fatty acid receptor Human Nuclear Factor 4 (HNF4α, HNF4γ) and orphan receptors such as Chicken Ovalbumin Upstream Promoter Transcription Factor (COUP-TFI, COUP-TFII). The NRs from this third class act as monomers or homodimers bound on direct repeat response elements. Finally, the fourth class is made of orphan nuclear receptors such as Estrogen Related Receptors (ERRα, ERRβ, ERRγ), Retinoid-related Orphan Receptors (RORα, RORβ, RORγ), Nurr1, NOR1, Nurr77 and the steroidogenic factor 1 SF-1. Therefore, NRs represent a crucial superfamily of transcriptional factors whose transcriptional activity may be modulated by specific natural or synthetic ligands, identifying NRs as promising therapeutical targets in numerous diseases, and especially in NLRP3-driven diseases as described below.

Structure and Molecular Functions of Nuclear Receptors

NRs consist of modular domains, including a variable amino N-terminal activation domain (AF-1), a highly conserved DNA-binding domain (DBD), a conserved hinge region linking the DBD with the conserved ligand-binding domain (LBD) (72) (**Figure 2A**). The DBD mediates the specific recruitment of NR monomers, homodimers, heterodimers to their DNA response element and is involved in the dimerization of NRs with their partner altogether with the hinge region and the LBD. In addition, the LBD mediates ligand-dependent interactions with transcriptional co-activators such as p300/CBP or co-repressors such as NCoR or SMRT (**Figure 2**). These interactions are controlled, at the structural level, by ligand-dependent

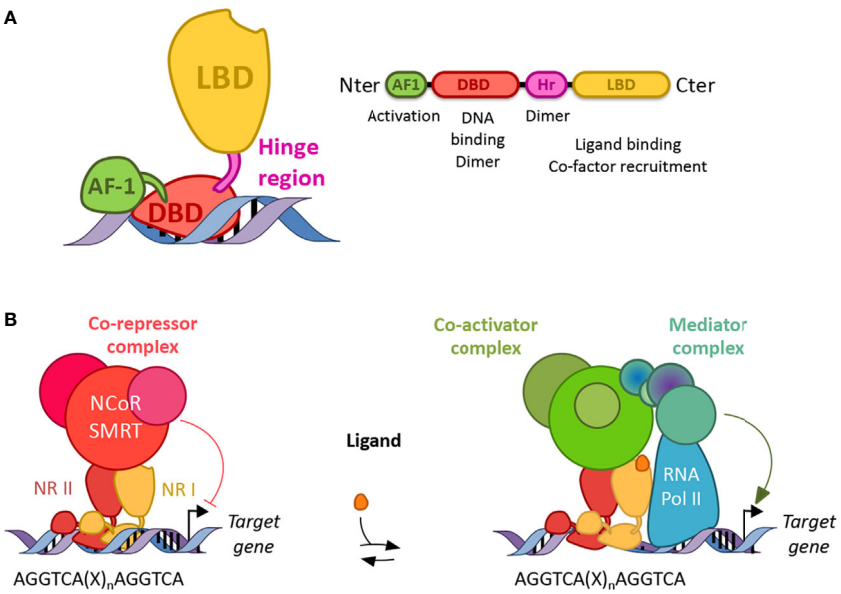

FIGURE 2 | Structure and function of nuclear receptors. **(A)** Canonical structures of nuclear receptors. Nuclear receptors are composed of a N terminal activation function domain whose activity is independent of ligand binding, a DNA binding domain (DBD), a hinge region (Hr) and a ligand binding domain (LBD). Their respective activity is mentioned accordingly. Dimer: dimerization **(B)** Nuclear receptors work as homo or heterodimers which bind a response element present in the promoter of their target genes. Response elements are composed of two AGGTCA half-sites separated by one to four nucleotides (X). In the absence of ligand, NRs (except class I) preferentially bind co-repressor and inhibit gene transcription. In the presence of a ligand, co-repressors are degraded by the proteasome and co-activators are recruited, which then allows the binding of a mediator complex and the ARN polymerase II.

conformational changes in the last α-helix 12 (αH12) of the LBD known as AF2 (73). In the absence of a ligand, co-repressors are preferentially bound to NRs, especially those of class II, while ligand binding induces a conformational change of the αH12 helix which then triggers the release of co-repressors, allowing co-activator binding (**Figure 2B**). If several NRs, especially those of the class II including PPARs, LXRs, RARs are then able to bind target genes in the absence of a ligand and recruit co-repressors to actively repress gene expression, class I steroid hormone receptors are usually sequestered into the cytoplasm in the absence of ligands and are translocated into the nucleus to bind their target genes in the presence of a ligand. Finally, the Rev-erb subfamily, Rev-erbα and Rev-erbβ, lacks the αH12, which then prevents the recruitment of co-activators (74, 75). Instead, although Rev-erbs are able to recruit co-repressors and actively repress gene expression in the absence of a ligand, ligand binding enhances co-repressor recruitment and the transcriptional activity of Rev-erbs to further inhibit the expression of their target genes (74, 75). Their transcriptional activity might be regulated by post-translational modifications including phosphorylation, ubiquitination, and SUMOylation (76–85).

Nuclear Receptors and the Innate Immune System

NRs are involved in the control of numerous physiological activities including metabolism (86, 87), reproduction (88, 89), cell cycle (90), vasculature (91, 92), brain activity (93, 94), circadian rhythm (95–97) and immunity (98–103). NRs have then been widely implicated in the control of inflammatory processes and the control of immune cell activity (99). In macrophages, many NRs display anti-inflammatory activities by quenching the NF-κB dimer into the cytoplasm (99). For instance, GR inhibits the expression of TNFα and COX2. In addition, iNOS expression is inhibited by both PPARγ and GR, while TLR4 expression is dampened by Rev-erbα and PPARγ (78, 104). In the same manner, IL-6 expression is reduced by both GR and Rev-erbα (99, 105). Interestingly, LXRα is able to induce TLR4 in human macrophages only, emphasizing the species-specificity of such regulatory pathway and also induces its own negative regulatory loop by enhancing Rev-erbα expression to avoid TLR4 lasting expression (106). In addition, nuclear receptors such as PPARγ (107), LXRα (101, 108, 109), Nurr77 (110) and Rev-erbα (104, 111) also control the skewing of pro-inflammatory macrophages toward anti-inflammatory macrophages. Finally, nuclear receptors including LXR (112), GR (113), and Rev-erb (105, 114) regulate macrophage recruitment by controlling the production of adhesion molecules or the secretion of chemokines such as Monocyte Chemoattractant Protein 1 (MCP1).

NUCLEAR RECEPTORS IN THE PRIMING OF NLRP3

Nuclear Receptors Control NF-κB-Dependent Regulation of NLRP3 Inflammasome

Many NRs have been shown to interact with the NF-κB complex and to inhibit this pathway either by directly interacting with the NF-κB complex in the cytoplasm, a mechanism known as quenching (98, 99), or by preventing the polyubiquitination of the IKK complex, which then promotes NF-κB inhibition (98). However, although these regulatory processes are known, only few studies demonstrate the direct link between NR-controlled NF-κB pathway and NLRP3 priming. For instance, dexamethasone, a GR synthetic ligand, and cortisol treatments in human THP1 macrophages and in BMDMs induce the expression of NLRP3 mRNA and proteins in a GR-dependent manner but not those of *Casp1* and *Il1β* (115) (**Figure 1**). Nevertheless, glucocorticoids enhance the secretion of mature IL-1β by these cells (115), thus demonstrating the ability of glucocorticoids to set up an active NLRP3 inflammasome pathway. Although the molecular mechanisms involved therein were not investigated in this report, this regulatory effect may be due, at least partially, to the activation of the NF-κB pathway. Indeed, dexamethasone as well as chronic stress, which triggers the production of cortisol, induce the NF-κB pathway in hippocampal neuroinflammation and depression-like behavior (116). In addition to GR, PXR agonists altogether with PXR overexpression induce NLRP3 and NLRP2 mRNA levels in endothelial HUVEC cells (117) (**Figure 1**). Interestingly, oxLDL has been shown to induce NLRP3 expression in a LOX1- and NF-κB-dependent manner (118). LOX1 is the main endothelial oxLDL receptor, whose stimulation by oxLDL induces NF-κB pathway (119), a mechanism reminiscent to the CD36-dependent one in macrophages (19). Interestingly, statins inhibit the activated NF-κB pathway and NLRP3 inflammasome by oxLDL in vascular endothelial cells through a PXR-dependent mechanism as well (118). Intriguingly, PXR blocks NF-κB binding in oxLDL-primed HUVEC, thus suggesting that PXR activation inhibits NLRP3 activation (118). Furthermore, epleronone-mediated inhibition of MR suppresses the expression of NLRP3 and Caspase 1 both in the liver and epididymal white adipose tissue (eWAT) (120) (**Figure 1**). However, whether these epleronone-mediated effects on NLRP3 pathway are dependent on MR remain to be confirmed. For instance, it is unknown whether MR-response elements are present in the promoter of inflammasome component coding genes. Accordingly, MR knock-down impairs aldosterone regulatory effect on IL-1β expression in LPS-stimulated BMDM, but this effect was likely due to an inhibition of NF-κB phosphorylation instead of a direct effect on NLRP3 gene expression (120). It is noteworthy that increased MR expression is associated with an increase in NLRP3 expression and altered microglia phenotype in hippocampus from spontaneously hypertensive rats (121). However, the actual functional impact of MR in this process needs further investigation to prove the implication of MR in this context. Furthermore, NRs such as RXRs and RAR are activated by retinoic acids including 9-*cis*-retinoic acid (9-*cis*-RA) and all-*trans*-retinoic acid (ATRA). Interestingly, human LPS-primed macrophages treated with ATRA exhibit elevated NLRP3 RNA and protein levels associated with an increase in caspase 1 and pro-IL-1β maturation. At the molecular level, ATRA alone induces NLRP3 expression and enhances LPS-induced NRLP3

and IL-1β mRNA levels by upregulating the phosphorylation of IκB, ERK, and p38 (122). Therefore, stimulation of GR, MR, PXR, and RAR induces NLRP3 priming. Besides, PPARγ also controls the NF-κB-dependent NLRP3 priming in different contexts including astrocytes and retinal ischemia/reperfusion (123, 124). Here, IL4-activated PPARγ inhibits NLRP3 protein levels in an NF-κB-dependent manner in High Mobility Group Box-1 (HMGB-1)-stimulated astrocytes (124), while treatment with pioglitazone, a PPARγ agonist, ameliorates retinal ischemia/reperfusion-mediated inflammatory response by suppressing NLRP3 activation in an NF-κB-dependent manner (123) (**Figure 1**). Furthermore, GW4004-mediated activation of FXR also inhibits the expression of TLR4 and Myd88 in ileum (125). The gene expression of the NLRP3 inflammasome pathway components was altered accordingly, although the direct impact of GW4004 on NF-κB activation was not reported in this context (125). Finally, in addition to PPARγ, Rev-erbα may also inhibit NLRP3 priming, at least partially, *via* the inhibition of p65 expression in mouse RAW264.7 macrophage cell line (126). Accordingly, modulation of Rev-erb activity revealed that Rev-erbs may inhibit p65 and IκB phosphorylation in RAW264.7 cells thus inhibiting NF-κB activity (127). Together these data indicate that PPARγ and Rev-erbα may inhibit NK-κB-dependent NLRP3 priming (**Figure 1**). Overall, as many NRs including the Constitutive Androstane Receptor (CAR) (128, 129) and PPARα (130, 131), have been demonstrated to control the NF-κB pathway (103), it could be anticipated that they may also be involved in NF-κB-dependent NLRP3 priming processes, although this still needs to be proven.

Nuclear Receptors Directly Regulate NLRP3 Priming

NRs are also able to directly control NLRP3 transcription. For instance, Rev-erbα, a transcriptional repressor, is directly recruited to four distinct Rev-erb Response Elements (RevRE) into the *Nlrp3* gene promoter and actively inhibits *Nlrp3* expression in both human and mouse primary macrophages (132) (**Figure 1**). Furthermore, the deletion of RORγ or the use of a RORγ inverse agonist decreases NLRP3 mRNA and protein levels, which is associated with a reduction of IL-1β secretion in LPS-primed BMDM (133). ROR and Rev-erb share the same consensus sequence allowing them to bind the same RORE/RevRE response elements (**Figure 1**). Accordingly, RORγ was found to be recruited to the same Rev-erbα sites in the *Nlrp3* promoter (132, 133). Finally, although poorly investigated, NRs also control *Nlrp3* mRNA post-transcriptional stability through the regulation of miRNA. Indeed, the PPARβ/δ agonist, GW0742, significantly reduces the number of activated pro-inflammatory microglial cells after hypoxia–ischemia in neonatal rat brain (134). This effect is mainly due to a decrease in TXNIP, NLRP3, IL-6 and TNFα (134). At the molecular level, the PPARβ/δ antagonist GSK3787 and the miR-17-5p inhibitor abolish GW0742 effect, thus demonstrating the dependency of GW0742 on the PPARβ/δ-miR-17-5p axis (134) (**Figure 1**). However, the identification of the precise mechanisms by which PPARβ/δ controls the regulation of miR-17-5-p still needs further investigations. It is not excluded either that other NRs may regulate miRNA expression implicated in the post-transcriptional regulation of *Nlrp3* mRNA stability.

NUCLEAR RECEPTORS REGULATE THE NLRP3 ACTIVATION STEP

In addition to NLRP3 priming, nuclear receptors are also able to control NLRP3 activation, *ie* the second step of NLRP3 regulation. For instance, deletion of *Rev-erbα* increases nigericin- and ATP-induced ASC speck formation in mouse primary macrophages, thus suggesting that Rev-erbα prevents NLRP3 inflammasome assembly and its activation (132). However, the underlying mechanisms still need to be uncovered, and it cannot be excluded that this effect on NLRP3 activation reflects only the increase of *Nlrp3* gene expression triggered after Rev-erbα deficiency. However, because Rev-erbα regulates mitochondrial function and autophagy processes in skeletal muscle (135), we may speculate that the inhibition of NLRP3 assembly by Rev-erbα could be mediated by a decrease in ROS production and an enhancement of mitochondrial function.

Interestingly, the bile acid receptor FXR is also able to physically interact with NLRP3 and Caspase1 thus inhibiting NLRP3 activity (136) (**Figure 1**). In addition, bile acids behave as DAMPs and inhibit the priming and activation of the NRLP3 inflammasome in the context of cholestatic and septic mice (136). At the molecular level, bile acids induce a prolonged Ca^{2+} influx and activate NLRP3 synergistically with ATP administration (136). It is noteworthy that these effects are independent of ROS production and K^+ efflux (136). In this context, FXR deletion increases endotoxemia sensitivity while FXR overexpression increases mice resistance to endotoxemia, thus suggesting an FXR-independent effect of bile acids action in sepsis (136). Such FXR-independent effect of bile acid on NLRP3 inflammasome may be mediated by the membrane receptor Takeda G coupled Receptor 5 (TGR5), another bile acid receptor. Indeed, treatment of BMDM with bile acids suppresses LPS/Nigericin-mediated NLRP3 activation in a TGR5-cAMP-PKA dependent by inducing NLRP3 ubiquitination and phosphorylation (137–140).

Furthermore, vitamin D enhances VDR-mediated inhibition of NLRP3 activation (141). Indeed, vitamin D3 (VitD3) inhibits NLRP3 activation in LPS-primed mouse peritoneal macrophages in the presence of nigericin, MSU or alum (142). In addition, vitD3 dampens ASC speck formation by preventing the NLRP3/NEK7 interaction (142). Interestingly, vitD3 also promotes NLRP3 ubiquitination. Indeed, the LBD of VDR is able to physically interact with the NACHT-LRR domain of NLRP3 thus inhibiting the association of NLRP3 with BRCC3 and preventing NLRP3 deubiquitination (141) (**Figure 1**). Particularly, VDR has been shown to prevent NLRP3 modification on K63 and its subsequent activation (141). Finally, vitD3 also increases VDR-controlled UCP2 expression thus inhibiting ROS accumulation in LPS-primed peritoneal

macrophages (141). Altogether, VDR inhibits NLRP3 inflammasome by favoring NLRP3 ubiquitination, preventing NLRP3 assembly and reducing ROS-mediated NLRP3 activation.

LXRs have also been shown to modulate the NLRP3 pathway. In colon cancer cells for instance, LXRβ activates NLRP3 inflammasome by inducing Pannexin1-dependent ATP release and autocrine P2x7R activation, which in turn leads to anti-tumoral effect of LXR agonists (143) (**Figure 1**). By contrast, LXRs have also been shown to inhibit Casp1, IL-1β and IL-18 expression through a direct DNA-dependent mechanism in human and mouse primary macrophages (109). In addition, LXRs enhance expression of IL-18BP, the decoy receptor of IL-18, through an indirect IRF8-dependent mechanism (101, 108, 109). In this study, LXRs did not appear to control *Nlrp3* mRNA levels in macrophages. Instead, they inhibit the expression of other inflammasome components such as pro-casp1, pro-IL18 and pro-IL1β and they induce the expression of inhibitory factor including IL18BP. On the contrary, LXRα was recently shown to decrease NLRP3 mRNA and protein levels in renal cell carcinomas metastasis *in vivo* and *in vitro*, thus resulting in the reduction of pro-IL1β and pro-caspase1 protein levels and the inhibition of IL1β secretion (144). Finally, lysosomal acid lipase (LIPA)-mediated 25- and 27-hydroxycholesterol (OHC) production, two LXR natural agonists, decreases efferocytosis and metabolic inflammation by activating LXR and by inhibiting NLRP3 in THP1 human macrophages (145). However, the interdependency of each pathway needs further investigations as results from Viaud et al. suggest that 25-OHC dampens inflammasome function independently from LXR activation (145) (**Figure 1**). Instead, it may be due to reduced MAM-dependent mitochondrial repurposing leading to NLRP3 inhibition (145). Therefore, it seems that LXR activity on the NLRP3 inflammasome depends on the cellular and tissular context, underlying cell-specific and context-specific mechanisms that still need to be explained. Interestingly, ERRα and PPARβ/δ increase Mitofusin 2 expression (146, 147). Although the link between the regulation of MAM and NLRP3 has not been established yet, we may anticipate that both NR may be involved in NLRP3 activation.

Epleronone is an antagonist of MR while aldosterone is a MR activator. Interestingly, epleronone-mediated inhibition of MR inhibits IL-1β secretion from eWAT (120). At the molecular level, epleronone treatment prevents ROS production and ATP- or nigericin-induced IL-1β secretion in LPS-primed BMDM, thus suggesting an effect on NLRP3 activation (120). Accordingly, aldosterone induced renal tubular cell injury by activating NLRP3 in a mtROS-dependent manner. Aldosterone-induced IL-1β and IL-18 maturation was then inhibited by NLRP3 knock-down or epleronone-mediated MR inhibition. Epleronone abolishes aldosterone-induced NLRP3, ASC, Casp1, and IL-18 maturation in mouse kidney, but the mechanism is still uncovered (148).

PXR activation with xenobiotics also induces Caspase1 maturation and IL-1β secretion in human THP1 and mouse primary macrophages (149). At the molecular level, PXR promotes rapid ATP release thus acting as an activation signal 2 (149). In this context, SRC kinase (SFK) promotes Pannexin1 phosphorylation thus triggering rapid ATP release (149) (**Figure 1**). It is, however, uncertain whether PXR controls Pannexin1 and SFK at the genomic or non-genomic levels (149). However, since the release of ATP occurs only 15 seconds after PXR agonist stimulation, this effect is unlikely transcriptional but instead it may be due to post-translational modification, advocating for a non-genomic effect of PXR in the regulation of NLRP3 activation step.

Finally, glycolysis and metabolic intermediates were shown to impact NLRP3 activation and ROS production (150). Interestingly, ATRA treatment induces hexokinase 2 expression in human LPS-primed monocyte-derived macrophages, thus shifting the metabolism of macrophages toward glycolysis and activating the NLRP3 inflammasome (122). Imbalance of metabolic homeostasis then appears to be directly linked to the NLRP3 inflammasome activity and the innate immune system thus emphasizing the importance of metabolic sensors in the control of inflammatory pathway. As exemplified here, NRs play an important role in such regulatory processes by bridging metabolism sensing and immunity.

NLRP3 IN THE REGULATION OF NR ACTIVITY

Until now, we have reviewed the regulatory effect of NRs on NLRP3. Interestingly, the NLRP3 pathway can also control the activity of NRs. For instance, the NLRP3/Caspase1 complex is able to cleave GR, thus impairing glucocorticoid activity in acute lymphoblastic leukemia (ALL) patients (151). Two cleavage sites of caspase 1, LLID and IKQE, have been identified in GR. Accordingly, increase in caspase 1 induced GR cleavage, decreased GR transcriptional activity and promoted glucocorticoid resistance (151). Interestingly, the comparison of NLRP3 and Caspase 1 expression between glucocorticoid sensitive and resistant primary leukemia cells isolated from 444 patients shows that high expression of Caspase 1 and NLRP3 is associated with an increase in glucocorticoid resistance (151). It is noteworthy that the higher expression of NRLP3 and Caspase 1 observed in glucocorticoid-resistant cells is likely due to lower somatic methylation of their respective promoter (151). Conversely, inhibition of Caspase 1 restores glucocorticoid sensitivity. Similar mechanisms were observed for AR (152).

Finally, the 17-oxo-DHA is a bioactive electrophilic α,β-unsaturated keto-derivative of the ω3 fatty acid docosahexaenoic acid (DHA) that is endogenously generated by COX2 in activated macrophages (153). The nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that binds antioxidant response element (ARE) to control antioxidant and detoxifying enzyme transcription including heme oxygenase 1 (HO-1) and glutathione S-transferase (GST). 17-oxo-DHA displays anti-inflammatory and cytoprotective activities by inducing Nrf2-dependent anti-oxidant response and by suppressing NF-κB-dependent inflammatory reactions. Interestingly, 17-oxo-DHA inhibits nigericin-induced ASC speck formation in human THP-1 macrophage cell line. In the context of cigarette smoke-driven chronic obstructive

pulmonary disease (COPD), the 17-oxo-DHA compound prevents inflammasome-dependent GR degradation in human peripheral blood mononuclear cells (PBMCs) (153). Although the underlying mechanisms are uncovered, we may speculate that 17-oxo-DHA controls Caspase 1 activity.

REGULATORY FUNCTION OF NR IN NLRP3-DRIVEN DISEASES AND THEIR THERAPEUTIC POTENTIAL

NLRP3 inflammasome upregulation is involved in numerous inflammatory diseases including joint, intestinal, respiratory, brain, hepatic, kidney, sexual organ and cardiometabolic diseases. Strikingly, NRs were widely involved in the regulation of these diseases through the control of NLRP3 (**Table 1**). It is then not surprising that the modulation of NR activity by specific agonists or antagonists regulates NLRP3 priming or activation and improves or worsens such diseases depending on the context.

Brain Diseases

Cerebral ischemia is a particular condition promoting neuroinflammation (154, 155). The 17β-Estradiol (E2), an ER agonist, display neuroprotective effect in the context of global cerebral ischemia, a well-known condition in which NLRP3 pathway components are induced (154). In this context, E2 inhibits the expression of NLRP3 inflammasome components and NLRP3 activation by decreasing P2xr7 expression in protein and proline-glutamic acid and leucine-rich protein 1 (PELP1)-dependent manner (154) (**Table 1**). Accordingly, nicotine attenuates ERβ action on inflammasome activity and exacerbates ischemic brain damage (155). Indeed, nicotine inhibits ERβ protein levels in hippocampus and cortex while it increases ASC, IL1-β and Caspase 1 protein levels in brain of female rats (155). However, further investigations are needed to demonstrate whether nicotine regulatory effects on the NLRP3 pathway are mediated by ERβ and NLRP3 instead of a direct activation of the non-canonical or alternative pathway. In addition to ERs, a PPARβ/δ agonist significantly reduces neuroinflammation after hypoxia–ischemia by inhibiting the expression of TXNIP and NLRP3 (134). Furthermore, temporal lobe epilepsy (TLE) is characterized by spontaneous recurrent seizures leading to neuroinflammation features such as astrocytosis associated with microglia activation and inflammatory cytokine production (156) (**Table 1**). In the context of human and mouse TLE, the Rev-erb ligand, SR9009, prevents neuroinflammation by inhibiting NLRP3 mRNA and protein levels, reducing astrocytes and microglial activation and decreasing apoptosis, which then preserves neurons and provides neuroprotection (156). Finally, glucocorticoids induce NLRP3 in an NF-κB-dependent manner in hippocampal microglial cells, which mediates chronic stress-induced depressive-like behavior in rats (116) (**Table 1**). Altogether, these data demonstrate that NRs such as ERs, Rev-erbs, and GR play a regulatory role on NLRP3-induced brain disease such as cerebral ischemia, epilepsy and depressiveness. As such, the modulation of their activity with ligands may dampen the severity and the progression of such diseases.

Intestinal Diseases

Colitis is an inflammatory disease of the colon whose causes are still uncertain. We may differentiate acute ulcerative colitis from chronic Crohn's disease. Strikingly, NLRP3 inflammasome is induced in dextran sulfate sodium (DSS)-induced colitis mouse model. Numerous NRs have then been shown to control DSS-induced colitis severity by modulating NRLP3 inflammasome pathway. For instance, the FXR agonist GW4064 exerts mild effect on colitis reduction by decreasing NLRP3 expression in LPS-induced ileum injury (125) (**Table 1**). However, GW4064 rapidly dampens both canonical and non-canonical NLRP3 activation in an FXR-independent manner, thus questioning the underlying mechanism involved in this fast response (171). Nevertheless, it is not excluded that FXR mediates GW4064 effect after a prolonged exposure to the agonist in this context (172). In obese patients, VDR polymorphisms were associated with increased inflammasome component expression, pro-inflammatory cytokine secretion and gut permeability, or dysbiosis, raising circulating LPS (173). Additionally, VitD3-activated VDR and SR9009-activated Rev-erbs also protect from DSS-induced colitis (126, 142), which then emphasizes the use of such NR-targeted approaches to control inflammatory bowel diseases (**Table 1**). It is noteworthy that numerous compounds derived from Chinese medicine are able to control the inflammasome pathway. For instance, Berberine, isolated from *Rhizoma Coptidis*, has been used for centuries in Chinese medicine to treat gastrointestinal disorders. Intriguingly, Berberine inhibits NLRP3 activation in DSS-induced colitis in a Rev-erbα-dependent manner (174). Naringin is a flavonoid extracted from grapefruit, sour orange and citrus seed that display anti-inflammatory properties (175). Interestingly, PPARγ mediates the anti-inflammatory effects of Naringin on DSS-induced ulcerative colitis (175). Whether thiazolidinediones, a PPARγ agonist class, prevent colitis progression as well remains to be determined (**Table 1**). Finally, LXRβ activates NLRP3 inflammasome in colon cancer cells leading to anti-tumoral effect of LXR agonists (143) (**Table 1**).

Kidney Diseases

Podocytes are important glomerular cell types playing a key role in blood filtration by the kidney. Aldosterone, a MR agonist, drives NLRP3-dependent podocyte dysfunction *in vivo* and *in vitro* by inducing oxidative stress (158) (**Table 1**). Remarkably, eplerenone, that inhibits MR, protects podocytes from aldosterone-induced injury (158). However, the dependency of MR in this context still needs to be addressed. In addition to podocytes, aldosterone also induces renal tubular cell injury. These cells play a pivotal role in the absorption of glucose, amino acids and ions by the renal tubule. In this context, aldosterone promotes mtROS production and subsequent NLRP3 activation (159). Strikingly, aldosterone induces NLRP3, IL1β, IL18 and CASP1 expression in human immortalized normal kidney cells

TABLE 1 | Activity of NRs in NLRP3-driven diseases.

Diseases	NR	Compounds	Effect on inflammasome	Effect on disease	Mechanism	Reference
Brain diseases						
Cerebral ischemia	ER	17β-Estradiol	Inhibition	Neuroprotection	Decreases P2xr7	154, 155
	PPARβ/δ	GW0742	Inhibition	Decrease neuroinflammation	Decreases TXNIP and NLRP3	134
Depression	GR	dexamethasone	Activation	Increase depressive-like behavior	NF-κB-dependent ROS production	116
Temporal lobe epilepsy	Rev-erb-α, -β	SR9009	Inhibition	Preserve neurons	Decreases NLRP3	156
Intestinal diseases						
Colitis	FXR	GW4064	Inhibition	Protection	FXR-independent? Inhibition of NF-κB?	125
	PPARγ	n/a	Inhibition	Protection	PPARγ mediates naringinin protection	157
	VDR	VitD3	Inhibition	Protection	Inhibits NEK7-mediated NLRP3 activation	142
	Rev-erb-α, -β	SR9009	Inhibition	Protection	Decreases NLRP3 in a NF-κB-dependent and independent manner	126
Colon cancer	LXRβ	T091317, GW3945, 25OH-Chst	Activation	Anti-tumoral effect	Interaction with Pannexin-1 and ATP release	143
Kidney diseases						
	MR	aldosterone	Activation	Podocyte dysfunction	mitROS production-mediated NLRP3 activation	148, 158, 159
	PPARγ	pioglitazone	Inhibition	Protects renal tubular cells	Inhibits NLRP3 and IL-1β transcription	157
Respiratory diseases						
Acute lung injury	Rev-erb-α, -β	SR8278 (antagonist)	Activation	Increases lung water content	Rev-erb inhibition induces NLRP3 inflammasome pathway	127
P. aeruginosa infection	PPARα	n/a	Inhibition	Induces complications	Increases NLRP3, ASC, Casp1, and p65 protein level	160
Cardiometabolic diseases						
Atherosclerosis	LXR	GW3965	Activation	Human study: not defined	IL1-β increases, HIF1α-dependent NLRP3 activation (?)	161
I/R	Rev-erb	SR9009	Inhibition	Prevents heart failure	Inhibits CCL2 and NLRP3 expression	162
Diabetic hypertension	MR	aldosterone	Activation	Increases hypertension and fibrosis	Induces mitROS-mediated NLRP3 activation	163
Diabetic retinopathy	PPARα	Fenofibrate	Inhibition	Improves retinopathy	Nrf2-dependent NLRP3 inhibition	164
	Nurr1	n/a	Inhibition	Inhibits Müller glia cells	NF-κB-dependent NLRP3 activation	165
Hepatic diseases						
Fulminant hepatitis	Rev-erb-α, -β	SR9009	Inhibition	Decreases Fulminant hepatitis	Inhibits CCL2/MCP1, NLRP3, IL-18, and IL-1β expression	132
	RORγ	SR1555, SR2211	Inhibition	Decreases Fulminant hepatitis	Inhibits NLRP3 and IL-1β expression	133
Cholestasis	FXR	GW4064 (?)	Inhibition	Improves cholestasis-potentiated sepsis	Physically interacts with NLRP3	136
	VDR	Calcipotrion	Inhibition	Alleviates cholestatic liver injury	Inhibits NLRP3 pathway and hepatic stellate cell activation	166
NASH	PPARβ/δ	GW501516	Inhibition	Prevents NASH pathogenesis	Inhibits NLRP3, NLRP6, NLRP10, Casp1 and IL-1β expression	167
I/R	Rev-erb-α, -β	SR9009	inhibition	alleviates hI/R-induced hepatic damage	Inhibits NLRP3 and IL-1β expression	168
Sexual organ diseases						
Endometriosis	ERβ	n/a	Activation	Activate cellular proliferation and adhesion	Inhibits TNF-driven apoptosis and activates NLRP3	169
Endometrial cancer	ERβ	Estrogen	Activation	Progression of endometrial cancer	Enhances NLRP3 and IL-1β expression	170

isolated from proximal tubules (HK-2 cells) in a dose- and time-dependent manner, thus inducing a phenotypic switch from HK-2 to fibroblast/pericyte cells in a MR and NLRP3-dependent manner (159). Accordingly, eplerenone abolishes these aldosterone-mediated effects. In addition, NLRP3 deletion in mice attenuates aldosterone-induced renal injury by protecting cells from apoptosis/pyroptosis and by preventing this phenotypic switch (159). Finally, aldosterone also induces tubulointerstitial fibrosis leading to kidney failure (148). As above, eplerenone abolishes aldosterone-induced macrophage

infiltration, tubulointerstitial fibrosis in a MCP1- and ICAM1-dependent manner (148). Precisely, macrophage inflammasome was required to induce renal fibrosis and kidney dysfunction after aldosterone administration, whereas renal cells were involved in MCP1 expression, showing a cell-specific aldosterone action in renal failure. However, the dependency on MR in renal fibrosis is still elusive (148). Finally, PPARγ activation with pioglitazone inhibits MSU-induced NLRP3 and IL-1β mRNA and protein levels in HK-2 cells (157) (**Table 1**). Intriguingly, MSU and LPS were able to induce PPARγ expression in HK-2 cells after a short exposure, but not a long exposure, thus suggesting that PPARγsets up a negative feedback loop to inhibit NLRP3 activation (157).

Respiratory Diseases

Acute lung injury is a severe IL-1β-associated complication that occurs after pulmonary inflammation and increases the mortality rate in patients. In mice, the Rev-erb antagonist SR8278 exacerbates LPS-induced lung permeability, which increases lung water contents (127) (**Table 1**). In this context, SR8278 increases macrophage recruitment in the lung and enhances IL-1β production in bronchioalveolar lavage fluid (127). In addition, PPARα ablation in mice increases NLRP3, ASC, Caspase 1 and p65 protein levels in the lung after infection with *Pseudomonas aeruginosa* (PA), which then promotes lung complications and subsequently worsens the pathophysiology of PA lung diseases (160) (**Table 1**).

Cardiometabolic Diseases

Cardiometabolic diseases include hypertension, diabetes, non-alcoholic fatty liver diseases, vascular dysfunction, and heart failure. They share common inflammatory features including NLRP3 inflammasome activation. We may distinguish atherosclerosis, heart ischemia–reperfusion, obesity, type 2 diabetes, and diabetic retinopathy. Atherosclerosis is a lipid-driven inflammatory disease of the vascular wall during which infiltrating LDLs are eventually modified, triggering their uptake by macrophages. Oxidized LDLs are indeed internalized and promote both priming and cholesterol crystals-mediated activation of NLRP3 in a CD36-dependent manner (19, 20). Accordingly, ablation of the NLRP3 inflammasome pathway decreases atherosclerosis progression (176, 177). Numerous NRs have been shown to be involved in atherosclerosis development including PPARs, Rev-erbα, LXRs, and Nur77 (3, 86, 108, 178). Interestingly, an LXR agonist has lately been shown to increase IL-1β protein levels in an HIF1α-dependent manner in human atherosclerotic lesions. It is however unknown whether it relies on an LXR-dependent mechanism (161) (**Table 1**). However, as HIF1α induces NLRP3 inflammasome activation (179–181), such regulatory mechanism may then account for LXR-dependent activation of IL1-β production in hypoxic atherosclerotic lesions.

Diabetes and hypertension are common coexisting diseases that accelerate micro and macrovascular complication occurrence. Different groups evidenced that aldosterone-activated MR increases hypertension and fibrosis through mtROS-mediated NLRP3 activation (163). In a model of obese diabetic *db/db* mice, spironolactone-mediated MR inhibition ablates inflammasome activation in mesenteric arteries (163) (**Table 1**). In addition, spironolactone treatment ameliorates glucose homeostasis without affecting body mass and mesenteric artery KCl-induced contraction. However, spironolactone ameliorates acetylcholine-activated vasorelaxation in phenylephrine-contracted mesenteric artery *ex vivo* (163). Accordingly, the NLRP3 inhibitor MCC950 mimics spironolactone effect in this vasoreactivity model, then suggesting that NLRP3 controls vasoreactivity in a MR-dependent manner (163). Diabetic retinopathy is a common neurovascular complication of diabetes that represents the most frequent cause of vision loss and blindness worldwide. In early non-proliferative stages, hyperglycemia causes glucotoxicity and damages retinal small vessels. As the disease progresses, alteration of small vessels triggers hypoxia and the development of small, fragile neovessels that can bleed, clot, and alter the retina. Because of cell death, diabetic retinopathy may also be considered as a chronic low-grade inflammatory disease in which the NLRP3 inflammasome is activated (54, 182). Strikingly, treatment with the PPARα ligand fenofibrate (FF) ameliorates diabetic retinopathy by inducing Nrf2 signaling and inhibiting NLR3 inflammasome (164) (**Table 1**). FF inhibits Nrf2 expression in mouse retinal Müller glial cells and attenuates gliosis in diabetic retina (164). However, it is uncertain whether FF effect is mediated by PPARα activation. Finally, Nurr1 deficiency promotes high glucose-induced Müller glial cell activation by inducing NF-κB and the NLRP3 inflammasome axis (165) (**Table 1**).

Post-ischemia reperfusion (I/R), after a heart ischemic episode, triggers a profound inflammatory response called reperfusion injury, which provokes adverse cardiac remodeling and heart failure. Consistently, MCC950-mediated NLRP3 inhibition lowers infarct size and areas at risk (183). Remarkably, administration of SR9009 Rev-erb agonist, one day after myocardial I/R, prevents heart failure by targeting cardiomyocyte inflammasome in a Rev-erb-dependent manner (162) (**Table 1**). In addition, Rev-erb activation inhibits CCL2 secretion and leucocyte recruitment at ischemic sites, thus lowering cardiac inflammation that would prevent cardiac remodeling (162).

Hepatic Diseases

Cholestasis is a common liver complication in patients with extrahepatic infection or sepsis and consists in bile acid accumulation in liver and serum. Intriguingly, on the one hand, BAs behave as DAMPs which activate both priming and activation of NLRP3, while on the other hand, the BA receptor FXR inhibits NLRP3 activation by physically interacting with NLRP3 (136) (**Table 1**). However, because the GW4064 compound may modulate NLRP3 activity in an FXR-independent manner (171), we may anticipate that BA effects on NLRP3 in cholestatic mice may also occur in an FXR-independent manner, thus explaining this apparent discrepancy. However, as FXR expression is down-regulated in endotoxic mice, FXR synthetic ligands display a poor effect on cholestasis (136), thus advocating for the identification of an alternative therapeutic strategy such as promoting the increase of FXR expression. Finally, the VDR agonist calcipotriol is also able to alleviate cholestatic liver injury and fibrosis by inhibiting the NLRP3

inflammasome pathway involved in inflammation, and hepatic stellate cells activation likely responsible of fibrosis (166) (**Table 1**).

Non-alcoholic fatty liver diseases (NAFLD) are common chronic liver diseases, ranging from hepatic steatosis to non-alcoholic steatohepatitis (NASH), which is characterized by lipid accumulation, inflammation, and fibrosis (184). NASH may eventually progress to irreversible cirrhosis and hepatocarcinoma (184). Remarkably, inhibition of the NLRP3 inflammasome pathway reduces liver inflammation and fibrosis in an experimental mouse NASH model (185). Interestingly, the dual PPARα and PPARβ/δ agonist GFT505/Elafibranor displays hepatoprotective effects in different rodent models of NASH by reducing fibrosis and cytokine secretion including IL-1β (167). Consistently, administration of the PPARβ/δ agonist GW501516 inhibits Caspase 1 and IL-1β hepatic mRNA levels in mice fed a high fat diet (HFD) and co-treated with LPS (186) (**Table 1**). In human hepatic hepG2 cell line, palmitic acid and LPS co-treatment induces the expression of NLRP3, NLRP6 and NLRP10 as well as Caspase 1 and IL-1β (186). Consistently with *in vivo* data, GW501516 prevents palmitate/LPS-induced inflammasome component gene expression (186). Intriguingly, although GW501516 accordingly impairs Caspase 1 maturation, it does not control IL-1β secretion (186).

Fulminant hepatitis (FH) is a life-threatening condition characterized by fast evolving hepatic dysfunction associated with tissue necrosis, inflammation and hepatic encephalopathy (187). Albeit numerous factors including fungi intoxication, viral infection, and metabolic diseases trigger FH, the main cause of FH nowadays is drug overdose with acetaminophen as the main one (187). Acetaminophen accumulation induces P450-mediated overproduction of toxic metabolites leading to oxidative stress, mitochondrial membrane potential loss and hepatocellular death. Tissue necrosis is then responsible of the release of DAMPs such as ATP and subsequent NLRP3 inflammasome activation (188, 189). Strikingly, *Rev-erbα*-deficiency aggravates FH in a mouse model of LPS-galactosamine (GalN)-induced liver injury. This occurred in an NLRP3-dependent manner by alleviating its inhibitory effect on Caspase 1 activity and on IL-1β expression and secretion (132) (**Table 1**). As Rev-erbα also impairs CCL2/MCP1 chemokine expression, ablation of Rev-erbα worsened neutrophils and monocytes infiltration in LPS/GalN-challenged mice, thus contributing to increased liver injury (132). Consistently, pre-treatment with the Rev-erb agonist SR9009 prevents LPS/GalN-induced FH pathogenesis by inhibiting the NLRP3 inflammasome pathway and CCL2 expression, thereby delaying death and improving the survival rate from 10% in the control to 70% in the SR9009-treated mice (132). Finally, the RORγ inverse agonists SR1555 and SR2211 reduce the expression and secretion of IL-1β in LPS/GalN-induced FH and exert a hepatoprotective effect that improves the survival rate of treated FH mice (133) (**Table 1**). However, whether RORγ mediates SR1555 and SR2211 effect on NLRP3 pathway and FH protection still needs to be proven. Nevertheless, RORγ deletion in LPS-primed BMDM inhibits NLRP3 and IL-1β secretion, which is consistent with a RORγ-inhibiting effect of SR1555 and SR2211 on these processes (133).

Rev-erb-α has also been highlighted lately in the context of hepatic ischemia–reperfusion (hI/R). hI/R is a complex phenomenon during which hepatocyte damage hits when blood supply returns into the ischemic liver after a liver transplantation, hepatectomy, and ischemic shock (190). Inflammatory responses play an important role in hI/R injury during which activated Kupffer cells release ROS and pro-inflammatory cytokines including IL-1β. Consistently, NLRP3 deficiency protects against liver I/R injury in mice (191). Accordingly, *Rev-erbα* deletion sensitizes mice to hI/R and is accompanied by exacerbated NLRP3 activation and pro-inflammatory cytokine secretion (168). On the contrary, SR9009 treatment alleviates hI/R-induced hepatic damage by inhibiting IL-1β expression (168). In conclusion, Rev-erbs, RORγ, VDR, PPARβ/δ, and FXR then exhibit hepatoprotective effects in acute liver inflammatory diseases by dampening the NLRP3 inflammasome activity.

Sexual Organ Diseases

Endometriosis is a sexual organ disease originating from abnormal deposition of endometrial cells that grow outside from the uterine cavity. It affects 6–10% of reproductive-aged women. Endometriosis causes pelvic pain in 50% of cases and fertility problem in 40–50% of cases (169). Endometriosis is likely due to high production levels of 17β-estradiol that could play a role in the proliferation of endometriotic tissues (169) (**Table 1**). Compared to ERα, ERβ expression is significantly higher in endometriotic tissue than in normal uterine endometrium in human. In addition, the role and the specific expression of ERα in endometriotic tissues are controversial (169). Interestingly similar patterns were observed in the mouse (169). Interestingly, *NLRP3*$^{-/-}$ mice exhibit smaller ectopic lesions compared to wild type mice, thus suggesting that NLRP3 induces endometriosis (169). Strikingly, ERβ inhibits TNFα-driven apoptosis and activates NLRP3 in endometriotic tissues (169). Accordingly, ERβ then increases IL-1β secretion, which enhances cellular adhesion and proliferation (169). Consistently, NLRP3 inflammasome activation has been shown to promote the progression of human endometrial cancer in an ERβ-dependent manner (170) (**Table 1**). At the molecular level, ERβ interacts with the NLRP3 inflammasome in the cytoplasm (169). However, the exact regulatory mechanism still needs to be investigated.

NR-DEPENDENT CONTROL OF NLRP3 CIRCADIAN RHYTHMICITY AND CHRONOTHERAPY

Our ability to anticipate environmental changes imposed by the rotation of the Earth is controlled by the circadian clock, which properly gates many, if not all, physiological processes to the most appropriate time window (192). Among these physiological pathways, immune functions vary according to the time of day (3, 193), a process described as circadian immunity, in which innate immune cells such as macrophages harbor an intrinsic

clockwork that drives circadian transcription of genes involved in the response to bacterial challenge (105, 194, 195). Pioneer studies have demonstrated that important features of the immune system such as trafficking and abundance of blood leucocytes, their recruitment to tissue, their ability to respond to pathogens and to secrete immune molecules vary in a circadian manner (196, 197). At the molecular level, the biological clock is a complex network of transcription factors and interlocked transcriptional feedback loops that orchestrate cellular circadian rhythms. Among the core clock components, the ligand-activated nuclear receptors Rev-erbs and RORs participate in the circadian control of the immune system (104, 106), whereas pharmacological activation of Rev-erbα and ROR modulates the expression and release of key pro-inflammatory cytokines (105, 133). It is noteworthy that Rev-erb nuclear receptors, altogether with RORα are the only core clock components whose activity may be directly modulated by a synthetic compound, thus representing an interesting therapeutical approach to directly modulate immune circadian behavior (198).

Over the past 100 years of global industrialization, mankind underwent some important changes in its lifestyle including its food habits, the ease of travel, the increase in shift work and social demands, and erratic exposure to artificial light from luminescent screens, which have dramatically altered circadian rhythms. It is now well-recognized that disruption of the intrinsic molecular clock impedes a proper immune response (199) and has severe repercussions on health. Indeed, numerous clinical studies have demonstrated that disruption of circadian rhythms in human represents an additional risk factor for neurological, metabolic and chronic inflammatory disorders (200–202) such as asthma, rheumatoid arthritis, atherosclerosis, type 2 diabetes or Alzheimer Disease (193, 202). Most of the clock-driven diseases demonstrate a chronic inflammatory component, either infiltration of macrophages in the vascular wall due to an accumulation of non-infectious DAMPs such as cholesterol crystal causing atherosclerosis, hydroxyapatite in joints leading to rheumatoid arthritis or the deposit of β-amyloid fibers, which activates microglial cells in Alzheimer disease (197).

Remarkably, clock disruption alters NLRP3 circadian oscillations in a mouse model of jetlag or in genetic and pharmacological models of clock alteration, thus modulating the progression of inflammatory diseases (3) including colitis (126), myocardial infarction/ischemia–reperfusion injury (162), lung injury (127) and fulminant hepatitis (132, 133). At the molecular level, NLRP3 expression altogether with IL-1β and IL-18 mRNA levels oscillate in a daily manner under the control of Rev-erbα *in vivo* and *in vitro* (132). Indeed, Rev-erbα ablation abolishes circadian oscillations in *Nlrp3* gene expression in peritoneal macrophages and in serum shock-synchronized human and mouse primary macrophages, with functional repercussions on IL-1β and IL-18 oscillatory secretion (132). *In vitro*, Rev-erbα deletion promotes elevated expression of *Nlrp3*, *Il1β* and *Il18*, which is accompanied by an increase in IL-1β and IL-18 secretion (132). By contrast, activation of Rev-erbs with heme, their natural ligand, or with synthetic ligands reduces the secretion of these cytokines by inhibiting the expression of NLRP3 inflammasome component genes (132). Strikingly, the susceptibility to fulminant hepatitis and hepatic ischemia reperfusion injury is time-of-day dependent, upon the control of the molecular clock with Rev-erbα as an important regulator of the inflammasome (132, 168). Remarkably, pharmacological activation of both Rev-erbs and ROR reduces liver injury and improves the survival time and rate in a NLRP3-dependent manner in treated mice (132, 133). Consistently, time of cardiac ischemia/reperfusion and subsequent SR9009 treatment affect heart function recovery, the best response being obtained when Rev-erb expression is at its highest, *ie* when NLRP3 expression is at its lowest (162). Interestingly, the NF-κB-driven long non-coding RNA *Lnc-UC* has lately been shown to be induced by the core clock component Bmal1, thereby generating circadian expression of *Lnc-UC* (203). Then, *Lnc-UC* physically interacts with Cbx1 protein to reduce its gene silencing activity *via* H3K9me3, thereby enhancing Rev-erbα expression in an epigenetic manner (203). Then, by inducing Rev-erbα expression, *Lnc-UC* ablates NF-κB signaling and NLRP3 inflammasome signaling in macrophages (203). Consistently, *Lnc-UC* deletion disrupts clock gene expression, sensitizes mice to DSS-induced colitis and disrupts the diurnal rhythmicity in disease severity (203). Additionally, Rev-erbα-mediated effect of Berberine on DSS-induced colitis shows better effect when administered at ZT10 (late resting phase) compared to ZT2 (early resting phase), thus acknowledging the rationale to target core clock components in the control of NLRP3-driven diseases (174). Such circadian effect of drug efficiency might be explained by the lower severity of colitis at ZT10, which coincides with the maximum expression of Rev-erbα. In conclusion, circadian pharmacological effects of compounds on different diseases likely result from diurnal rhythms of both disease severity and daily oscillations of the drug target expression. Altogether, these observations advocate for chronotherapeutic practice on NLRP3-driven diseases.

CONCLUDING REMARKS

NLRP3 inflammasome deregulation drives numerous diseases. Inhibition of NLRP3 using MCC950 demonstrates beneficial effects in fulminant hepatitis and in myocardial ischemia reperfusion (3). However, MCC950 displays hepatotoxic properties advocating for the development of alternative NLRP3 inhibitory strategies (3). Here, we provide the first extensive review showing the close links between nuclear receptors and the NLRP3 inflammasome pathway. Indeed, NRs are able to either activate the NLRP3 inflammasome or inhibit both priming and activation steps of the NLRP3 inflammasome pathways, acting at different levels, which offers numerous possibilities to modulate NLRP3-driven disorders. Indeed, the activity of NRs can be modulated by a plethora of synthetic, but also natural ligands. As such, NRs should be considered as sensors of environment changes including metabolic alterations, hormonal signal, pollutions and circadian rhythmicity. As NRs are able to control similar processes, we

may consider that the entire NR family integrate these different environmental modifications, that may occur simultaneously, to deliver the best response. We may then anticipate that depending on their environment, NRs cooperate to appropriately modulate the NLRP3 inflammasome. NRs would then allow the adaptation of the innate immune system and the NLRP3 inflammasome to adjust its response from cytokine secretion to pyroptosis-induced cell death. Finally, nuclear receptors, including Rev-erb and ROR, control the circadian expression of NLRP3. As such, NLRP3 protein amounts are not equal across the day, thereby emphasizing the necessity of a chronotherapeutic approach. In the case of clock disruption as observed in shift workers or in elderlies for instance, targeting clock components to re-entrain the molecular clock and sustain circadian amplitude of NLRP3 expression may also be considered as an alternative or an additional approach.

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BP and HD wrote and edited the manuscript and the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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PPARgamma in Metabolism, Immunity, and Cancer: Unified and Diverse Mechanisms of Action

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The proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily, is one of the most extensively studied ligand-inducible transcription factors. Since its identification in the early 1990s, PPARγ is best known for its critical role in adipocyte differentiation, maintenance, and function. Emerging evidence indicates that PPARγ is also important for the maturation and function of various immune system-related cell types, such as monocytes/macrophages, dendritic cells, and lymphocytes. Furthermore, PPARγ controls cell proliferation in various other tissues and organs, including colon, breast, prostate, and bladder, and dysregulation of PPARγ signaling is linked to tumor development in these organs. Recent studies have shed new light on PPARγ (dys)function in these three biological settings, showing unified and diverse mechanisms of action. Classical transactivation—where PPARγ activates genes upon binding to PPAR response elements as a heterodimer with RXRα—is important in all three settings, as underscored by natural loss-of-function mutations in FPLD3 and loss- and gain-of-function mutations in tumors. Transrepression—where PPARγ alters gene expression independent of DNA binding—is particularly relevant in immune cells. Interestingly, gene translocations resulting in fusion of PPARγ with other gene products, which are unique to specific carcinomas, present a third mode of action, as they potentially alter PPARγ's target gene profile. Improved understanding of the molecular mechanism underlying PPARγ activity in the complex regulatory networks in metabolism, cancer, and inflammation may help to define novel potential therapeutic strategies for prevention and treatment of obesity, diabetes, or cancer.

Keywords: PPARy, adipocyte, immune cell, cancer cell, mechanism

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INTRODUCTION: PPARG

General Modes of Action

Since its discovery in the early 1990s by Tontonoz et al (1)., the nuclear receptor PPARγ, encoded by the *PPARG* gene on chromosome 3p25.2 in humans (**Figure 1A**) (2), has been recognized as the master regulator of adipose tissue biology. The human *PPARG* gene, encompassing 9 exons, generates four PPARG splice variants (PPARG1-4) encoding for two protein isoforms *via* differential promoter usage and alternative splicing (**Figure 1B**) (3). The mRNAs PPARG1,

PPARG3, and PPARG4 all give rise to the PPARγ1 isoform. PPARγ1 is a 477 amino acid protein that is broadly expressed with relative high levels in the adipose tissue, liver, colon, heart, various epithelial cell types, and skeletal muscle. In addition, PPARγ1 is expressed in numerous cells of the immune system, including monocytes/macrophages, dendritic cells, and T lymphocytes. The PPARG2 mRNA transcript translates into the PPARγ2 isoform. PPARγ2, containing an additional 28 amino acids in its NH2-terminus, is almost exclusively expressed in adipose tissue. This isoform is also expressed in urothelial cells (4, 5), which are highly specialized transitional epithelial cells that line the organs of the urinary system, including the bladder, and in regulatory T cells (Tregs) and other T cell populations, albeit that total PPARγ expression is low in non-Tregs (6). Recently, a third and fourth PPARγ protein isoform, denoted as PPARγ1Δ5, and PPARγ2Δ5, respectively, have been reported (**Figure 1B**) (7). PPARγ2Δ5 is endogenously expressed in adipose tissue and lacks the entire ligand binding domain (LBD) due to physiological exon 5 skipping (7). The endogenous expression PPARγΔ5 positively correlates with body mass index (BMI) in overweight or obese and type 2 diabetic patients. The naturally occurring PPARγΔ5 isoforms impair the adipogenic potential of adipocyte precursor cells by dominant-negative inhibition of PPARγ, which possibly contributes to adipose tissue dysfunction in obesity (7).

PPARγ is a representative member of the nuclear receptor (NR) superfamily. To date, 48 NRs have been identified in human. NRs regulate various critical aspects in development, physiology, reproduction, and homeostasis. NRs are multi-domain ligand-inducible transcription factors that share a structural homology to a varying extent (8). Alike other NRs, PPARγ contains an autonomous transactivation domain 1 (AF-1) in the unstructured N-terminus (**Figure 2**). The AF-1 domain is implicated in the constitutive ligand-independent activation of PPARγ target genes. Juxtaposed to the AF-1 domains is the DNA binding domain (DBD) that contains two zinc fingers required for DNA binding. The DBD connected to the ligand binding domain (LBD) *via* a flexible hinge region. In the case of PPARγ, this hinge region physically interacts with the DNA (9). The ligand binding domain (LBD) is situated in the C-terminus. The LBD is a complicated structure that is arranged in a conserved three-layered α-helical sandwich containing 12 α-helices and 4 β-strand elements (8). The LBD overlaps with the ligand-dependent transactivation domain 2 (AF-2). The LBD is a key domain for transactivation of PPARγ target genes as it is implicated in ligand binding, heterodimerization with binding partner retinoid X receptor alpha (RXRα), and interactions with transcriptional co-regulators.

PPARγ exerts its gene regulatory potential *via* transactivation and transrepression (**Figure 3**). Transactivation involves a mechanism by which PPARγ binds as a heterodimer complex with RXRα to PPAR response elements (PPREs) (10). PPREs consist of a hexameric repeat (AGGTCA) spaced by one or two nucleotides (referred to as DR1 and DR2 elements) (11), which are situated in promoter and enhancer regions of PPARγ target genes (12). Noteworthy, enhancers may not only loop to the nearest promoters, but can also increase transcription of their target genes *via* looping to promoters at greater genomic distances.

In the last decade, genome-wide binding profiles of PPARγ have been mapped in different cell types, including adipocytes and macrophages (13–17). These binding profiles have not only indicated that PPARγ binds to thousands of sites in the genome, of which many binding sites are located far from proximal promoters, but also that the PPARγ binding is highly context-dependent as binding sites differ between cell types and even between adipocytes from different anatomical locations (13–17). The context-dependency of PPARγ binding is at least in part mediated by cooperative binding to the chromatin with other adipogenic transcription factors, such as C/EBPα, followed by cooperative recruitment of coactivators (15).

Transcriptional control of the target genes by PPARγ furthermore depends on multiprotein coregulatory complexes that are recruited to the PPREs (18). In basal conditions, i.e., in absence of ligand, PPARγ/RXRα favors stable interactions with corepressor complexes, containing NCoR or SMRT, which recruit chromatin-modifying enzymes such as histone deacetylases that make the chromatin inaccessible to binding of transcription factors or resistant to their actions and thereby actively repress transcription (**Figure 3A**). Upon ligand binding, the PPARγ/RXRα heterodimer undergoes a conformational change that promotes corepressor release and recruitment of coactivators, like SRC1 and CBP. Coactivators enhance PPARγ transactivation by facilitating acetylation of the histone tails, making the chromatin less restrictive, and assembly of general transcriptional machinery. Next to the "classical" transactivation mechanism described above, PPARγ can also negatively regulate gene expression by a mechanism referred to as ligand-dependent transrepression (**Figure 3B**). This mechanism involves antagonizing the NF-κB and AP-1 pro-inflammatory signaling pathways, and has been mostly described in immune cells (19–23). In this case, PPARγ does not bind to DNA itself, and several studies indicate that PPARγ transrepresses genes as a monomer, i.e., independent of RXRα (23). While various mechanisms have been postulated for transrepression by different NRs (24–26), the most detailed mechanism proposed for PPARγ involves inhibition of co-repressor degradation. Pascual et al. (27) showed that clearance of NCoR/SMRT-HDAC3 complexes by proteosomal degradation from various AP1- and NFkB-regulated promoters (e.g., IL-8, Mmp12, and iNOS) upon activation is prevented in the presence of liganded, monomeric PPARγ.

Interestingly, the transrepression mechanism described above involves a specific post-translational modification, SUMOylation of lysine 365. In fact, to adequately processes external signals and adapt to relevant gene expression programs PPARγ activity is regulated by several, probably interconnected, post-translational modifications, including phosphorylation, acetylation, and the aforementioned SUMOylation [reviewed in (28)]. Depending on cellular context and the kinases involved, phosphorylation of PPARγ S112 can either impair or increase PPARγ activity (29). Phosphorylation of PPARγ S273 by Cdk5 does not affect its adipogenic capacity, but affects many PPARγ target genes

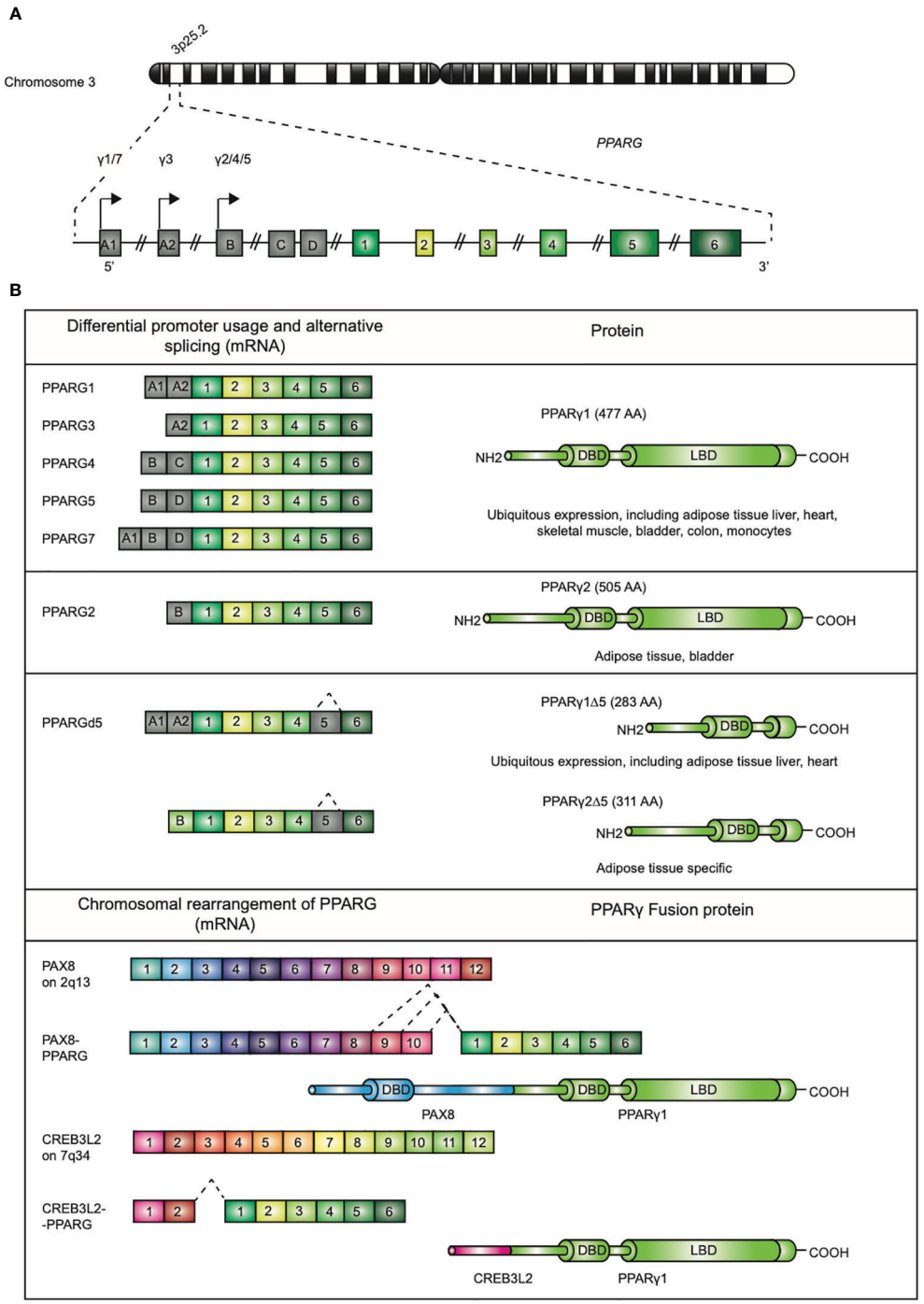

FIGURE 1 | Genomic map of the PPARG gene on chromosome 3p25 and structure of PPARγ isoforms. **(A)** The gene *PPARG* is situated on chromosome 3p25. The gene encompassed 9 exons (exon A1-2, exon B-D, and exons 1-6). **(B)** Alternative promoter and mRNA splicing give rise to several PPARγ mRNA and protein isoforms. The mRNAs PPARG1, -3, and -4 translate into PPARγ1 (477 amino acids; AA). mRNA PPARG2 gives rise to PPARγ2 (505 AA). A third and fourth PPARγ protein isoform, denoted as PPARγ1Δ5 and PPARγ2Δ5, have been reported. These isoforms lack the ligand binding domain (LBD), which is due to alternative splicing. Chromosomal rearrangement of PPARγ leading to PAX8/PPARγ and CREB3L2/PPARγ fusion proteins, contains functional DBDs of both proteins, have been described in carcinogenesis.

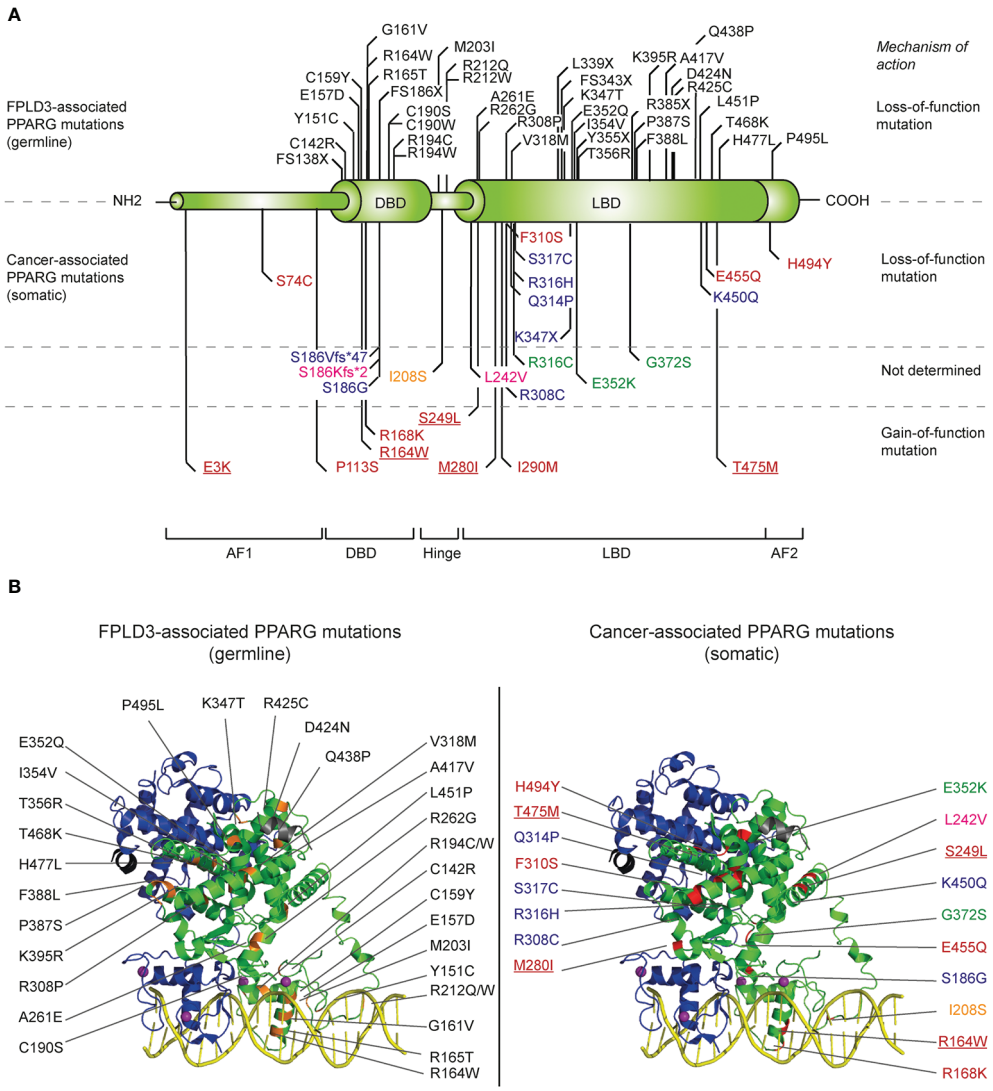

FIGURE 2 | Overview of identified natural *PPARG* mutations implicated in FPLD3 and cancer. **(A)** Schematic representation of the distinct domains of PPARγ. Mutations indicated above the PPARγ structure are mutations are germline loss-of-function mutation, implicated in FPLD3. Mutations depicted below the PPARγ structure are somatic loss-of-function or gain-of-function mutations identified in different cancer types. Mutations have been identified in tissue form digestive tract (colon, stomach, oesophagus, and pancreas; indicated in blue), melanoma (green), breast cancer (pink), prostate cancer (yellow), and bladder cancer (red). Some bladder cancer-associated PPARγ mutations (underscored in figure) have also been identified in other types of cancer, including lung cancer (E3K), kidney cancer (R164W), endometrium cancer (S249L), melanoma (M280I), and diffuse glioma (T465M), respectively. **(B)** FPLD3 (orange, left panel) and cancer associated mutations (red, right panel) indicated in 3D representation, based on the crystal structure of PPARγ (green)-RXRα (blue) on DNA (yellow) with Rosiglitazone, 9-cis retinoic acid and NCOA2 peptide (grey) (PDB entry 3DZY).

that have been shown to be dysregulated in obesity (30). In addition, acetylation of K268 and K293 correlates with the phosphorylation status of S273 and favors lipid storage and cell proliferation (31). Selective adipocyte deletion of the deacetylase Sirt1 that deacetylates PPARγ K268 and K293 leads to dephosphorylation of S273 and improve metabolic functions (32).

Alike other NRs, PPARγ governs nutrient- and hormone-mediated responses. Despite intensive efforts, it is not clear whether PPARγ is *in vivo* activated by a specific, high-affinity, and endogenous ligand. PPARγ LBD crystal structures reveal a large ligand binding pocket (LBP), which not only allows for promiscuous binding of ligands with lower affinity, but also allows ligands to occupy the canonical LBP in different conformations (33). Indeed, the activity of PPARγ can be modulated by a variety of natural compounds, including polyunsaturated fatty acids (34), eicosanoids (35, 36), and oxidized lipid components (discussed below) (37), suggesting that PPARγ functions as a general lipid or nutrient sensor (34). However, the physiological relevance of these compounds is not exactly clear. Endogenous ligands not only bind with low affinity for PPARγ, also the physiological concentrations in mammalian

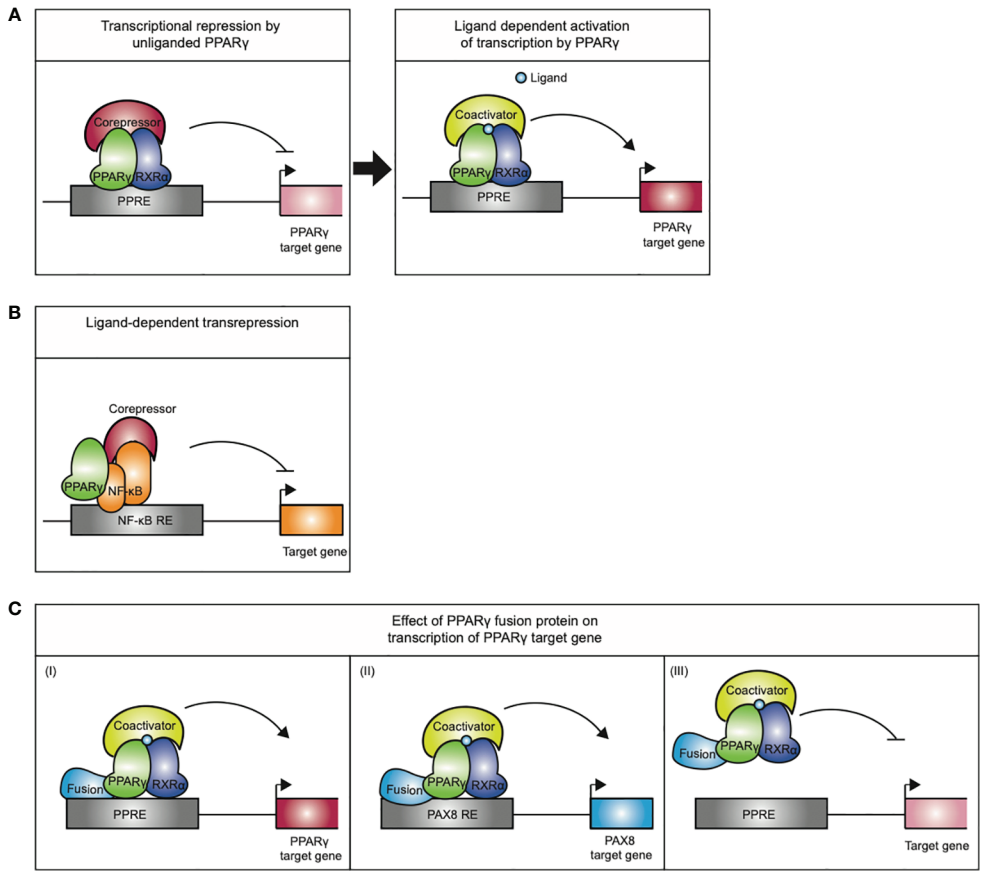

FIGURE 3 | Mechanisms of action exerted by the PPARγ/RXRα heterodimer. **(A)** Transcriptional repression by unliganded PPARγ. Upon ligand binding the PPARγ/RXRα heterodimer undergoes a conformational change that promotes corepressor release and recruitment of coactivators, initiating transcription. **(B)** Ligand-dependent transrepression by antagonizing the NF-κB (and AP-1, not indicated) pro-inflammatory signaling pathways. This effect does not require DNA binding by the PPARγ. **(C)** The mode of action performed by PPARγ-fusion proteins in carcinogenesis is not completely understood. (I) altered expression of PPARγ target genes, (II) altered expression of PAX8 target genes (III) PPARγ fusion protein may act as a negative inhibitor of tumor suppression by inhibiting PPARγ target gene expression.

cells are often insufficient to function as a physiological ligand (38). Alternatively, the physiological activation of PPARγ could be the resultant of combined effects of multiple ligands that simultaneously bind with different affinities to distinct subregions in the LBP (39), thereby inducing different PPARγ conformations with potential different biological outcomes (39).

PPARγ is the cognate receptor for thiazolidinediones (TZDs), a class of anti-hyperglycaemic drugs, including rosiglitazone and pioglitazone (40). TZDs stimulate adipogenesis (40) and cause a metabolically beneficial shift in lipid repartitioning from storage in visceral to subcutaneous adipose tissue depots as well as from ectopic storage in non-AT organs (e.g., liver muscle) to AT (41–43). TZDs and endogenous ligands have overlapping binding sites in the LBP, which potentially allows for binding competition to the same site. TZDs occupy the canonical LBP of PPARγ and by interacting with residues in helices 3, 5, 6, and 7 and the β-sheet, stabilizes the dynamics of helix 12 and the AF2 surface (44, 45).

Whereas TZDs are commonly referred to as full classical PPARγ agonists, TZDs have a separate biochemical activity: inhibition of the Cdk5-mediated phosphorylation of PPARγ at serine residue 273 (30). Phosphorylation of PPARγ S273 requires a physical interaction between CDK5 and PPARγ (46). The transcriptional corepressor NCoR is an adaptor protein for the physical interaction between CDK5 and PPARγ. Upon rosiglitazone the interaction between NCoR and PPARγ is reduced, which leads to i) derepression of PPARγ and activation of the PPARγ transcriptional program and ii) attenuation of the psychical interaction between CDK5 and PPARγ and subsequent reduced phosphorylation of S273 (46). Interestingly, MRL24 that displays poor agonistic activity but robust anti-diabetic activity in mice (47), was also very effective in inhibiting the Cdk5-mediated phosphorylation (30). This suggests that new classes of antidiabetic drugs that i) bind with high affinity to PPARγ, ii) specifically target the Cdk5-mediated phosphorylation of S273, and iii) completely lack the classical

transcriptional agonism, hold promise for treatment of T2DM. The PPARγ ligand SR1664 was essentially displayed no transcriptional activity and was very effective in blocking the Cdk5-mediated phosphorylation (48). In obese mice, SR1664 displayed strong antidiabetic effects without adverse effects (48). However, unfavorable pharmacokinetic properties of SR1664 preclude its administration in human (48). Therefore, SR1664 should rather be considered as a proof-of-principle.

In addition to binding in the canonical LBP, a recent structure-function study shows that some PPARγ ligands denoted as noncanonical agonist ligands (NALs), like the aforementioned compound MRL24, and SR1664, can also bind to an alternate site of PPARγ (49). TZDs, including rosiglitazone and pioglitazone display less prominent alternate site functional effects (49). The alternate binding of PPARγ ligands can occur when the canonical LBP is occupied by the covalent antagonists or endogenous ligands. Although the exact mechanisms are not clear, alternate site binding stabilizes the AF2 surface, most likely indirectly *via* stabilization of helix 3. Furthermore, coregulator-binding assays indicate that alternate site binding has an impact on coregulator interactions, transactivation, and target gene expression (49). The identification of the alternate binding site has three important implications. Firstly, compounds that block phosphorylation of S273 with little transactivation might be complicated by alternate site binding if this site *in vivo* contributes to classical PPARγ agonism. Secondly, it needs to be defined whether some of the supposed PPARγ-independent effects of TZDs could in fact be mediated by the alternate site binding. Lastly, allosteric modulators that target the alternate site might be particularly relevant for obese individuals in which the probability that canonical LBP is occupied by oxidized fatty acids due to increased bioavailability of endogenous ligands is increased (49).

PPARγ IN ADIPOSE TISSUE

White, beige, and brown adipocytes have been identified in mammals. Although these three type of adipocytes rise from different precursors and differ significantly in their morphology and function, the cells all go through a well-orchestrated differentiation process to become mature and fully functional (50). During the various stages of the adipocyte lifespan, PPARγ is a well-established key player. Recently, a fourth type of adipocyte, denoted as pink adipocytes, has been described in in mammary glands of pregnant mice (51). During pregnancy, lactation, and post-lactation subcutaneous white adipocytes in murine mammary gland undergo a transdifferentiation process ending in milk-producing epithelial glandular cells that contain abundant cytoplasmic lipid droplets to meet the nutritional needs of the pups (51, 52). As the number of studies in pink adipocytes is limited so far, we will focus in this review on the role of PPARγ in white, brown, and beige adipocytes. In these cells, PPARγ exerts its essential functions primarily *via* "classical" transactivation of target genes.

White Adipocytes

White adipose tissue (WAT) is the most abundant adipose tissue in the human body (53). Mature white adipocytes are unilocular cells composed of a large lipid droplet occupying ~95% of the cellular volume. Depending on the size of the lipid droplet, the cell size varies from 20 to 200 μM (54). The *in vivo* regulation of adipocyte development, including the stem cell commitment toward white adipocytes, is poorly understood. Adipocyte-lineage tracing, which so far can only be performed in mice, indicate that white adipocytes can be derived from both $Myf5^-$ and $Myf5^+$ precursor cells (55). The Myf5-lineage distribution in adipose tissue is dynamic and can be affected by ageing and diet. The $Myf5^-$ and $Myf5^+$ white adipocytes can compensate for each other during development, reflecting adipose tissue plasticity (55). In mice, depot-dependent variations were observed among the degree of plasticity (55). Although it remains to be defined whether this concept also applies to human adipocytes, a heterogeneity in adipocyte origins may explain the heterogeneity in adipose tissue depot function and contribute to adipose tissue patterning variations in the human population (55). After stem cell commitment toward white adipocyte lineage, the expression and activation of PPARγ is both sufficient and crucial to initiate the adipogenic differentiation program and maintain adipocyte phenotype, integrity, and function, based on a large set of different genetic mouse models (56). PPARγ primarily regulates the expression of genes implicated in adipocyte differentiation and adipocyte maintenance. In addition, PPARγ governs the expression genes involved in various processes in lipid and glucose metabolism including lipogenesis (e.g., *LPL, ANGTPL4*, and *CIDEC*), fatty acid transport (e.g., *FABP4*), and gluconeogenesis (e.g., *PEPCK, GYK*, and *AQP7*).

The importance of PPARγ for white adipose tissue biology in humans is underscored in patients suffering from familial partial lipodystrophy subtype 3 (FPLD3), a rare autosomal dominant inherited condition caused by loss-of-function mutations in the *PPARG* gene [reviewed in (28)]. Patients with FPLD3 lack subcutaneous adipose tissue in the extremities and gluteal region combined with lipohypertrophy in the face, neck, and trunk, and suffer from multiple metabolic complications including type 2 diabetes mellitus (T2DM). Since the first report of a germline loss-of-function mutation in *PPARG* in patients with FPLD3 (57) an increasing number of FPLD3-associated mutations in *PPARG* has been identified [reviewed in (28)]. The FPLD3-associated PPARγ mutations are mainly situated in either the DBD or LBD (**Figure 2**). Mutations in the DBD interfere in efficient DNA binding. Mutations affecting the LBD—which are scattered over the whole LBD, based on crystal structures (**Figure 2**)—often cause multiple molecular defects by impairing heterodimerization with RXRα, ligand- and/or cofactor binding (18).

Taken together, genetic mouse models together with the FPLD3-associated PPARγ mutations indicate that PPARγ plays a key role in white AT differentiation, function, and maintenance. The dominant mode of action in this biological

setting appears to be "classical" transactivation: the majority of genes regulated by PPARγ in white adipocytes rely on direct DNA binding, and FPLD3-associated PPARγ mutations do not alter transrepression, although this is not studied frequently (58).

Brown Adipocytes

Brown adipose tissue (BAT) emerged approximately 150 million years ago in mammals (59). BAT is unique for endothermic placental mammals and makes it possible to maintain a body temperature that is higher than the ambient temperature by producing heat independently of shivering and locomotor activity. This process is also referred to as non-shivering thermogenesis (59). BAT is richly innervated and vascularized and is composed of brown adipocytes (~40 μM in size) that contain multilocular lipid droplets and a large number of mitochondria (54). BAT derives its brown color from the conspicuous iron-rich mitochondrial mass. BAT uniquely expresses the gene *UCP1*, which encodes for uncoupling protein 1 (UCP1), located in the inner mitochondrial membrane. When activated, UCP1 mediates non-shivering thermogenesis by uncoupling of the oxidative phosphorylation from ATP synthesis, thereby provoking 1) dissipation of chemical energy in the form of heat and 2) stimulating high levels of fatty acid oxidation (60).

BAT is present in dedicated depots. In rodents, BAT is abundantly present throughout life. In human adults, BAT is located mainly cervical/axillary, perirenal/adrenal, and in the mediastinum along large blood vessels, trachea, and surrounding the intercostal arteries (59). In new-born infants, BAT is also situated between the shoulder blades as a thin kite-shaped layer (60). Although BAT depots regress with increasing age and can become even indistinguishable from WAT, healthy adults retain metabolically active BAT (61–63). For instance, positron emission tomography (PET) and computer tomography (CT) in human indicated that BAT-mediated thermogenesis is activated and increases in size by cold exposure (61–63). This process is also known as BAT recruitment. Depending on the size of the BAT depots, thermogenesis can account for up to approximately 15% of the total daily energy expenditure (64). Therefore, increasing energy expenditure by activation of BAT has been suggested as a therapeutic strategy for treating obesity (65).

Mice studies indicate that PPARγ functions is a master regulator in BAT (66). BAT-specific PPARγ knock out mice showed reduced wet weight of BAT, smaller brown adipocytes, and smaller lipid droplets when compare to wild type animals. However, there was no difference in total body weight or body composition (67). Furthermore, it was also shown that loss of PPARγ inhibited the ability of brown adipocytes to respond to β -adrenergic stimulus in *in vitro* cultures (67). An increase in non-shivering thermogenesis was observed in mice treated with TZDs (68, 69), and *in vitro* studies showed that activation of PPARγ in brown adipocytes leads to increase in adipogenesis and increase in lipid metabolism (70). Additional studies pointed at PPARγ as crucial regulator of UCP1 expression and BAT function (71). Specific BAT PPARγ target genes have been described (FABP3 and GYK), and particularly the de-acetylation of K268 and K293 of PPARγ by SIRT1 have been linked to BAT (32). De-acetylation of these residues is required for the recruitment of Prdm16, an essential cofactor in BAT (72). Moreover PGC1a, one of the most well-known regulators of BAT, has also been identified as a cofactor of PPARγ in BAT (73).

Collectively, PPARγ plays a key role in BAT differentiation and function, which most likely relies on "classical" transactivation, although transrepression cannot be excluded given the limited number of studies. BAT-specific molecular mechanisms, which may be different from WAT, could involve for example specific transcriptional cofactors (73), but details remain to be fully elucidated.

Beige Adipocytes

Mammals possess a second type of thermogenic adipocytes: beige adipocytes, also denoted as "brite" (brown-like in white) adipocytes (74). Beige adipocytes are inducible thermogenic cells that are sporadically located in white adipose tissue depots (74). Beige adipocytes share many morphological and biochemical features with brown adipocytes (**Figure 1**) (60). Alike brown adipocytes, beige adipocytes contain multiple small lipid droplets and a large number of mitochondria that express UCP1. Recruitment of beige adipocytes, referred to as "browning" or "beigeing/beiging" of white adipose tissue, is induced in response to environmental conditions, including chronic cold exposure, exercise, long-term treatment with PPARγ agonists or β3-adrenergic receptor agonists, cancer cachexia, and tissue injury (75). It is currently unknown whether beige adipocytes arise through transdifferentiation from pre-existing white adipocytes or by *de novo* adipogenesis from a precursor cell pool, or both (76).

Although, the exact mechanism by which PPARγ agonists induce browning of white adipocytes is not exactly known, PPARγ agonist require full agonism to activate the browning fat program. The effect is at least in part mediated by PRDM16, a factor that as described above is essential in the development of classical brown fat (77). Therefore, it is likely that in beige adipocytes, alike brown adipocytes, "classical" transactivation by PPARγ is an important mechanism of action.

PPARγ IN IMMUNE CELLS

Even though PPARγ is the master regulator of adipocyte differentiation and function (78), already in one of the first publications showed high PPARγ expression in mouse spleen (79) suggesting a role for PPARγ in immune cells. In fact, PPARγ is expressed in a variety of immune cells and its role and importance have been investigated during the last twenty years (80–82). Although PPARγ expression have been described in several types of immune cells we will focus on monocyte/macrophages and dendritic cells as part of the innate immune system, and T cells of the adaptative immune system.

As described above for adipocytes, PPARγ plays a role in determining the cellular phenotype by regulating differentiation (adipogenesis) and function (e.g., lipid metabolism and secretome) by directly activating the transcription of so-called PPARγ target genes. Similar molecular mechanisms are in place in immune cells, and also here PPARγ can deterimine cellular phenotype: amongst

others, PPARγ 1) regulates macrophage differentiation, 2) regulates classical/alternative macrophage activation ("polarization"), 3) controls lipid metabolism in multiple immune cell types, and 4) plays an immune-modulatory role. PPARγ function in immune cells could also be categorized according to its mechanism of action, with the regulation of lipid metabolism and the ability to induce differentiation of immune cells more linked to "classical" transactivation, while the transrepression activity of PPARγ is more important in its immunomodulatory role and both mechanisms are involved in macrophage activation.

Transactivation by PPARγ in Immune Cells

PPARγ can directly activate the transcription of target genes in immune cells through direct DNA binding, similar to its activity in adipocytes described above. As mentioned earlier, the genomic locations where PPARγ binds and the target genes partly overlap between, for example, adipocytes and macrophages, but cell-type specific regulation may depend on cooperation with other transcription factors like PU.1 and STAT6 (17, 83).

PPARγ expression is highly induced during monocyte to macrophage differentiation (84–86), and although initial studies using embryonic stem cells suggested that PPARγ is dispensable in this process (87), more recent studies have demonstrated that PPARγ is essential for the differentiation of fetal monocytes into alveolar macrophages (88). In mature macrophages, PPARγ was found to cooperate with *PU1* specifically on monocyte-unique target genes (17), reminiscent of the interplay between PPARγ and C/EBPa in adipocytes mentioned earlier. PPARγ is also expressed in several dendritic cell (DC) subtypes and is also highly upregulated in monocyte-derived DC differentiation (89, 90). Although the importance of PPARγ in immune cell differentiation is evident, little is known about the exact function of the receptor in these differentiation processes. Better models are required as well as studying the contribution of PPARγ in a more cell-type specific way.

Next to macrophage differentiation, PPARγ is also an important regulator in macrophage polarization, where PPARγ activation drives the alternative M2 macrophage phenotype (91–93). Alternatively activated macrophages (M2 phenotype) can be induced by IL-4, IL-10, and IL-13 and are characterized by the expression of several genes including Arg1 and Mgl1/CD301a, CD-204 and mannose receptor/CD163, and IL-10 and transforming growth factor beta (TGF-β). Some of these, including Arg1 and Mgl1 (94), are direct PPARγ target genes. Furthermore, PPARγ expression is induced by IL-4/STAT6 signaling as well as IL-13 (95), and STAT6 functions as a "facilitator" of PPARγ signaling, all supporting the idea that PPARγ is crucial for the anti-inflammatory M2 phenotype in macrophages. It was recently found that PPARγ contributes to maintain a chromatin structure that facilitates the binding of STAT6 and polymerase II upon repeated IL-4 treatments. PPARγ recruits the coactivator P300 and RAD21 to the DNA and thus reinforcing a M2-like phenotype in macrophages (96), is worth mention that this function of PPARγ is independent of ligand binding.

Next to macrophage and DC differentiation and macrophage polarization, PPARγ can also directly regulate lipid metabolism in immune cells (37, 87, 92, 97, 98), reminiscent of its role in white and brown adipocytes. In monocytes, macrophages, and dendritic cells, PPARγ directly regulates the expression of genes involve in lipid transport and metabolism such as the class B scavenger receptor *CD36* (99), *FABP4, LXRA, and PGAR* (86). The use of PPARγ ligands in these cells has shown that the expression of these genes is upregulated upon treatment and downregulated when treated with PPARγ antagonists (100). The CD36 protein is also involved in macrophage uptake of oxLDL, but at the same time PPARγ directly activates an LXR-ABCA1 pathway for cholesterol efflux (97). In DCs PPARγ also plays a key role in lipid homeostasis by directly regulating many "known suspects" (101) but it also regulates another aspect of lipid homeostasis and lipid antigen presentation. Activation of PPARγ gives higher expression of CD1d, a molecule involved in the presentation of lipid antigens to T cells, resulting in a DC subtype with increased potential to activate iNKT cells (100, 102, 103). These findings indicate that PPARγ has a functional role in the modulation of the immune response through DCs beyond regulation of more classical lipid metabolism pathways.

Changes in the lipid microenvironment can trigger different DC functions that regulate the immune response (104). PPARγ classical transactivation role bridges the lipid microenvironment and the DC function by activating genes involve in lipid transport, metabolism, and presentation.

The classical role of PPARγ as a gene activator has also been studied in T cells and again relates to lipid metabolism (81, 82). T cells can be subdivided into cytotoxic T cells, T helper, and regulatory T cells (Treg), and the T helper cells can be further classified depending on the phenotype into Th1, Th2, and Th17; less well characterized are Th9 and Th22 subsets. Regardless of the subtype of T cell, activation of PPARγ is linked to an activation of genes related to lipid metabolism (CD36 and FABPs) indicating the importance of PPARγ in this process. Special mention deserves the visceral adipose tissue resident regulatory T cells (VAT Tregs), in which PPARγ has been implicated in its function and development (6). VAT Tregs represents a unique subtype of cells in which the expression of PPARγ positively correlates with the expression of chemokines and chemokines receptors (Ccr2, Cxcl3, and Cxcr6) that regulates leukocyte migration and infiltration, lipid metabolism genes, and IL10. Interestingly, the PPARγ1 and PPARγ2 isoforms induce the same genes upon activation in VAT Tregs (mainly related to lipid metabolism) but differ in the genes that they downregulate (6), the latter happening most likely through the mechanism of transrepression.

Transrepression by PPARγ in Immune Cells

The role of PPARγ as an immune-modulator, and in particular a repressor of inflammation, has been studied in most detail in macrophages and T cells (19–22, 93). Although the transrepression activity of PPARγ is probably not exclusive to immune cells, this immunomodulatory role is a good example of the importance of this specific mechanism of action of PPARγ.

In macrophages it has been shown that activation of PPARγ using TZDs suppresses the production of pro-inflammatory

cytokine, such as TNFα, IL-1B, and IL-6 (19, 93) and the expression of other genes involved in inflammation, including iNOS and MMP9, in a dose-dependent manner. As described above, inhibition of the transcription factors NFkB and AP-1 is the most widely studied mechanism, but other mechanisms are also possible (23). Similarly, in DCs PPARγ ligands downregulate chemokines and receptors (IL-12, CD80, CXCL10, RANTES) that recruit Th1 lymphocytes (100, 102). In addition, PPARγ activation in DC may impair the migration of these cells to the lymph nodes, and this might be partially due to inhibition of CCR7 by PPARγ (102, 105).

The role of transrepression by PPARγ in T cells has been the object of intensive discussion during the last two decades (81, 82, 106), as this mechanism of action was implicated in seemingly conflicting biological processes. Initial studies suggested that PPARγ had an inhibitory effect on T cell proliferation (107), and that the underlying mechanism involved transrepression of the IL2 gene: activated PPARγ was shown to bind to nuclear factor of activated T cells (NFAT) and repress its activity and binding to the IL-2 promotor (107, 108). Besides T cell proliferation, PPARy-mediated transrepression was reported as a repressor of excessive Th1 response, by on the one hand inhibiting production of the Th1 cytokine and antigen-specific proliferation and on the other hand controlling Th2 sensitivity to IL-33 (109, 110). In fact, Cunard and colleagues showed that PPARγ binds to the IFNγ promoter and is able to repress its expression when T cell were treated with PPARγ ligands, and that IFNγ expression was enhanced when cells were treated with PPARγ antagonist GW9662 (111). The underlying mechanism was proposed to be inhibition of AP-1 activity, similar to the transrepression mechanism in macrophages. However, while these studies suggest a pro-Th2 role for PPARγ mediated transrepression, PPARγ was also reported to be involved in the downregulation of well-known Th2 cytokines like IL-4, IL-5, and IL-13, again through interaction with NFAT (112). Altogether, these studies indicate that the role of PPARγ in the modulation of the Th2 response in T cells remains unclear and further research is needed to fully elucidate its function. Finally, PPARy-mediated repression is important for Th17 differentiation, as lack of PPARγ leads to increased Th17 differentiation while activation of PPARγ was shown to have inhibitory effects (22). PPARγ recruits NCoR and SMRT to the *Rorc* promoter, thereby inhibiting IL-17a expression, and blocks IL-6 signaling by inhibiting the DNA binding activity of STAT3 (20, 21).

In summary, transrepression by PPARγ—where it counteracts other transcription factors like NFkB, AP-1, NFAT, and STAT3—may be a major molecular mechanism that drives the functional phenotype(s) and secretory output of macrophages, dendritic cells, and T cells. Findings in T cells appear sometimes conflicting, which makes it difficult to assign a clear pro-Th1 or pro-Th2 role to PPARγ activation. It also indicates that the use of ligands in these cells might "hide" some of the PPARγ functions and more subtle approaches, such as the use of cels harboring specific PPARγ mutations or selective PPARγ modulators, must be used in order to fully elucidated PPARγ role in immune cells, taking the complex interactions between immune cell population into account.

PPARγ IN CANCER

Cancer is driven by the acquisition of genome instability. The cancer genome landscape contains an enormous diverse repertoire of amplifications, deletions, inversions, translocations, point mutations, loss of heterozygosity, and epigenetic changes that collectively result in tumorigenesis. The role of PPARγ in tumorigenesis is controversial. A large body of evidence suggests that PPARγ functions as a tumor suppressor, as activation of the PPARγ/RXRα signaling pathway in different types of cancer, including colon (113), lung (114, 115), pancreatic (116), prostate (117), and breast (118, 119) cancers, leads to inhibition of cell growth, decreased tumor invasiveness, and reduced production of proinflammatory cytokines. In addition, treatment with TZDs was shown to increase sensitivity to chemotherapy through downregulation of Metallothionein genes (120) and/or endotrophin (121), which may be linked to ligand-mediated prevention of S273 phosphorylation (122).

Furthermore, in lung cancer cells, a tumor suppressive function of PPARγ was contributed metabolic reprogramming (123), an essential biochemical adaptation required for cancer viability that is considered to be a crucial emerging hallmark of cancer (124). In contrast, a protumorigenic role for PPARγ has been suggested in a variety of cancers as well (5, 125, 126). Here, we will discuss several loss-of-function and gain-of-function mechanisms by which PPARγ can be implicated in tumor initiation and progression in several major cancers. In addition, we will address the yet partly undefined role of PPARγ fusion proteins in cancer.

Transactivation by PPARγ

Loss-of-function Mutations

As discussed above, the PPARγ1 isoform is highly expressed in colon epithelial cells. The role of PPARγ in the development of normal colon epithelium and colorectal cancers is not completely understood and seems to be dual. The growth and differentiation of many colorectal cancers can be considerably inhibited upon ligand activation of PPARγ1 (113). This finding suggests that PPARγ functions as a tumor suppressor during colorectal carcinogenesis. In line with this, somatic *PPARG* mutations have been reported in ~8% of sporadic colorectal cancers (**Figure 2**). Genetic and epigenetic phenomena due to genetic alterations in other genes, like *RAS*, can further decrease PPARγ function in colon cancer. Activating mutations in *RAS* for example can result in hyperactivation of ERK1/2 and JNK pathways and ultimately impair PPARγ activity (28). Whereas all FPLD3-associated *PPARG* mutations that have been reported to date lead to mutant proteins that show a consistent and profound impairment in the transcriptional activity of PPARγ, the functional effects of colon cancer-associated *PPARG* mutations vary considerably (127). So far, six unique somatic *PPARG* mutations in colorectal cancers have been reported (128, 129). A side-by-side analysis of these colon-cancer associated mutants with some FPLD3-associated PPARγ mutants, shows that the colon-cancer associated mutants do not consistently

display profound intra- and/or intermolecular defects (127). Moreover, while the abovementioned studies suggest that PPARγ functions as a tumor suppressor during colorectal carcinogenesis, it should be noted that other studies suggest that PPARγ activation increases the risk of developing colorectal cancer. Ligand-activation of PPARγ in *min* mice, an animal model for familial adenomatous polyposis due to mutations in the *APC* gene, results in a considerably greater number of polyps in the colon (125). Follow-up studies are clearly needed to reconcile these apparently conflicting findings and assign a clear role to PPARγ in colon cancer.

In basal bladder tumors, four non-recurrent loss-of-function PPARγ mutations (S74C, F310S, E455Q, and H494Y, **Figure 2**) have been identified (130). All four PPARγ mutants display significantly reduced transcriptional activities. Biochemical and biophysical analysis of amino acid residues F310 and H494, situated in helix 3 and 12, respectively, indicated that both residues are essential for proper stabilization of helix 12. F310S and H494Y favor an inactive conformation, impairing both a proper release of corepressors and recruitment of coactivators (130). Basal tumors rely on EGFR signaling for growth (131). Interestingly, in basal cell lines the overexpression of wildtype but not H494Y, downregulates EGFR signaling.

Although the cancer-related PPARγ mutants—which are mainly scattered throughout the LBD (**Figure 2**)—may display variable and more subtle, i.e., context-dependent, intra- and/or intermolecular defects than the FPLD3-associated PPARγ, the cancer-related PPARγ mutants (**Figure 2**) are impaired in their ability to exert "classical" transactivation.

Gain-of-Function Mutations

In addition to its well-established role as master regulator in adipocyte biology, PPARγ has also been shown to be involved in the terminal differentiation of urothelium (4), a layer of specialized epithelial cells lining the lower urinary tract. However, little is known about its function in the bladder and in the pathogenesis of bladder cancer. In 12–17% of the muscle-invasive bladder carcinomas (MIBC) and in 10% of the non-muscle-invasive bladder carcinomas, *PPARγ* focal amplifications leading to PPARγ overexpression have been reported, suggesting a role for PPARγ in the initiation and maintenance of bladder cancer. MIBC are biologically heterogeneous and can further be grouped into basal and luminal subtypes (132). PPARγ has a protumorigenic role in luminal MIBCS, as the loss of PPARγ expression impairs the bladder cancer cell viability (133). These luminal tumors maintain molecular urothelial differentiation, even in the loss of morphological differentiation (133). This molecular differentiation depends on PPARγ (133).

In approximately 5% of the MIBCs and the luminal subgroup of MIBCs hotspot mutations of RXRα (S427F/Y) has been identified. These RXRα mutations rely on the introduction of an aromatic amino acid residue that enhances the ligand-independent activation of PPARγ (134). Tumors harboring RXR S427F/Y display enhanced expression of genes implicated in adipogenesis and lipid metabolism, including *ACOX1, ACSL1, ACSL5*, and *FABP4* (135). In addition, the RXRα hotspot mutations stimulate the proliferation of urothelial organoids, render bladder tumor cell growth PPARγ-dependent, and favor tumor evasion by the immune system.

Recently, seven recurrent driver gain-of-function PPARγ mutations have been identified in luminal bladder tumors (E3K, S249L, M280I, K164W, and T475M) (5). The mutations occur throughout the protein, affecting the N-terminus, DNA-binding domain, and ligand-binding domain (**Figure 2**). One recurrent mutation (E3K) was specific to the PPARγ isoform as it was situated in the N-terminal end. Functional analysis indicates that five mutations promote the transcriptional activity of PPARγ, which renders PPARγ-dependence to the cells. The three recurrent LBD-mutations promote, in absence of PPARγ ligands, the adoption of the active conformation of PPARγ by stabilizing helix 12 and induce recruitment of co-activators. Interestingly, four of the seven recurrent PPARγ mutations have also been identified in other types of cancer, including lung cancer, kidney cancer, cutaneous melanoma, and diffuse glioma (**Figure 2**) (5). Furthermore, other recurrent mutations that have not been identified in bladder cancer, have been identified in other types of cancer, including melanoma and prostate cancer (**Figure 2**) (5). Surprisingly, one of these recurrent PPARγ mutations, which are yet functionally uncharacterized, results in the same amino acid changes as FPLD3-associated loss-of-function PPARγ mutations (e.g., R164W and E352Q/K). This may indicate that a potential loss-of-function or gain-of-function effect is context dependent.

Although, not all recently identified gain-of-function PPARγ mutants have extensively been characterized and even affect different domains in the protein, at least some of the mutants have implications for "classical" transactivation of PPARγ target genes in bladder cancer.

Somatic PPARγ Fusion Proteins in Cancer

Besides the loss- and gain-of-function mechanisms described above, a third way in which PPARγ may be involved in carcinogenesis is represented by PPARG gene fusions observed in follicular thyroid carcinomas (FTCs). The t(2;3)(q13;p25) chromosomal translocation results in a *PAX8/PPARG* fusion gene that is detected in approximately 35% of FTCs and in a subset of follicular variant of papillary thyroid carcinomas (136). This chromosomal rearrangement is occasionally present in follicular adenomas as well (137). The gene paired-box gene 8 (*PAX8*) encodes for a member of the paired box (PAX) family of transcription factors and is a critical regulator in physiological thyroid development (138). In addition, PAX8 promotes the thyroid progenitor survival en in the mature thyroid it drives the expression thyroid specific genes, including genes encoding for thyroglobin and thyroid peroxidase (138, 139). The endogenous expression of *PPARG* in the thyroid is extremely low and it remains to be defined whether PPARγ has a physiological function in the thyroid (140). The translocation t(2;3)(q13;p25) results in a fusion transcript, driven by the *PAX8* promoter, wherein most of the coding sequence of PAX8 is fused in-frame to the entire coding sequence of PPARγ1 (141). The PAX8-PPARγ fusion protein (PPFP) contains functional DBDs of both the PAX8 and PPARγ (142). *In vitro* and *in vivo* evidence indicates that the PAX8-PPARγ fusion protein can function as

an oncoprotein i) by acting as a negative inhibitor of tumor suppressor PPARγ or as ii) a novel transcriptional factor with proto-oncogene activity. Nevertheless, the expression of *PAX8-PPARG* in FTCs does not affect prognosis (143).

A second chromosomal translocation, t(3;7)(p25;q34) resulting in a *CREB3L2/PPARG* fusion gene, is a low incidence fusion mutation that is found in <3% of the FTCs (144). The gene cAMP Responsive Element Binding Protein 3 Like 2 (*CREB1L2*) encodes for a member of the bZIP transcription factor family. The CREB3L2/PPARγ fusion protein consists of amino acids 1 to 106 of wildtype CREB3L2, a new glutamic acid at position 107 juxtaposed to the all 477 amino acids of wildtype PPARγ1 (144). The CREB3L2/PPARγ fusion protein stimulates cell growth of transduced primary thyroid cells by inducing proliferation (144). The fusion protein seems to be unresponsive to thiazolidinediones. In addition, CREB3L2/PPARγ interferes in the CRE-related transcription as overexpression of CREB3L2/PPARγ inhibits the transcription of native cAMP-responsive genes in normal thyroid cells (144). The impaired ability to stimulate transcription is consistent with the loss of CREB3L2 bZIP domain, implicated in dimerization and DNA binding, in the CREB3L2/PPARγ fusion protein (144). The oncogenic activities of the CREB3L2/PPARγ fusion protein are most likely (at least in part) due to 1) disruption one functional CREB3L2 allele and 2) inhibition of cAMP responsive genes by interfering in CREB3L2 DNA-binding (144).

Taken together, the PPARγ fusion proteins display a third mode of PPARγ action, as they potentially alter the target gene profile of both parent proteins in the chimeric protein (**Figure 3C**) and will target multiple signaling pathways implicated in cancer.

Since the identification of the *PPARG* gene in the early 1990s the role of PPARγ in cancer has extensively been studied in many different human cancer cells and animal models. However, the biological significance of PPARγ in cancer development and progression is far from completely understood and for some cancers appears to be even inconsistent and contradicting. At best, the overall conclusion from these studies is that the context, e.g., specific tumor type, tumor stage, and tumor microenvironment, determines the exact role and function of PPARγ in human cancer. Therefore, cell-culture studies are limited in representing the complex gene-gene and gene-environment molecular interactions that are implicated in cancer onset and progression.

FUTURE PERSPECTIVES

For many years, PPARγ was referred to mainly as the master regulator of adipocyte function, and although its expression in the immune system was already described in early research, its actual role in these cells only became apparent later (**Figure 4**). Nowadays, the immunomodulatory role of PPARγ in several immune cells is well-established as described in this review. While PPARγ clearly functions in gene transactivation in both adipocytes and immune cells, gene repression by PPARγ has been predominantly investigated in immune cells. PPARγ has also emerged as a factor involved in cancer onset and progression of several cancer types in recent years. Also, in this case, transactivation mechanisms are clearly relevant, underscored by both loss-of-function and gain-of-function mutations. It should be noted however that no single unifying role for PPARγ in human cancer emerges, and that transrepression has not always been studied specifically. Finally, gene fusions with other gene products (PAX8, CREB3L2) as reported in specific carcinoma presents a third way in which PPARγ regulates gene expression, resulting in either altered target gene sets and/or loss of activation.

It is well known that PPARγ is the molecular target for TZDs, these drugs have been widely used for the treatment of hyperglycemia and T2DM. TZDs stimulate the expression of genes implicated in lipid uptake and storage (145) and consequently the levels of ectopically stored and circulating lipids are decreased. In addition, TZDs also increase the expression of adiponectin, which contributes to enhance insulin sensitivity of the liver, and improves hepatic steatosis (145). Given its central role in adipocyte biology and energy homeostasis, there is a clear rationale behind therapeutically targeting PPARγ and improving insulin sensitivity. However, the use of TZDs is curtailed due to serious side-effects [review in (146)]. Although some side-effects, such as troglitazone-associated hepatotoxicity and rosiglitazone-associated myocardial infarction have been solved (147), others are still present. These common side-effects include weight gain, fluid retention, and osteoporosis. These unwanted side-effects are due to the ubiquitous expression of PPARγ1 in combination with the full agonism characteristics of TZDs. As indicated earlier, new generations of ligands, referred to as noncanonical agonist ligands (NALs) and selective PPARγ modulators (SPPARMs), hold promise in that respect. In fact, very recently, it has been shown how selective modulators of PPARγ can improve liver histology without affecting body weight in biopsy-confirmed mouse model of nonalcoholic steatohepatitis (NASH) (148).

Similar to being a potential drug target in metabolism, PPARγ could represent a therapeutic target for a variety of cancers because of its ability to be selectively activated through its LBD. As indicated above, various parameters including tumor type and genetic background must be taken into account, as PPARγ displays oncogenic and tumor suppressor roles. Nonetheless, targeting PPARγ in the cancer context can be effective. In pancreatic ductal adenocarcinoma for example, the fourth most frequent cause in cancer-related deaths, PPARγ ligands have shown promising results *in vitro* and *in vivo* increasing apoptosis and reducing tumor growth, respectively (149, 150).

While we have described above that PPARγ is expressed in multiple cancer cell types, and PPARγ ligands can affect cancer cell function and behavior (e.g., proliferation and sensitivity to chemotherapy), some of the anti-cancer effects may actually occur indirectly through adipocytes surrounding the tumor or distal adipose tissue. PPARγ plays a crucial role in AT, and as it has been shown before, AT influences cancer initiation and progression through several mechanisms (151).

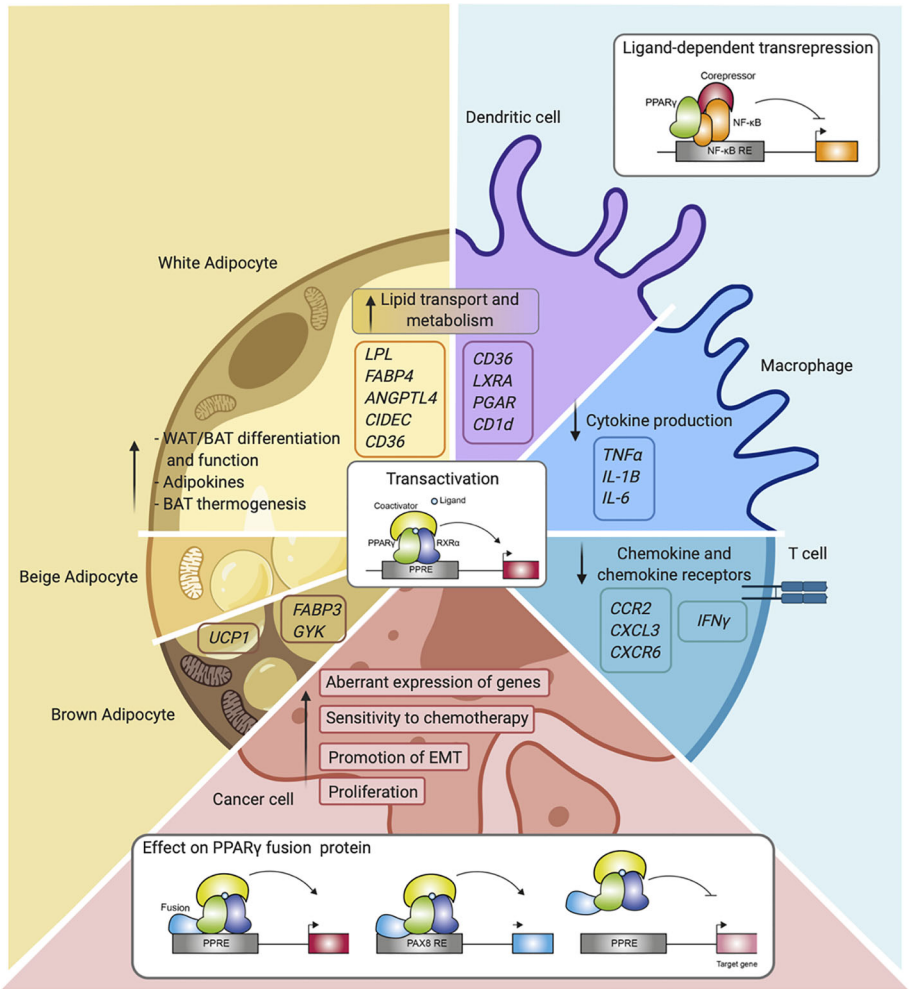

FIGURE 4 | Overview of PPARγ function and mechanisms in the different cell types. Schematic representation of PPARγ in adipocytes (white, beige, and brown adipocytes), immune cells (macrophages, dendritic cells, and T cells), and cancer cells. Indicated are different cellular processes and mechanisms of action in which PPARγ is involved.

It is estimated that obesity contributes to up to 20% of cancer-related deaths. Obesity is associated with increased risk of cancer development (i.e., colorectal, post-menopausal breast, and kidney among others) but the association with poor prognosis is even stronger for some of these cancer types. Obese AT is characterized by a chronic low-grade inflammation that leads to dysfunctional adipocytes, metabolic dysregulation, and secretion of pro-inflammatory cytokines are some of the factors that have been correlated with increased risk of cancer death. A clear example of this is the adipokine endotrophin (152), a cleavage product of the collagen VIα3 chain. Endotrophin has been shown to promote tumor growth by enhancing the ability of breast cancer cells to undergo epithelial to mesenchymal transition (EMT) in mice and humans (153). Interestingly, TZDs have been shown to decrease levels of endotrophin in obese patients (154).

A second exciting option to consider when considering the use of PPARγ ligands in cancer treatment is the role of the receptor in epithelial to mesenchymal transition (EMT). Epithelial cells that undergo EMT in the primary tumor acquire crucial features that increase their invasiveness, migratory phenotype, and resistance to apoptosis that are essential for the development of metastasis (155). Transdifferentiation of breast cancer epithelial cells undergoing EMT into post-mitotic adipocytes cells using TZDs and MEK inhibitors have been shown to be a promising therapeutic approach to repress primary tumor invasion and metastasis formation (156). The ability of PPARγ to drive or inhibit EMT might be subjected to the specific cell type from which the tumor arises however, as for example different studies in lung cancer cells have shown PPARγ ligands to inhibit and promote EMT (157). More research is needed to study the implication of PPARγ in

EMT to fully determine its role and if it can be a real cancer treatment option.

PPARγ plays a pivotal role in the crossroad between obesity, immunity, and cancer. Understanding the common and unique molecular mechanism underlying the function of PPARγ in these situations will allow the development of new therapies. In order to do so, some challenges have to be overcome; achieving a selective modulation of PPARγ and a cell-specific delivery of these modulators are two of them. In order to maximize the beneficial effects of targeting PPARγ, the key might be that PPARγ has to be targeted in one specific cell type, and not indiscriminately throughout the whole body. The use of nanoparticles coupled to biological ligands that binds to specific membrane receptors for drug delivery is a technique that is been study for cancer treatment and it could have a bright future in the nuclear receptor field if its proven successful. Given the different and complex roles of PPARγ in metabolism, immunity, and cancer, which rely on overlapping and diverse mechsmisms of action, cell-specific delivery of PPARγ ligands, especially noncanonical agonist ligands (NALs) and selective PPARγ modulators (SPPARMs), represent a promising field of study for future research.

AUTHOR CONTRIBUTIONS

MH-Q, MB, and EK drafted, edited, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Disrupted Lipid Metabolism in Multiple Sclerosis: A Role for Liver X Receptors?

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Multiple sclerosis (MS) is a chronic neurological disease driven by autoimmune, inflammatory and neurodegenerative processes leading to neuronal demyelination and subsequent degeneration. Systemic lipid metabolism is disturbed in people with MS, and lipid metabolic pathways are crucial to the protective process of remyelination. The lipid-activated transcription factors liver X receptors (LXRs) are important integrators of lipid metabolism and immunity. Consequently, there is a strong interest in targeting these receptors in a number of metabolic and inflammatory diseases, including MS. We have reviewed the evidence for involvement of LXR-driven lipid metabolism in the dysfunction of peripheral and brain-resident immune cells in MS, focusing on human studies, both the relapsing remitting and progressive phases of the disease are discussed. Finally, we discuss the therapeutic potential of modulating the activity of these receptors with existing pharmacological agents and highlight important areas of future research.

Keywords: liver X receptor, multiple sclerosis, lipid metabolism, nuclear receptor, cholesterol

INTRODUCTION

Multiple sclerosis (MS) is a chronic degenerative disease of the central nervous system (CNS) and a major cause of neurological disability amongst young adults (1). The disease course is heterogeneous, characterized by acute onset neurological symptoms (relapses) and steady accrual of disability (progression). The underlying pathophysiology is complex and differences exist in the mechanisms causing relapse-predominant MS (RMS) and progressive neurodegeneration (either primary progressive where progression occurs from disease onset or secondary progressive where progression follows a period of relapsing disease) (2). In RMS, relapses are associated with auto-inflammatory processes driven by defects in immune regulation and activation and, migration of multiple effector immune cells across the blood brain barrier (BBB) into the CNS. Interactions between autoreactive immune cells and CNS resident cells, such as microglia and astrocytes, result in the release of inflammatory mediators that exacerbate localized inflammation. These inflammatory episodes resolve and lesions remyelinate, however subsequent neuronal degeneration can lead to persistent disability (3, 4).

The mechanisms driving accrual of disability in progressive MS are not well characterized but include neuro-axonal, oligodendrocyte and astrocyte damage leading to neurodegeneration. This is mediated by compartmentalized chronic inflammation within the CNS, involving the formation of CNS lymphoid-like structures and activation of CNS-resident innate cells (including microglia); notably, unlike RMS, the BBB is less permeable to immune cells migrating from the periphery (5–7).

Evidence supports a role for lipid metabolism (including changes in cholesterol, oxysterols, sphingolipids and fatty acids) not only in MS pathogenesis, but also as biomarkers of disease activity and progression and as treatment targets (8–14). One hypothesis is that abnormal lipid-mediated signaling in immune cells could contribute to MS pathogenesis (15). Lipid metabolism plays a crucial role in immune cell activation, differentiation and effector function (16). For example, activated T-cells have higher plasma membrane cholesterol (17) and fatty acid levels (18) and, fatty acid synthesis controls lineage differentiation into pro-inflammatory T-helper (Th)17 cells (19). Furthermore, modulation of plasma membrane lipid rafts, signaling microdomains in the plasma membrane enriched with lipids such as cholesterol and glycosphingolipids, influence immune cell differentiation and function (20, 21) with potentially pathogenic consequences (22). Conversely, manipulation of plasma membrane lipids can restore immune cell function in autoimmunity and cancer (23–25).

Interestingly, statins, inhibitors of the cholesterol biosynthesis enzyme 3-Hydroxy-3-Methylglutaryl-CoA Reductase-a widely used class of lipid lowering therapy, have been extensively studied in MS (26). Notably, a phase-II clinical trial showed that high dose simvastatin (CNS-penetrant statin) attenuated brain atrophy and disease progression without adverse effects in secondary progressive MS patients (27). A phase-III clinical trial is underway (MS-STAT2; NCT03387670, http://www.isrctn.com/ISRCTN82598726). Statins have pleiotropic effects on the immune system through the simultaneous promotion of Th2 differentiation, inhibition of Th1 mediated damage and reduction of neurotoxic pro-inflammatory molecules (28). Simvastatin also inhibits secretion of cytokines necessary for Th1 and Th17 differentiation in RMS patients (29) by inhibiting the interferon regulatory factor-4 transcription factor (30). Statins may also work through inhibition of mevalonate pathway-derived isoprenoids that mediate membrane association of certain signaling proteins, rather than direct inhibition of cholesterol itself (31, 32).

How disrupted lipid metabolism influences disease processes in MS remains uncertain. The lipid-activated nuclear receptors, liver X receptors (LXRs) and peroxisome proliferator-activated receptors (33, 34), are responsible for integration of lipid metabolism signaling in multiple immune and neuronal cell types, and could both play an important role (33, 35). This mini review presents evidence to support a role for LXRs in dysregulated lipid metabolism and immunopathogenesis in MS.

LIVER X RECEPTORS

LXRs are nuclear transcription factors with key functions in lipid metabolism and cholesterol homeostasis (36–39). Two isoforms exist, LXRα and LXRβ, encoded by *NR1H3* and *NR1H2* genes respectively (40). They share 78% of their amino acid sequence identity but are differentially expressed; LXRα in metabolically active tissues (including liver, adipose tissue, macrophages, lung, intestine) while LXRβ is expressed ubiquitously. LXRs are activated by oxidized derivatives of cholesterol (oxysterols) (41–43) and intermediates of cholesterol biosynthesis (44, 45). Synthetic ligands for LXRs have been developed and used to understand LXR function, the most common being GW3965 and T0901317 (later reported to also act on other nuclear receptors) (46–49).

Cholesterol forms an essential component of cellular membranes and its oxysterol derivatives regulate many cellular processes. Cholesterol overload is toxic to cells, therefore pathways responsible for its generation are coupled to those responsible for cellular efflux (removal) and are tightly controlled, to ensure homeostasis (17). LXRs regulate intracellular lipid (including cholesterol) metabolism through a number of pathways including reverse cholesterol transport *via* the ATP binding cassette transporters (ABC)A1 (50) and ABCG1 (51) which promote cholesterol removal to the liver for catabolism and excretion by high density lipoprotein (HDL) particles. LXRs regulate the transcription of numerous genes involved in this process including, apolipoprotein-A1 (Apo-A1), apolipoprotein-E (Apo-E) (52, 53) and cholesteryl ester transfer protein (54). Other processes regulated by LXRs include; inducible degrader of the LDL receptor (55); Niemann Pick type-C proteins-1 and 2 involved in the lysosomal/late endosomal trafficking and recycling of intracellular lipids (56); fatty acid metabolism both *de novo* synthesis or through the Sterol Regulatory Element Binding Protein (SREBP)1, fatty acid synthase (FASN (57)) and fatty acid desaturation (FADS1, FADS2), elongation (elongation of very long-chain fatty acids protein) and phospholipid remodeling (Phospholipid transfer protein and lysophosphatidylcholine acyltransferase-3) (58–60).

The brain contains 20% of body cholesterol and ~70-80% of cholesterol in the brain comprises an essential component of myelin in neuronal cells (61). The BBB prevents cholesterol transfer from the circulation into the brain, therefore brain cholesterol is synthesized *de novo* (62) *via* the 3-hydroxy-3-methylglutaryl-coenzyme-A reductase pathway. Cholesterol produced by glial cells is effluxed *via* ABCA1 to HDL-like molecules such as Apo-E, where it is taken up by LDL-receptors and other lipoprotein receptors in neurons (which have a high demand for cholesterol due to the large area of membrane in axons and dendrites). Intracellular cholesterol is transported *via* Niemann Pick type-C proteins. Conversely, excess cholesterol is eliminated *via* hydroxylation to 24(S)-hydroxycholesterol (catalyzed by cholesterol 24-hydroxylase), a polar oxysterol and the most abundant oxysterol in the brain, which crosses the BBB, enters the circulation, and is eliminated by the liver (61, 63) (**Figure 1A**).

Oligodendrocytes maintain myelination and remyelination processes within the CNS and LXR-regulated lipid metabolism

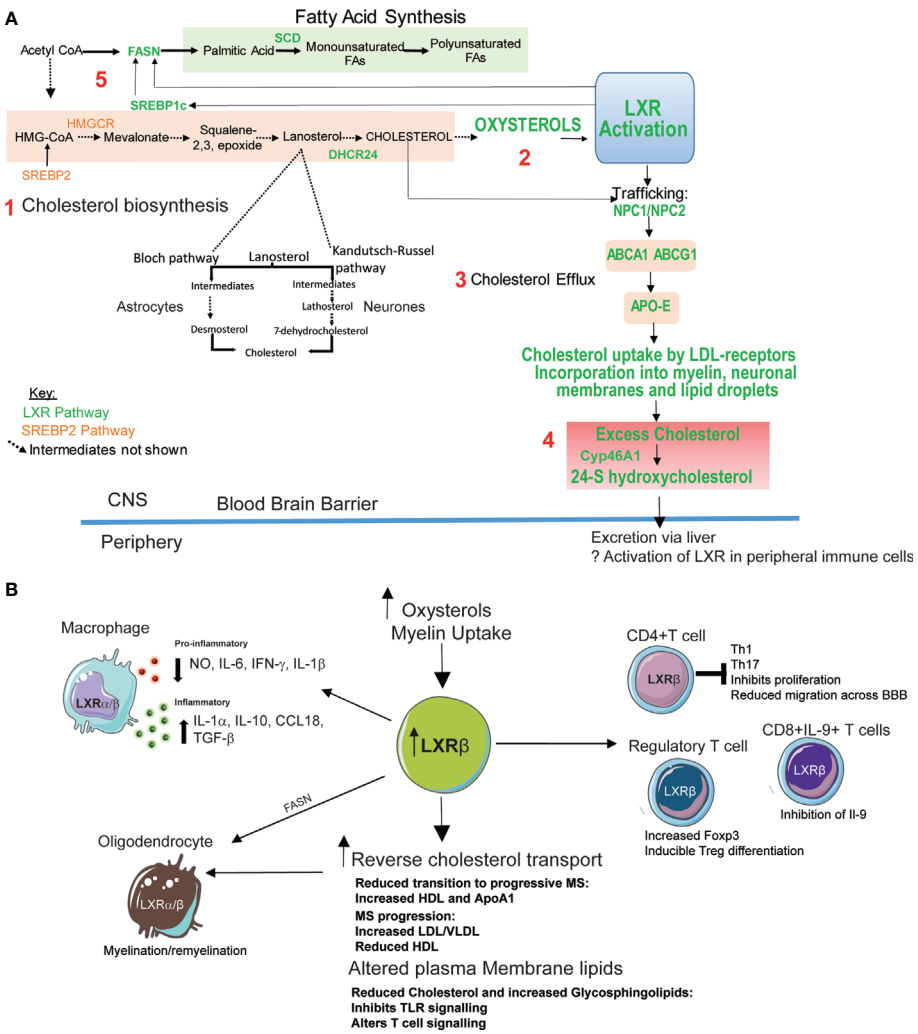

FIGURE 1 | Potential therapeutic roles of LXR activation in MS. **(A)** Intracellular cholesterol levels in the brain are tightly regulated by two transcription factors (61): 1. Liver-X-receptor (LXR) and sterol response element binding-protein 2 (SREBP-2). SREBP2 upregulates genes involved in cholesterol biosynthesis. Cholesterol in the brain is produced *de novo* mainly by glial cells such as astrocytes using the Bloch pathway. Neurons which have a high cholesterol requirement produce less cholesterol *via* the Kandutsch-Russell pathway. 2. LXR is activated by by-products of cholesterol synthesis (oxysterols). 3. LXR activation promotes cholesterol export *via* intracellular cholesterol transporter Niemann Pick Type C1 and 2 (NPC1/NPC2), and ATP binding cassette (ABC) A1 and ABCG1 which efflux cholesterol from the plasma membrane to high density lipoprotein (HDL)-like lipoproteins including apolipoprotein-E (Apo-E). 4. Cholesterol is taken up by cells *via* lipoprotein receptors. Excess cellular cholesterol (potentially generated by neurodegeneration processes) is stored in lipid droplets or converted into oxysterols. 24-S hydroxycholesterol is the most abundant oxysterol in the brain and its production is catalyzed by the enzyme Cyp46A1 (cholesterol 24S-hydroxylase). 24-S hydroxycholesterol is able to cross the blood brain barrier to the periphery where it is degraded in the liver. 5. LXR also promotes fatty acid synthesis through its target genes SREBP1c, fatty acid synthase (FASN) and stearoyl-CoA desaturase (SCD). Plasma membrane levels of cholesterol and fatty acids can influence lipid rafts-membrane microdomains important for immune synapse formation and immune cell activation and function. Fatty acid (glycosphingolipid) abundance and composition can also influence plasma membrane fluidity (64). **(B)** LXRβ expression is elevated in peripheral blood mononuclear cells from MS patients potentially due to increased levels of oxysterols including 24S-hydroxycholesterol. Increased LXR activation can also be triggered by myelin uptake by glial cells in the central nervous system (CNS). LXR activation induces reverse cholesterol transport (**A**, 4). Patients with MS have altered lipoprotein profiles which may reflect defects in the efficacy of this process. MS progression is associated with reduced levels of high density lipoproteins (HDL)- responsible for effective cholesterol efflux. LXR activation also induces fatty acid and glycosphingolipid biosynthesis (**A**, 5). Changes in cellular cholesterol and glycosphingolipids can alter immune cell function by altering cell signaling and downstream functions including proliferation and cytokine production. In T-cells LXR activation reduces T-cell infiltration into the CNS (65) and inhibits naïve CD4+ T-cell differentiation towards an inflammatory Th17 phenotype (66) and suppressed IL-9 producing CD8+ T cells during anti-tumor responses (67). LXR activation is crucial for Treg function (68). LXR activation stimulates oligodendrocyte myelin production and remyelination processes (69). Mechanisms include stimulation of reverse cholesterol transport and fatty acid synthesis. LXR activation leads to the repression of inflammatory responses through the downregulation of pro-inflammatory genes including inducible nitric oxide synthase (NO), interleukin (IL)-1β, IL-6 and tumor necrosis factor-α. Myelin uptake by macrophages activates LXR and suppresses the production these pro-inflammatory mediators These myelin-laden macrophages, express high levels of anti-inflammatory IL-1-receptor-α, IL-10, CC-chemokine ligand-18 and transforming growth factor-β (70).

pathways are crucial to their function (71, 72). CNS myelination is reduced in LXR knockout mice, conversely LXR activation stimulates oligodendrocyte maturation, myelin production and remyelination processes (69). Mechanisms include stimulation of reverse cholesterol transport *via* LXR target genes including ABCA1 and Apo-E, which restore remyelination in aged mice (12) and fatty acid synthesis; depletion of the LXR-target gene FASN blocked oligodendrocyte myelination and remyelination in the murine CNS (73).

Cholesterol, Oxysterols and LXR in MS

The relationship between disrupted serum cholesterol levels and adverse clinical outcomes in MS has been observed in several studies (74). Notably elevated apolipoprotein-B (Apo-B) (the major component of low/very low density lipoprotein cholesterol, LDL/VLDL) in clinically isolated syndrome (before confirmed MS diagnosis) correlated positively with increased Expanded Disability Status Scale (EDSS) indicating that cholesterol levels could serve as biomarkers for disease progression (74, 75), even accounting for age as a confounder. Similarly, in RMS, elevated serum LDL correlated positively with disease activity assessed by new MRI lesions (10, 11); increased LDL, total cholesterol and Apo-B levels were independently associated with higher EDSS score (9, 76); as were elevated VLDL subset levels (77). Conversely, high serum HDL was associated with reduced BBB injury and reduced inflammatory infiltrate in the cerebrospinal fluid (78). In RMS, increasing HDL and Apo-AI levels over time predicted a reduced likelihood of transition to secondary progressive disease and reduced brain atrophy (79). Also a greater reduction in HDL following interferon-β treatment in RMS patients predicted lower rates of future brain atrophy (10).

Differential patterns of oxysterol expression are also described in MS depending on the stage of disease (80, 81). Higher circulating oxysterols, notably, 24S-hydroxycholesterol, are thought to reflect elevated brain cholesterol metabolism and ongoing neurodegeneration (74, 81, 82). RMS patients progressing to secondary progressive disease over 5 years had higher CNS-derived serum 24S-hydroxycholesterol and Apo-B and reduced 7-ketocholesterol (83). While one study shows increased serum 7-ketocholesterol in patients with primary progressive disease (80). In older patients with RMS and those with primary progressive MS, serum 24S-hydroxycholesterol levels are low (84, 85) most likely due to increased brain atrophy and neuronal loss.

How changes in systemic cholesterol and oxysterols relate to LXR function in MS remain uncertain. Changes in oxysterol availability in MS (83) could lead to modulation of LXR signaling and influence subsequent immune cell function. For example, Th17 cells upregulate an enzyme that sulfates oxysterols (SULT2B1), thereby inactivating them as LXR ligands and driving preferential activation of RORγt (essential for Th17 function) instead of LXR (86). Also cholesterol/oxysterols are tightly suppressed in a subset of IL-9 producing $CD8^+$ T cells to prevent transrepression of the *Il9* locus by LXR (67) and differentiated type-1 regulatory T-cells (Tregs) upregulate 25-hydroxycholesterol to limit IL-10 production (87).

LXRβ expression is elevated in peripheral blood mononuclear cells from MS patients compared to healthy controls supporting a role for LXR in immune cell dysregulation (88) and LXR signaling was upregulated in T-cells during the adoptive transfer EAE (experimental autoimmune encephalomyelitis) model of MS (89). Interestingly, absence of LXRα in brain endothelial cells in EAE resulted in more severe disease, increased BBB permeability and CNS inflammatory infiltrate (90).

MS patients are also characterized by other defects in lipid metabolism. A lipidomic analysis of $CD4^+$ lymphocytes from MS patients identified altered phospholipids and elevated cardiolipins, potentially reflecting mitochondrial dysfunction (91). Glycosphingolipids (including ceramides and downstream metabolites hexosylceramide and lactosylceramide) are dysregulated in MS serum, plasma and immune cells (92–94). For example, decreased ceramides in white blood cells from MS patients were associated with impaired granulocyte-colony stimulating factor signaling and impaired neutrophil migration (93) and altered glycosphingolipid synthesis induced pathogenic inflammatory processes in astrocytes in a murine model of secondary progressive MS (95). Our recent work shows that LXR activation accelerates the conversion of ceramide to hexosylceramide (a key event in glycosphingolipid biosynthesis) in human $CD4^+$T-cells. LXR stimulation regulated $CD4^+$T-cell function in part by upregulating plasma membrane glycosphingolipids and reducing cholesterol thereby altering T-cell receptor-mediated signalling (96).

Collectively, these studies suggest that disrupted LXR function could be implicated in MS pathogenesis.

Anti-Inflammatory Effects of LXRs in Immune Cells

LXR activation leads to the repression of inflammatory responses through the downregulation of pro-inflammatory genes including inducible nitric oxide synthase, interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (97–100). This was thought to result from a transrepression mechanism involving SUMOylation of ligand-bound LXR. In macrophages, SUMOylation of LXR stabilizes corepressors on the nuclear factor kappa B (NF-κB) transcription factor, therefore dampening the transcription of target genes (101). However, a more recent study demonstrated LXRs ability to repress inflammatory genes in the absence of SUMOylation *via* the upregulation of the transmembrane cholesterol transporter ABCA1 which increases cholesterol efflux, alters plasma membrane lipid raft composition, and thereby inhibits Toll-like receptor signaling to downstream effectors NF-κB and mitogen-activated protein kinase (64).

The role of microglia (CNS-resident macrophages) in MS is complex; they can be both pathogenic (antigen presentation to T-cells and release of pro-inflammatory cytokines) and anti-inflammatory (clearing myelin debris and enabling remyelination) (102). LXR response genes ABCA1 and Apo-E are upregulated in microglia from active demyelinating MS lesions (103). The same study shows that myelin uptake induces production of 27-hydroxycholesterol oxysterol which

activates LXRα and induces ABCA1 and Apo-E upregulation in human monocyte-derived macrophages. Myelin uptake by macrophages also activates LXRβ and suppresses the production the pro-inflammatory mediators nitric oxide and IL-6 and interferon-γ/IL-1β signalling (104). These myelin-laden macrophages, termed foamy macrophages, similar to lipid-laden macrophages present in atherosclerotic plaques, and derived from either resident microglia or infiltrating monocytes, have a distinct phenotype characterized by enhanced expression of genes involved in migration, phagocytosis and inflammation as well as genes involved in LXR signaling and cholesterol efflux. Moreover, murine foamy macrophages within MS lesions, defined by elevated HLA-DR and neutral lipid content, express high levels of anti-inflammatory IL-1-receptor-α, IL-10, CC-chemokine ligand-18 and transforming growth factor-β (70). Thus the anti-inflammatory effects of foamy macrophages arise from their response to phagocytosis of myelin, at least in part *via* LXR activation which suppresses pro-inflammatory mediator release and also inhibits T-lymphocyte proliferation (105).

LXR activation ameliorates EAE severity, potentially by reducing infiltration of T-cells into the CNS (65). Activation of LXRα and LXRβ can also inhibit naïve $CD4^+$ T-cell differentiation towards an inflammatory Th17 phenotype. This occurs by activating SREBP1a and SREBP1c, which bind to the IL-17 promoter and the aryl hydrocarbon receptor (Ahr) (a positive regulator of Th17 differentiation), thus antagonizing Ahr-mediated IL-17 transcription (66). IL-17 suppression following LXR activation has been reproduced in splenocytes from the EAE model (106) and in in the context of other autoinflammatory diseases (107) such as Behcet's disease. In murine models, LXR is crucial for Treg function by increasing Foxp3 expression and promoting inducible-Treg differentiation (68, 108).Together, these studies demonstrate that activation of LXR influences macrophage and T-cell differentiation and polarization (66, 104, 106, 107). These actions may be protective in the context of MS (**Figure 1B**).

Therapeutic Activation of LXRs

Due to their actions on lipid and cholesterol metabolism and the immune system, LXRs have attracted interest as therapeutic targets in neurodegenerative diseases (109, 110). Despite numerous studies showing the benefits of LXR agonism with the first generation of these compounds in experimental models, their translation to clinical practice has proven difficult. Systemic LXR activation promotes hepatic lipid accumulation (steatosis) and hypertriglyceridemia, both risk factors for cardiovascular disease, through the induction of *de novo* lipogenesis by LXRα in the liver (39). This prompted the development of a new generation of selective agonists, including selective LXRβ-agonists, tissue-selective agonists or agonists targeting the trans-repression/anti-inflammatory actions of LXRs (109) although, to our knowledge, none of these have been tested in preclinical models of MS (**Table 1**).

Macrophage-selective LXR agonists such as N,N-dimethyl-3β-hydroxycholenamide (DMHCA) and the desmosterol mimetic methylpiperidinyl-3β-hydroxycholenamide (MePipHCA) are examples of transrepression-dissociated agonists that avoid SREBP1c-driven hypertriglyceridemia (114, 115), as does the ATI-111 compound (116). By activating reverse cholesterol transport-related LXR target genes while blocking the processing of SREBP-1c, they act similarly to the endogenous ligands (e.g., desmosterol and oxysterols), which inhibit SREBP

TABLE 1 | Summary of synthetic LXR agonist effects.

Compound	Activity	Status	Disease/Model	Actions	Reference
T0901317	LXRα/β dual agonist	Preclinical	EAE (MS model)	Reduced CNS inflammation Enhanced demyelination Reduced Clinical severity	(65, 66)
		Preclinical	WT mice	Enhanced Myelin gene/protein expression Increased Oligodendrocyte maturation Enhanced Remyelination	(69)
LXR-623	LXRα/partial/β full agonist	Clinical Trial-Phase 1-Discontinued	Atherosclerosis	Adverse neurological effects	(111)
		Preclinical	Glioblastoma	Enhanced cell death Increased cholesterol depletion Enhanced tumor regression Increased Survival	(112)
BMS-852927	LXRβ/selective partial agonist	Clinical Trial-Phase 1-Discontinued	Healthy subjects	Increased Cholesterol transport Enhanced Lipogenesis, triglycerides, LDL-C, apoB, apoE, CETP Decreased circulating neutrophils	(113)
DMHCA/ MePiPMHCA	Transrepression-selective	Preclinical	Colitis, brain injury	Reduced inflammation No induction of hepatic steatosis SREBP1c inhibition	(114, 115)
ATI-111	Transrepression-selective	Preclinical	Atherosclerosis (Ldlr-null mice)	Reduced atherosclerosis Lowers plasma triglycerides and cholesterol SREBP1c inhibition	(116)

activation through actions in the endoplasmic reticulum (117). More recent reports on T0901317 and GW3965 showing LXR-independent non-genomic effects in pancreatic β cells by interfering with mitochondrial metabolism and cytosolic calcium concentrations (118) highlights the importance of testing the impact of novel LXR agonists in appropriate cellular or experimental systems lacking the receptors or alongside validated LXR antagonists. Whether this is replicated in other cellular systems will require further investigation (119).

Studies with the first generation of LXR agonists suggested their use as novel therapeutic agents for the treatment of MS. LXR activation in EAE dramatically ameliorates demyelination and inflammation in an LXR-dependent manner (65, 66). LXR activation in cerebellar cultures, using T0901317 and 25-hydroxycholesterol, enhanced expression of myelin-associated proteins, likely through transcriptional changes, while reverting the demyelinating phenotype in an LXR-dependent fashion (69). This study points to a potentially beneficial effect of LXR agonists on CNS remyelination and reduced neuronal damage. Notably, a loss of function mutation in the *NR1H3* gene encoding LXRα in patients presenting with a rare genetic form of severe progressive MS, indicates that aberrant LXR signaling could be involved in MS progression (120). The synthetic LXR agonist T0901317 restored LXR-mediated ABCA1 expression in a cell-line transfected with the mutant LXR, suggesting that pharmacological activation of LXRs could be beneficial in progressive MS.

Strategies for tissue specific delivery are important in addressing the challenge of delivering therapeutic agents across the BBB during progressive MS, when inflammation is largely restricted within the CNS. Interestingly, a highly brain penetrant partial LXRα/full LXRβ agonist (LXR-623) had beneficial effects in a murine model of glioblastoma (112). However, in healthy volunteers LXR-623 showed adverse neurological effects at higher doses (111). Another study in healthy subjects using LXRβ selective agonist BMS-852927, showed enhanced cholesterol transport in human macrophages but also SREBP1c-induced lipogenesis which had not been predicted from primate models (113). Thus limitations exist using animal models to predict therapeutic responses in humans. Differences in TLR4 regulation between human and rodent cells (121, 122), treatment duration in culture (121) and differing eicosanoid regulation by LXR (58, 123) have been reported and could underpin some limitations of the first generation LXR ligands.

Targeting LXRs in specific cell types or tissues could yield promising results for LXR-based therapeutics. For instance, atherosclerotic plaque-targeting nanoparticles encapsulating LXR ligands upregulate LXR target genes (including cholesterol efflux genes) and downregulate proinflammatory mediators in macrophages *in vitro* and reduce macrophage frequency and promote regression in plaques without adverse effects on hepatic lipid metabolism *in vivo* (124–128). Similar strategies show promise at inhibiting inflammation (129), promoting resolution of inflammation (130) and improving apoptotic cell clearance through phagocytosis using gold nanocages loaded with LXR ligands (131). These recent advances could expedite the path to clinical translation for LXR agonists. There is a lack of MS-focused research exploring beneficial effects of LXR activation using these novel delivery approaches but they have paved the way towards further exploration of LXR ligands as effective therapeutics against MS.

DISCUSSION

In conclusion, further investigation into the role of LXRs in MS immunopathogenesis is warranted. Activation of these receptors can modify the expression of cytokines and other immune mediators and polarize immune cells towards pro or anti-inflammatory phenotypes (**Figure 1**). In experimental models, LXR activation can ameliorate clinical symptoms. The role of LXRs has focused primarily on $CD4^+$ T-cells and myeloid cells. However, the impact of lipid metabolism on other immune cells, particularly B-cells, is unexplored and could provide further insight into MS immunopathogenesis. Alternative strategies may focus on the modulation of immune cell function through lipid rafts.

Thus, dysregulated LXR-mediated pathways are likely to contribute to MS pathogenesis and provide a cohesive model describing the disease manifestations. A better understanding of LXRs in the context of MS is needed before their promising therapeutic potential can be fully realized.

AUTHOR CONTRIBUTIONS

SS researched and wrote a first draft of the review. KW, RF, IP-T, and EJ revised the manuscript. All authors contributed to the article and approved the submitted version.

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Crosstalk Between LXR and Caveolin-1 Signaling Supports Cholesterol Efflux and Anti-Inflammatory Pathways in Macrophages

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Macrophages are immune cells that play crucial roles in host defense against pathogens by triggering their exceptional phagocytic and inflammatory functions. Macrophages that reside in healthy tissues also accomplish important tasks to preserve organ homeostasis, including lipid uptake/efflux or apoptotic-cell clearance. Both homeostatic and inflammatory functions of macrophages require the precise stability of lipid-rich microdomains located at the cell membrane for the initiation of downstream signaling cascades. Caveolin-1 (Cav-1) is the main protein responsible for the biogenesis of caveolae and plays an important role in vascular inflammation and atherosclerosis. The Liver X receptors (LXRs) are key transcription factors for cholesterol efflux and inflammatory gene responses in macrophages. Although the role of Cav-1 in cellular cholesterol homeostasis and vascular inflammation has been reported, the connection between LXR transcriptional activity and Cav-1 expression and function in macrophages has not been investigated. Here, using gain and loss of function approaches, we demonstrate that LXR-dependent transcriptional pathways modulate Cav-1 expression and compartmentation within the membrane during macrophage activation. As a result, Cav-1 participates in LXR-dependent cholesterol efflux and the control of inflammatory responses. Together, our data show modulation of the LXR-Cav-1 axis could be exploited to control exacerbated inflammation and cholesterol overload in the macrophage during the pathogenesis of lipid and immune disorders, such as atherosclerosis.

Keywords: gene expression, caveolin-1, cholesterol efflux, inflammation, LXR, macrophage

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INTRODUCTION

Liver X Receptors (LXRα and LXRβ) are transcription factors that belong to the nuclear receptor superfamily. These are endogenously activated by oxidized forms of cholesterol (oxysterols), and function as intracellular sensors of cholesterol levels (1). The accumulation of cholesterol in macrophages, derived from the uptake of lipoproteins or cellular debris, leads to LXR activation and triggers the induction of a transcriptional program to promote cholesterol utilization. One of these pathways promotes the export of cholesterol and phospholipids outside the cell, through the transcriptional induction of members of the ATP binding cassette family such as ABCA1 and ABCG1 (2, 3). Indeed, the capacity of LXR synthetic ligands to inhibit the development of atherosclerosis in mice results, in part, by promoting the exit of excess cholesterol from lipid loaded macrophages or foam cells, known as cholesterol efflux (4). In addition to their important role in cholesterol metabolism, LXRs are also involved in inflammation and in the regulation of immune responses. Activation of LXR with synthetic ligands has been shown to repress the expression of inflammatory genes in macrophages such as inducible nitric oxide synthase (iNOS), COX-2 or pro-inflammatory cytokines including interleukin-6 (IL-6) and interleukin-1β (IL-1β) induced by bacteria or lipopolysaccharide (LPS) (5, 6). Several mechanisms have been proposed to explain the anti-inflammatory actions of LXR ligands, including transrepression, or the induction of anti-inflammatory molecules. Importantly, Ito et al. elegantly described a mechanism underlying this antagonism, which appears to involve the LXR ligand-dependent induction of ABCA1 and the redistribution of membrane lipids thereby resulting in reduced inflammatory signaling (7).

Caveolin-1 protein (Cav-1) is the most common isoform responsible for the formation of caveolae, a type of invaginated lipid raft microdomains between 50-100 nm of the plasma membrane enriched in cholesterol, which play an important role in the regulation of various cellular functions including endocytosis, transcytosis and cellular signaling (8). Cav-1 is also a high affinity cholesterol-binding protein. In fact, the formation of caveolae and the expression of caveolin-1 are highly dependent on the availability of cholesterol (9, 10). Previous studies have shown that these proteins participate in the regulation of plasma lipoprotein metabolism, as well as cholesterol homeostasis, a process that must be adequately controlled to limit and avoid cholesterol accumulation and, ultimately, prevent the development of atherosclerosis (11). In this context, Cav-1 has been shown to participate in intracellular trafficking of *de novo* synthesized cholesterol to the plasma membrane (12–15). Therefore, it is believed that the deficiency of Cav-1 would lead to the accumulation of cholesterol in certain intracellular compartments (16, 17). Other studies have also suggested that Cav-1 can participate in cholesterol efflux to extracellular acceptors (11, 18), however, very little is known about the role of Cav-1 in cholesterol efflux induced by LXR-ABCA1/G1 in macrophages. Despite that the presence of caveolae-like invaginations in immune cells has been a controversial topic (19), and that the major expression of Cav-1 is primarily found in vascular endothelial cells, there is ample evidence demonstrating that Cav-1 is also expressed and functional in different immune cells, including macrophages (20–26). Nevertheless, although several studies in the last two decades reported different roles for Cav-1 in the context of macrophage biology, the role of Cav-1 in macrophages is not completely understood. In these cells, Cav-1 appears to participate in apoptosis, lipid and cholesterol metabolism, as well as an anti-inflammatory mediator (27–29). Importantly, Cav-1 expression has been shown to increase in response to LPS but the overall participation of this protein in immune processes is not entirely clear (30, 31). Since dysregulation of lipid and immune homeostasis in macrophages are contributing factors for the development of several chronic diseases, our study aims to explore the possible participation of Cav-1 in the metabolic and inflammatory effects of LXR in macrophages.

MATERIAL AND METHODS

Animal Procedures

Cav-1 deficient mice (Cav-$1^{-/-}$, strain Cav-1tm1Mls/J, genetic background 129/Sv, C57BL/6J, and SJL) and their corresponding controls Cav-$1^{+/+}$ were obtained from the Jackson Laboratory (Bar Harbor, ME USA). LXRα$\beta^{+/+}$ and LXRα$\beta^{-/-}$ mice (Sv129/C57bl/6 background), were provided by David Mangelsdorf (University of Texas Southwestern, USA) and were maintained on standard chow under pathogen-free conditions. Mice aged 8-12 weeks were used for experimental procedures following Institutional Care Instructions (Bioethical Commission from Consejo Superior de Investigaciones Científicas).

Antibodies

Antibodies against caveolin-1, flotillin-1, clathrin, total STAT3 and STAT1 and their phosphorylated forms were obtained from BD Transduction Laboratories (Lexinton, KY). Monoclonal anti β–Actin was purchased from Sigma-Aldrich (St. Louis, MO) and polyclonal anti-iNOS, anti-GFP and anti-COX2 were obtained from Santa Cruz Biotechnology. ABCG1 Antibody (NB400-132) was from Novus Biologicals. ABCA1 and F4/80 were detected using specific anti serums, kindly provided by Michael L. Fitzgerald and Mason W. Freeman (MGH, Boston MA), and Siamon Gordon (Oxford University), respectively. Alexa Fluor-conjugated secondary antibodies were from Molecular Probes (Eugene, OR). Horseradish peroxidase HRP- and gold-conjugated secondary antibodies were from Jackson Inmmunoresearch Laboratories (West Grove, PA).

Cell Culture and Treatments

Macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection. RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) 100 units/ml penicillin and streptomycin and 2mM L-glutamine at 37°C in a humidified incubator at 5% CO_2 95% O_2. Culture reagents were purchased from BioWhittaker (Walkersville, MD). Mouse peritoneal macrophages were isolated from mice by peritoneal lavage using PBS 4 days after an intraperitoneal injection of 1.5 ml of sterile thioglycollate broth (thioglycollate-elicited).

Macrophages were seeded in 35-mm dishes at 3x 106/well in RPMI 1640 medium supplemented with 10% FBS and penicillin/streptomycin (each at 100 units/ml). Unattached cells were washed off after 3 h with RPMI (twice), and the remaining macrophages were incubated overnight in RPMI supplemented with 10% FBS. On the following day, cells were washed twice again, incubated in 0.5% FBS RPMI, and treated as indicated. For cell treatments we used the specific LXR agonists GW3965, provided by Tim Willson and Jon Collins (GlaxoSmithKline). T0901317 and the RXR agonist, LG268 obtained from TOCRIS. Ligands were dissolved in DMSO before use in cell culture. LXR ligands were used at 1 μM, whereas RXR ligand was used at 50 nM. LPS from E. Coli was purchased from Sigma.

Transfections

The full length murine Caveolin-1 or GFP cDNA were cloned in-frame in pCDNA3.1 vector (Invitrogen, Inc.). Twenty-four hours before transfection, $2x10^6$ RAW 264.7 cells were plated per 60-mm dish. On the day of transfection, 5 μg of plasmid DNA was diluted in 200 μl of serum-free DMEM media. In a separate tube, 20 μl of Lipofectamine 2000 was diluted in 200 μl of serum-free DMEM media. The diluted DNA and Lipofectamine were then gently mixed and incubated at 25°C for 30 min. After the incubation, 6 ml of serum-free RPMI media was added to the DNA/Lipofectamine mixture, mixed, and placed onto cells rinsed with serum-free DMEM media. The cells were incubated for 5 h at 37°C. Without washing, 3.6 ml of DMEM media containing 20% FBS was added. The cells were grown for 24 h. The media was removed, and DMEM media containing 10% FBS and 1.5 mg/ml Geneticin (G418) was added. Different clones of antibiotic-resistant cells were isolated and tested for Cav-1 and GFP expression. Cells were grown under constant selection in medium containing 500 μg/ml Geneticin.

Total Protein Extracts and Microsomal Purification

For total protein extracts, cells were washed in PBS and scraped with lysis buffer (62.5 mM Tris-HCl, 1% SDS, 60 mM octyl-glucoside 10% glycerol, pH 6.8) containing protease inhibitors (Roche, Boehringer Mannheim). To obtain liver protein extracts, the tissue was briefly dissected and washed thoroughly with ice cold phosphate buffered saline and snap frozen in liquid nitrogen. Frozen tissue was mechanically homogenized in lysis buffer with protease inhibitors. An aliquot of each extract was preserved for protein quantification by bicinchoninic acid assay (32). Five per cent β-mercaptoethanol and 0.001% bromophenol blue were then added, and samples were boiled at 95°C for 5 min. For microsomal fractionation, cells plated in three 100-mm wells were washed twice with ice-cold PBS and scraped down into PBS containing a protease inhibitor mixture. Cells were then sedimented and resuspended in 1 ml of hypotonic buffer (0.25 M sucrose, 20 mM Tricine, pH 7.8, 1 mM EDTA) with protease inhibitors. After 20 min on ice, cells were disrupted using a Potter-Elvehjem homogenizer. Nuclei and cellular debris were removed by sedimenting the homogenate at 1,000 g for 10 min at 4°C. The supernatant was ultracentrifuged 1h at 100,000 g in TLA-100.1 rotor (Beckman, Palo Alto, CA). The precipitated fraction was resuspended in lysis buffer and solubilized at 4°C.

Detergent-Free Caveolae Extraction

Caveolae extraction from mouse peritoneal macrophages and Raw 264.7 cell cultures were carried out following the procedure described previously were TX-100 was replaced by sodium carbonate and a sonication step was introduced to finely disrupt cellular membranes (33). Briefly, cells were homogenized using a loose-fitting Dounce homogenizer (10 strokes) and a sonicator (three 20-s bursts). 5mg of cellular homogenate was then adjusted to 45% sucrose by the addition of 2 ml of 90% sucrose prepared in MBS (25 mM MES, pH 6.5, 150mM NaCl) and placed at the bottom of an ultracentrifuge tube. 5mg of cellular homogenate was transferred to a SW41-Ti tube. A 5-35% discontinuous sucrose gradient was formed above and centrifuged at 39,000 rpms for 20 h in an SW41 rotor (Beckman Instruments, Palo Alto, CA) to obtain a total of 13 fractions A light-scattering band confined to the 5–35% sucrose interface was observed that contained caveolin but excluded most other cellular proteins. A volume of 20 μl form each sucrose fraction obtained was analyzed by western blot with specific antibodies.

Protein Analysis by Western Blotting and Bioplex ELISA

Equal amounts of each sample (25-50 μg) were electrophoresed on sodium dodecyl sulfate–polyacrylamide gels electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes. Membranes were pre-incubated with 5% blotting grade blocker non-fat dry milk (Bio-Rad Laboratories, Hercules, CA, USA) in TBS with 0.1% Tween 20 (TBS-T) at room temperature for 1 h and blotted over night at 4°C with the specific primary antibodies. Antibody-specific labeling was revealed by incubation with a HRP-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:5000) and visualized with the ECL chemiluminescence kit (Amersham Biosciences). Cytokine production *in vitro* by macrophages and its accumulation in the culture medium was quantified using an ELISA Bioplex kit (Bio-Rad) according to the manufacturer's instructions.

RNA Isolation and Gene Expression

RNA from liver and peritoneal macrophages were extracted using Trizol reagent (Life Technologies, Inc). Levels proinflammatory genes like IL-6, IL-1β, iNOS, COX-2, and other mRNAs (caveolin-1 and ABCA1) were determined by quantitative reverse transcription real time (RT)-PCR (SYBRgreen) containing sense and antisense primer sequences of the tested genes as well as of 36B4 ribosomal protein (housekeeping gene) as we have previously described (34).

Immunofluorescence Microscopy

Peritoneal macrophages and Raw 264.7 cells were seeded on coverslips at a density of 0.5 x 10^6/well. For fluorescent labeling of lipid rafts, the cells were incubated with 2 μg/ml of CTxβ Alexa 594 (Molecular Probes, Inc.) in 0.1% BSA-PBS for 20 min at 4°C prior to cell fixation. Cells were then fixed in 4%

paraformaldehyde (PFA) and permeabilized with 100% methanol for 5 min at -20°C. For liver immunohistochemical analysis, tissues were dissected from the animal, embedded in OCT compound (Tissue-Tek) and snap-frozen in liquid nitrogen and isopentane. 4 µm sections were air-dried, fixed with 4% PFA and permeabilized with 0.01% Triton X-100 in PBS. The sections and the coverslips were then sequentially incubated at room temperature in PBS containing 4% goat serum and 0.8% bovine serum albumin (BSA) for 60min, with the indicated primary antibody over night at 4°C and with the fluorescence-tagged secondary antibodies in 0.8% BSA-PBS for 60 min. The coverslips were then mounted on glass slides using Vectashield mountain medium fluorescence with DAPI (Vector Laboratories) and fluorescence signals were monitored using a Zeiss LSM 5 PASCAL Laser Scanning Microscope (Carl Zeiss, Germany).

Electron-Microscopy Procedures

Culture cells were chemically fixed at 4°C with a mixture of 2% PFA and 0.1% glutaraldehyde in PBS. After washing with PBS containing 50 mM glycine, cells were embedding in 12% gelatin and infused in 2.3 M sucrose. Mounted gelatin blocks were frozen in liquid nitrogen. Thin sections were prepared in an ultracryomicrotome (Leica EM Ultracut UC6/FC6, Vienna, Austria). Ultrathin cryosections were collected with 2% methylcellulose in 2.3 M sucrose. Cryosections were incubated at room temperature on drops of 2% gelatine in PBS for 20 min at 37°C, followed by 50 mM glycine in PBS during 15 min and 10% FBS in PBS during 10 min and 5% FBS in PBS 5 min. Then they were incubated with a mouse anti-ABCA1 and rabbit anti-caveolin 1 antibodies (1:50 both) in 5% FBS in PBS for 30 min. After three washes of PBS for 10 min, sections were incubated for 20 min with anti-mouse coupled to 12 nm and anti-rabbit coupled to 18 nm gold particles (Jackson ImmunoResearch, PA, USA). This was followed by three washes with drops of PBS for 10 min, two washes with distilled water. As a control for non-specific binding of the colloidal gold-conjugated antibody, the primary polyclonal antibody was omitted. The observations were done in an Electron Microscope Tecnai Spirit (FEI Company, The Netherlands) with a CCD camera SIS Megaview III.

Cholesterol Efflux Assays

Peritoneal macrophages from WT and Cav-1$^{-/-}$ mice were cultured at a density of 1 × 10^6 cells per well 1 day prior to loading with 0.5 mCi/ml [^{3}H]-cholesterol for 24 h with or without T0901317 (2 uM) for 12 h (35). Cells then were washed twice with PBS and incubated in RPMI 1640 medium supplemented with 2 mg/ml fatty acid-free bovine serum albumin (FAFA media) in the presence of an Acetyl-Coenzyme A Acetyltransferase (ACAT) inhibitor (2 mM; Novartis Corporation, New York, NY, USA) for 4 h prior to the addition of 50 ug/ml human ApoA1 in FAFA or HDL (Intracell) media. Supernatants were collected after 6 h and expressed as a percentage of [^{3}H]-cholesterol in the media per total cell [^{3}H]-cholesterol content (total effluxed [^{3}H]-cholesterol + cell-associated [^{3}H]-cholesterol).

RESULTS

Caveolin-1 Expression Modulates Inflammatory Responses and ABCA1 Expression in RAW264.7 Cells

Previous studies have shown that ligand-activated LXRs down-regulate the expression of inflammatory genes in macrophages (6, 36–38), and other studies identified Cav-1 as an immunomodulatory mediator in these cells (39, 40). In order to explore the possible implication of Cav-1 in the modulation LXR anti-inflammatory function, we analyzed the LPS-induced cytokine production in various clones of RAW264.7 macrophages expressing Cav-1 ectopically. RAW264.7 control cells (expressing green fluorescent protein, GFP) do not express Cav-1 basally (**Figure 1A**). Based on the expression levels of Cav-1 in purified fractions of plasma membrane assessed by Western blot (**Figure 1A**), and the co-localization with GM-1 ganglioside, a marker of lipid rafts labelled with a fluorescent choleric β-subunit toxin, we chose RAW Cav-1 clone 1 for further analysis (**Figure 1B**). Western blot and quantitative real-time PCR (qRT-PCR) experiments showed that LPS-dependent expression of iNOS and COX-2 (**Figure 1C**), as well as IL-6, IL-1β and MCP-1 (**Figure 1D**) was reduced in Cav-1 overexpressing cells compared with control GFP cells. Next, we explored whether Cav-1 expression could influence the expression of LXR target genes. RNA expression of Abca1, Abcg1, Apoe and Srebf1 was similar in RAW-GFP and RAW-Cav-1 cells (not shown). Since CAV-1 function has been associated to cholesterol trafficking within the plasma membrane, we explored whether expression of Cav-1 in RAW cells influences the localization of the key protein involved in cholesterol efflux ABCA1. Interestingly, we observed higher levels of ABCA1 protein in whole cell lysates of Cav-1 overexpressing macrophages compared to control cells (**Figure 1E**), as well as in lipid raft fractionation experiments, were ABCA1 co-fractionated with Cav-1 and other markers like Flotillin-1 (Flot-1) in rafts membranes (**Figure 1F**). Together, these results indicate that ectopic expression of Cav-1 in RAW264.7 cells reduced inflammatory gene expression and promotes ABCA1 protein localization within membrane raft microdomains. Altogether these results indicate that LXR and Cav-1 functions exhibit a reciprocal influence on each other and suggest a possible cross-talk between LXR and Cav-1 signaling in macrophages.

Caveolin-1 Expression Is Reduced in LXRαβ$^{-/-}$ Mouse Peritoneal Macrophages and Liver

Since Cav-1 overexpression enhanced ABCA1 protein in lipid rafts and promoted anti-inflammatory effects in macrophages, we assessed the abundance and distribution of Cav-1 in WT and *LXRαβ*$^{-/-}$ deficient mice. Our Western blot analysis in total cell lysates and in microsomal extracts from mouse peritoneal macrophages showed reduced Cav-1 expression in *LXRαβ*$^{-/-}$ cells compared to WT (**Figure 2A**), whereas no changes were observed in other lipid rafts marker such as Flot-1. Additionally, Cav-1 expression was also decreased in liver extracts from in *LXRαβ*$^{-/-}$ compared to WT mice (**Figure 2B**). Further, real-time qPCR

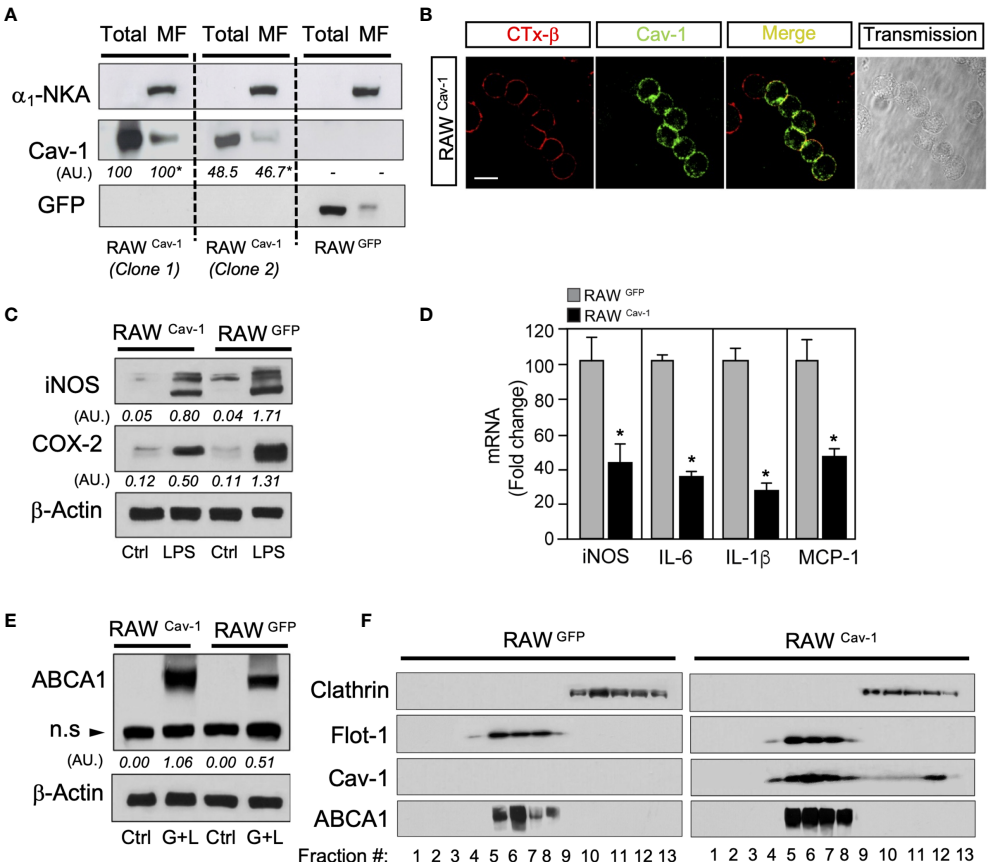

FIGURE 1 | Caveolin-1 expression modulates inflammatory genes and ABCA1 expression in RAW264.7 cells. **(A)** Western blot analysis of ectopically expressed Cav-1 in RAW264.7 cells ($RAW^{Cav\text{-}1}$) compared with RAW264.7 control cells overexpressing GFP (RAW^{GFP}). α1-Na/K-ATPase protein was used to detect plasma membrane enrichment in microsomal preparations. Densitometric analysis of Cav-1 are shown below each blot and are referred to Total or MF in $RAW^{Cav\text{-}1}$ cells clone 1. **(B)** Colocalization of Cav-1 with lipid rafts in the plasma membrane stained with CTx-β in $RAW^{Cav\text{-}1}$cells. Scalebar: 10μm. **(C)** Representative Western blot analysis of iNOS and COX-2 in $RAW^{Cav\text{-}1}$ and RAW^{GFP} cells. β-Actin was used as a loading control. Densitometric values of iNOS and COX-2 are shown below each blot. **(D)** qRT-PCR analysis of iNOS, IL-6, IL-1β and MCP-1 mRNA expression in $RAW^{Cav\text{-}1}$ and RAW^{GFP} cells treated with 100ng/mL of LPS for 6 hours. Data are expressed as relative expression levels and correspond to the means ± SEM from three independent experiments performed in triplicate $^{*}P < 0.05$ (significantly different from RAW^{GFP} cells). **(E)** Representative Western blot analysis of ABCA1 in $RAW^{Cav\text{-}1}$ and RAW^{GFP} cells in response to LXR/RXR ligands for 24h(GW3965 1μM+LG268 100nM). β-Actin was used as a loading control. Densitometric values of ABCA1 are shown below each blot. **(F)** Representative Western blot analysis of lipid raft fractionation in $RAW^{Cav\text{-}1}$ (left panel) and RAW^{GFP} (right panel) cells showing the expression of Cav-1 and ABCA1 in response to LXR/RXR ligands for 24h (GW3965 1μM+LG268 100nM). Flotillin-1 was used as positive control of raft fractions and Clathrin as non-raft protein.

analysis revealed a reduction of approximately 50% of Cav-1 mRNA levels in both peritoneal macrophages and liver samples from *LXRαβ* deficient mice (**Figure 2C**). Then, to better characterize the localization of Cav-1 within the plasma membrane, we observed the expression of the macrophage-specific membrane antigen F4/80 and Cav-1 in WT and $LXR\alpha\beta^{-/-}$ macrophages. Confocal images showed double positive immunostaining of F4/80 and Cav-1 in WT peritoneal macrophages, while the colocalization of both proteins was less evident in $LXR\alpha\beta^{-/-}$ macrophages due to reduced expression of Cav-1 in these cells (**Figure 2D**). Similar results were found in Kupffer cells from liver sections of $LXR\alpha\beta^{-/-}$ mice compared to WT controls (**Supplementary Figure 1**). These results indicate that LXR activity is important for the plasma membrane localization of Cav-1 in macrophages.

Subcellular Distribution Caveolin-1 and ABCA1 Is Controlled by LXR activity

Previous studies have reported contrasting data regarding the regulation of Cav-1 functions by cellular cholesterol in different mouse or human macrophage cell models (41). However, given the well-known role for Cav-1 in cholesterol transport from endoplasmic reticulum to the plasma membrane and the recent identification of the LXR targets, Gramd1/Aster, that control the movement of cholesterol accessible pools within the plasma membrane (42–44), we decided to explore whether LXR activation would affect Cav-1 expression and subcellular distribution in macrophages. To this end, we first performed microscopy analysis to examine the patterns of Cav-1 immunoreactivity within the plasma membrane during LXR

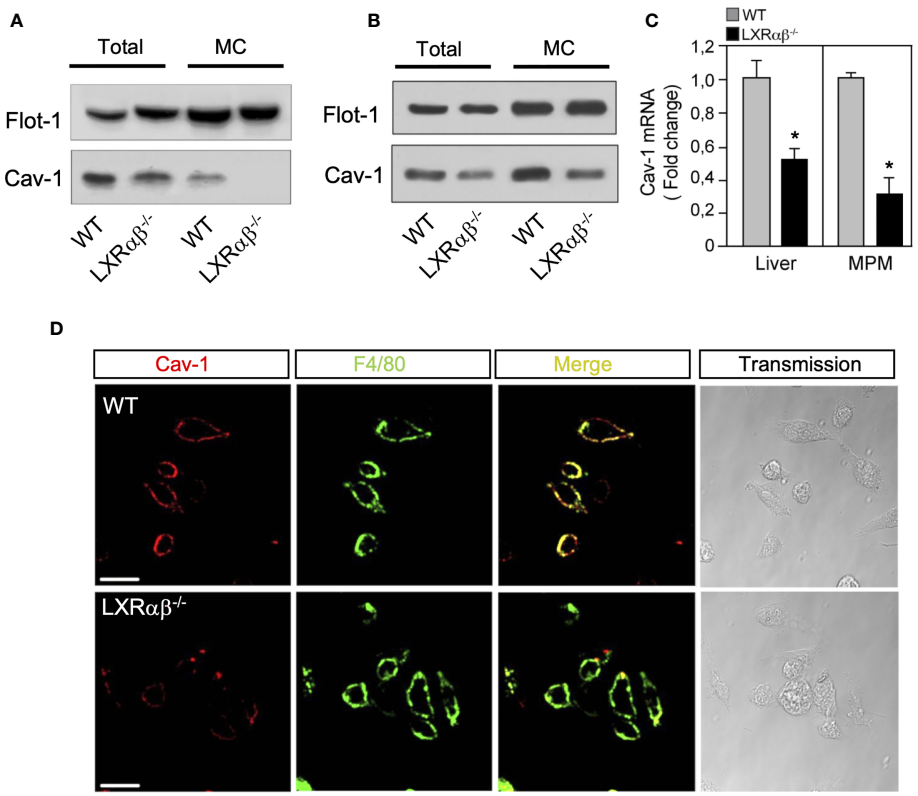

FIGURE 2 | Cav-1 expression is impaired in LXR$\alpha\beta^{-/-}$ mice. Representative Western blot analysis of Cav-1 in whole-cell lysates (Total) and microsomal fractions (MC) of peritoneal macrophages **(A)** and liver **(B)** from in WT and *LXR*$\alpha\beta^{-/-}$ mice. **(C)** mRNA expression of Cav-1 by real-time qRT-PCR in peritoneal macrophages and liver from WT and *LXR*$\alpha\beta^{-/-}$ mice. Data are expressed as relative expression levels and correspond to the means ± SEM from three independent experiments performed in triplicate *$P < 0.05$ (significantly different from WT [normalized to 1]). **(D)** Representative confocal images of Cav-1 expression (red) and F4/80 (green) in peritoneal macrophages from WT and *LXR*$\alpha\beta^{-/-}$ mice. Experiment was performed 3 independent times. Scalebar: 10μm.

activation. Interestingly, cells treated with a combination of the synthetic LXR and RXR ligands (GW3965 and LG268; GW+LG) showed an intense staining of plasma membrane Cav-1 compared to the vehicle-treated cells (**Figure 3A**, left panel), while such increase was not observed in LXR-null macrophages (**Figure 3A**, right panel). These results suggested a possible subcellular relocalization of Cav-1 in response to LXR activation. To further characterize the changes in Cav-1 localization in response to LXR activation, we used a sodium carbonate detergent-free method to purify lipid rafts from peritoneal macrophages. Western blot analysis from sucrose gradient fractions using specific antibodies against Cav-1, Flot-1, ABCA1 and ABCG1 showed that Cav-1 protein expression appeared mainly at light buoyant fraction (F4-F5) containing the lipid raft membranes together with the raft marker Flot-1, as well as in other intracellular organelles and membranes in peritoneal macrophages (**Figure 3B**, upper panel). Interestingly, after LXR activation, Cav-1 expression was slightly increased in the raft fraction but decreased in the non-raft compartments, consistent with an intracellular redistribution of Cav-1 dependent on LXR. Importantly, these effects of Cav-1 redistribution within raft fractions were abolished in *LXR*$\alpha\beta^{-/-}$ macrophages (**Figure 3B**, lower panel). Next, we also assessed the expression and localization of ABCA1 and ABCG1 under the same sucrose fractioning conditions. Western blot analysis showed that ABCA1 and ABCG1 proteins were mostly present in the lipid raft fraction and it co-fractionated with Cav-1 in wild type macrophages but not in *LXR*$\alpha\beta^{-/-}$ macrophages (**Figure 3B**, both top and lower panels). Moreover, in agreement with role of Cav-1 as a cholesterol transport protein and the capacity of LXR to control cholesterol pools within the cell, LXR activation promoted subcellular redistribution of cholesterol (stained with Filipin III), together with Cav-1, in WT macrophages but it remained and accumulated intracellularly in *LXR*$\alpha\beta^{-/-}$ cells (**Supplementary Figure 2**). This may represent an abnormal localization of this protein within the macrophage in cells lacking both isoforms of LXR. Together, these results indicate that LXR activation promotes ABCA1, ABCG1 and Cav-1 relocalization within the raft microdomains of the plasma membrane in murine macrophages.

Cav-1 and ABCA1 Co-Localize in Mouse Peritoneal Macrophages

Despite of the well-established importance of ABCA1 in cholesterol transport and role of Cav-1 in the cholesterol homeostasis, the possible colocalization of these two proteins has

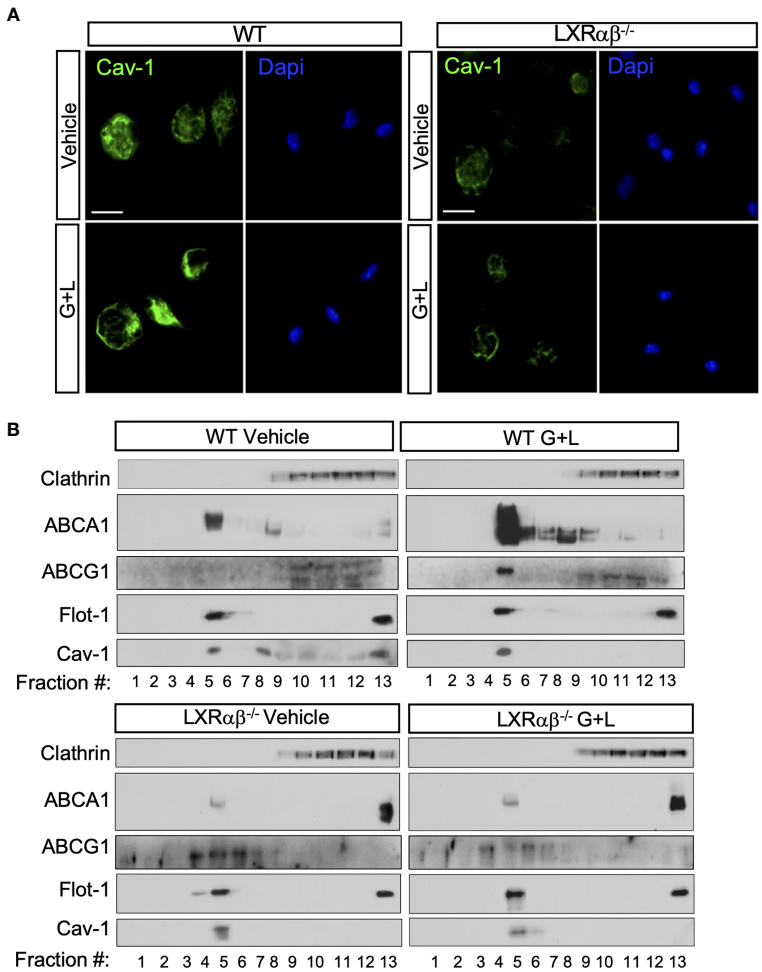

FIGURE 3 | Cav-1 cellular distribution is dependent on LXR. **(A)** Representative images showing the subcellular distribution of Cav-1 (green) by confocal microscopy in peritoneal macrophages from WT and *LXRαβ*$^{-/-}$ mice treated for 24 h with 1 μM GW3965 and 100 nM LG268 (G+L). Nuclei were stained with DAPI. Experiment was performed 3 independent times. Scalebar: 10μm. **(B)** Representative Western blot analysis of Cav-1 and ABCA1 and ABCG1 in lipid raft fractions from WT and *LXRαβ*$^{-/-}$ peritoneal macrophages treated for 24 h with G+L.

not been fully identified at the ultrastructural level in macrophages. Based on our previous results showing the co-fractionation of ABCA1 with Cav-1 we decided to further characterize in depth the colocalization of both proteins in mouse macrophages. To do so, we performed electron microscopy studies of colloidal ABCA1 and Cav-1 gold particles. Individual immunostainings revealed that both particles were primarily located in the caveolae-like structures and intracellular cytoplasmic vesicles, as well as in other non-caveolae membranes (**Supplementary Figure 3**). We then performed combined labeling of ABCA1 and Cav-1 in WT and *LXRαβ*$^{-/-}$ macrophages. **Figure 4** (upper micrographs) shows that ABCA1 and Cav-1 were consistently found decorating the membrane of the same vesicles. Intriguingly, we were not able to find colocalization of immunogold particles of ABCA1 and Cav-1 in *LXRαβ*$^{-/-}$ macrophages **Figure 4** (lower micrographs). These results are in agreement with our previous co-fractionation of Cav-1 and ABCA1 and suggest a possible reciprocal relationship of their functionalities, with possible implications of Cav-1 in LXR-dependent regulation of cholesterol metabolism and inflammation.

The Anti-Inflammatory Actions of LXR Agonists Are Decreased in Cav-1$^{-/-}$ Macrophages

We and others have previously reported that cultured WT macrophages with LXR agonists display reduced expression of inflammatory markers in response to LPS. These anti-inflammatory actions are abolished in macrophages from *LXRαβ*$^{-/-}$ mice (38, 45). In addition, given that overexpression of Cav-1 in macrophages partially inhibited inflammatory cytokine production and LXR activation promoted Cav-1 redistribution within the raft membrane domains, we hypothesized that Cav-1 could be involved in LXR-dependent regulation of inflammation. To asses this, we compared the inflammatory outcomes of primary

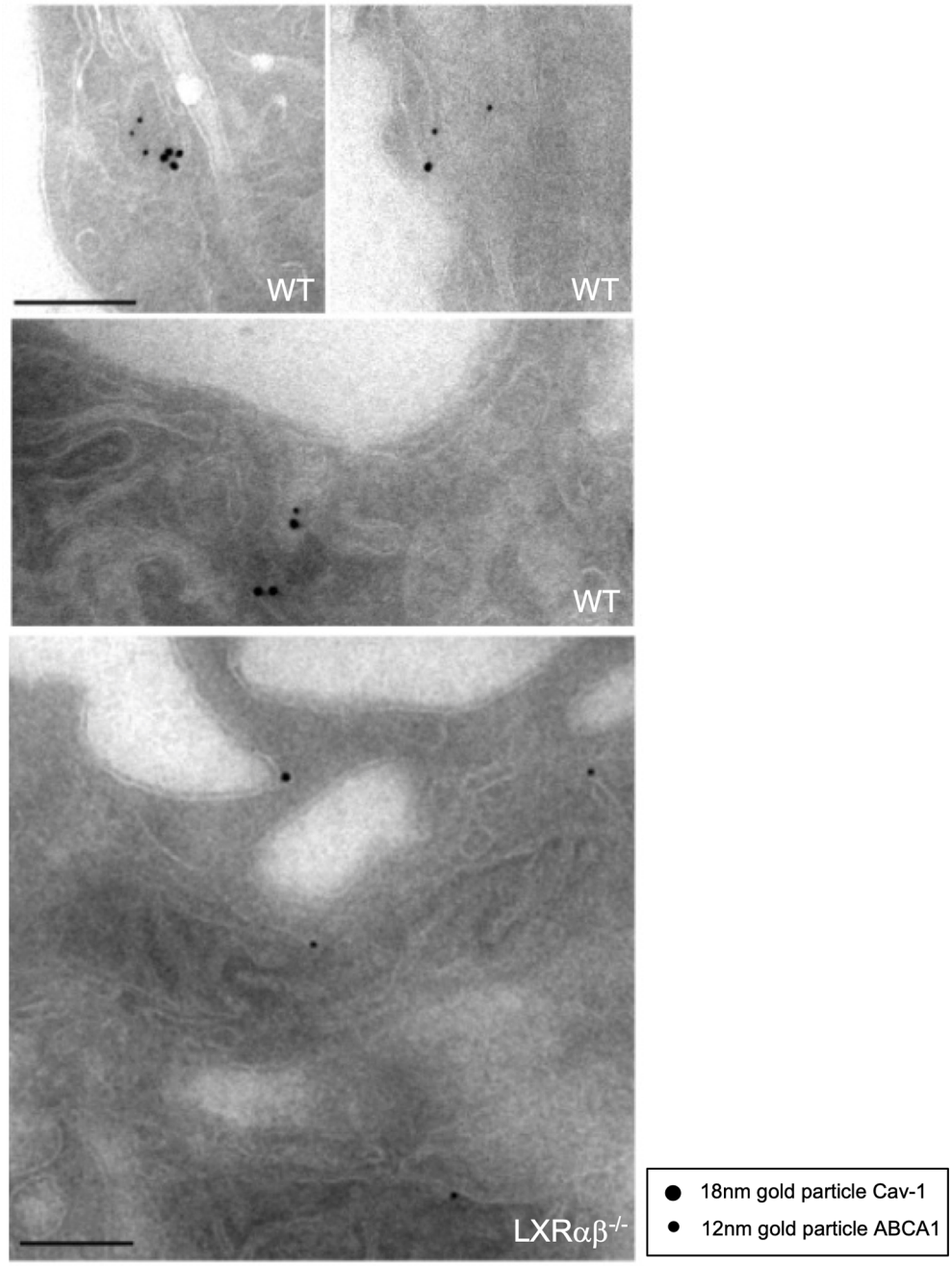

FIGURE 4 | Colocalization of Cav-1 and ABCA1 within sub-cellular domains of peritoneal macrophages. Representative immuno-electron microscopy images showing Cav-1 and ABCA1 expression in peritoneal macrophages from WT and *LXRαβ*$^{-/-}$ mice. Scalebar: 200nm.

peritoneal macrophages from wild type (WT) and *Cav-1*$^{-/-}$ mice treated with LPS alone, or in combination with LXR/RXR ligands. Interestingly, we found that the inhibitory capacity of LXR to block the production of pro-inflammatory cytokines, including TNF-α and IL-1β, as well as other mediators such as IFN–γ or GM-CSF was severely compromised in *Cav-1*$^{-/-}$ macrophages (**Figure 5A**). Similar results were observed by analyzing iNOS and COX-2 expression by Western blotting (not shown). Nevertheless, the ameliorated anti-inflammatory response of LXR in the absence of Cav-1 was not due to differences in the expression of LXRα, LXRβ or their target genes in Cav-1 deficient cells (**Figure 5B**). These results suggest that Cav-1 expression is an important element that facilitates full acquisition of LXR anti-inflammatory signaling in macrophages.

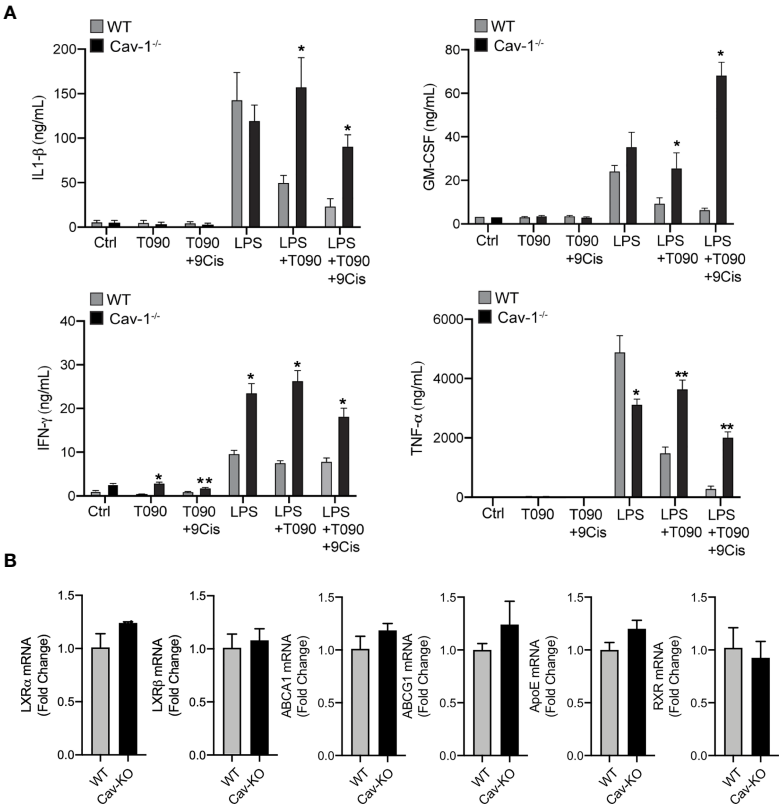

FIGURE 5 | Cav-1 influences anti-inflammatory effects of LXR in peritoneal macrophages. **(A)** Cytokine production in culture media of peritoneal macrophages from WT and *Cav-1*$^{-/-}$ mice pre-treated with 1 μM T0901317 and 1 μM 9-Cis Retinoic Acid for 18h hours prior to stimulation with LPS for another 24h. Data represent 3 independent experiments performed in triplicate $^{*}P < 0.05$, $^{**}P < 0.01$ (significantly different from WT in Ctrl conditions). **(B)** qRT-PCR analysis of LXRα, LXRβ, ABCA1, ABCG1 and ApoE mRNA levels in peritoneal macrophages form WT and *Cav-1*$^{-/-}$ mice. Data are expressed as relative expression levels and correspond to the means ± SEM from three independent experiments performed in triplicate compared to WT and normalized to 1).

Cholesterol Efflux to ApoAI and HDL Is Impaired in Cav-1 Deficient Macrophages

The induction of ABCA1 and ABCG1 expression and activation of the cholesterol efflux pathway (2, 3), together with inhibition of inflammation, are considered crucial steps for the atheroprotective functions of LXRs. Because our results suggest that ABCA1 and Cav-1 proteins co-localize in cellular membranes and that this mutual cooperation is promoted by LXR activity, we decided to explore whether alteration of Cav-1 expression could influence cholesterol efflux in macrophages. First, we assessed the expression of ABCA1 and ABCG1 in WT and Cav-1-/- macrophages. Although ABCG1 levels were slightly different in Cav-1 null macrophages, induction of both ABC transporters was appreciable in response to LXR agonists in WT and Cav-1 KO macrophages (**Figures 6A, C**). Next, we assayed *in vitro* the cholesterol efflux to ApoAI and HDL in primary peritoneal macrophages from WT and Cav-1-deficient mice. As shown in **Figures 6B, D**, the cholesterol efflux to ApoAI and HDL was significantly blunted in *Cav-1*$^{-/-}$ macrophages, indicating that Cav-1 expression is important for LXR-dependent cholesterol efflux. Similar results were obtained in bone marrow derived macrophages (not shown). Our results show that activation of LXR induced the co-expression of ABCA1, ABCG1 and Cav-1 in lipid raft microdomains within the plasma membrane. Since the overall ABCA1 protein content was similar to WT in Cav-1$^{-/-}$ macrophages, we reasoned whether lack of Cav-1 might have an influence in ABCA1 distribution within the membrane. To analyze this possibility, we cultured macrophages with LXR/RXR ligands and observed the localization of ABCA1 by confocal microscopy. As shown in **Supplementary Figure 4**, LXR activation induced a punctuate accumulation of ABCA1 protein in the membrane of WT macrophages, whereas this distribution was severely altered in Cav-1$^{-/-}$ macrophages. Taken together, this data indicate that Cav-1 participates in the distribution of ABCA1 within the plasma membrane and in ApoAI-dependent cholesterol efflux in response to LXR activation in macrophages.

DISCUSSION

LXRs are transcription factors that regulate crucial processes in lipid metabolism and also exert important functions in

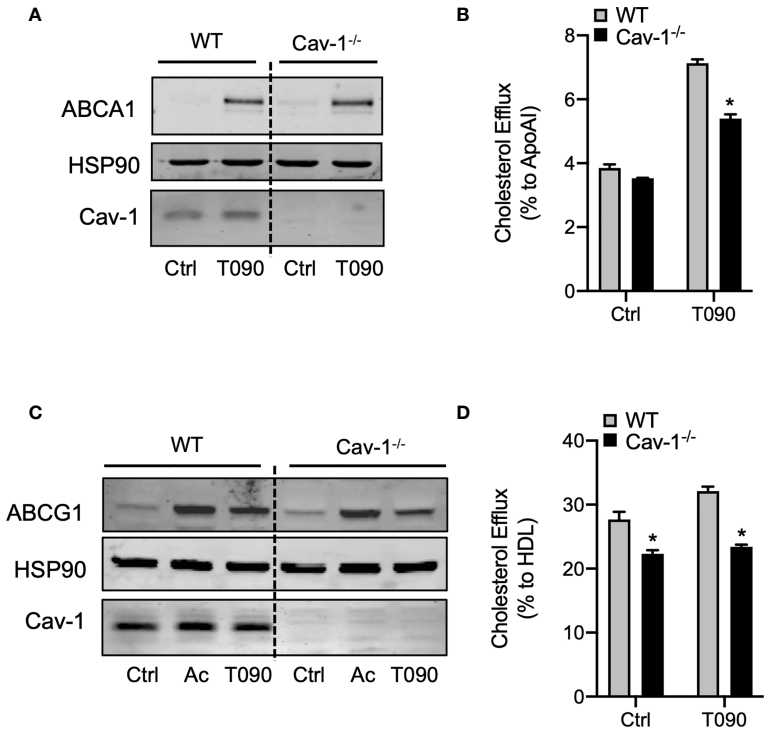

FIGURE 6 | Cav-1 influences subcellular distribution of ABCA1 and modulates ABCA1-dependent cholesterol efflux. **(A)** Western blot analysis of ABCA1 in peritoneal macrophages from WT and *Cav-1*$^{-/-}$ mice treated with or without or 1 μM T0901317 (T090) for 18 hours. Cav-1 was used to show its absence in the *Cav-1*$^{-/-}$ mice and HSP90 was used as a loading control. Experiment was performed at least 3 times. **(B)** Cholesterol efflux to apolipoprotein A1 (ApoA1) in peritoneal macrophages isolated from WT and *Cav-1*$^{-/-}$ mice stimulated with or without 3 μM of T090 for 16 hours. Data represent the mean ± SEM of triplicate samples (n = 3 per group; $P < 0.05$, (significantly different from WT in each treatment condition). **(C)** Western blot analysis of ABCG1 in peritoneal macrophages from WT and *Cav-1*$^{-/-}$ mice treated with or without or 1 μM T0901317 (T090) for 18 hours. Cav-1 was used to show its absence in the *Cav-1*$^{-/-}$ mice and HSP90 was used as a loading control. Experiment was performed at least 3 times. **(D)** Cholesterol efflux to HDL in peritoneal macrophages isolated from WT and *Cav-1*$^{-/-}$ mice stimulated with or without 3 μM of T090 for 16 hours. Data represent the mean ± SEM of triplicate samples (n = 3 per group; $P < 0.05$, (significantly different from WT in each treatment condition). *$P < 0.05$.

inflammation and host immunity (46, 47). LXRs have emerged as key regulators of whole body cholesterol homeostasis, in part through up-regulation of genes encoding plasma membrane transporters ABCA1 and ABCG1 that facilitate cholesterol efflux from macrophages and other cell types (1, 2, 48). Importantly, the same pathways that control cholesterol trafficking within the plasma membrane appeared as important regulators of inflammation and host defense (49, 50). In line with this, regulation of cholesterol efflux and the control of inflammatory and antimicrobial responses in macrophages have been shown to be partially dependent on ABCA1 (7, 50, 51). In the present study we provide an additional mechanistic clue that identifies Cav-1 as an important factor required for LXR-dependent functions in murine macrophages. Both the anti-inflammatory actions and cholesterol efflux to ApoAI or HDL promoted by LXR agonists are substantially diminished in the absence of Cav-1. Reciprocally, localization of Cav-1 within lipid-raft membrane microdomains is impaired in LXR-deficient macrophages. These findings reveal that a mutual cooperation between Cav-1 and LXR pathways participates in macrophage inflammatory and homeostatic responses.

Among caveolin proteins, Cav-1 is crucial for the formation of caveolae and participates in several cellular processes, including endocytosis, cholesterol trafficking and signal transduction (52, 53). However, the role of Cav-1 during atherosclerosis is context and cell-type dependent. Hypercholesterolemic mice with complete loss of Cav-1 or with Cav-1 targeting specifically in endothelial cells demonstrated that Cav-1 function accelerates atherogenesis by modulating endothelial lipoprotein transport and autophagy pathways (54, 55). In contrast, other studies using bone marrow transplant of *Cav-1*$^{-/-}$ hematopoietic progenitors into *LDLR*$^{-/-}$ mice indicated that an important contribution of Cav-1 in macrophages is generally atheroprotective through modulation of macrophage inflammation (56). Thus, these reports reconcile the apparent contrasting roles of Cav-1 in atherosclerosis and highlight the complexity and the importance of Cav-1 in lipid and inflammatory pathways in the context of atherosclerosis. Also, all these studies infer that LXRs and Cav-1 appear to participate in similar pathways in macrophages, including phagocytosis, inflammation and cholesterol efflux. However, a deeper comprehension of how LXR and Cav-1 pathways can affect each signaling reciprocally was not explored before.

The first experimental evidence that supported a mutual crosstalk between Cav-1 and LXR came from our experiments of forced Cav-1 expression in RAW264.7 macrophages that normally lack Cav-1. In this model, Cav-1 favors accumulation of ABCA1 mainly in lipid-raft domains in response to LXR ligands and a marked attenuation of inflammatory gene expression in response to LPS (**Figure 1**). Conversely, Cav-1 expression exhibited a reduction of ~50% in mRNA and total and microsomal protein expression in LXR-deficient macrophages and liver, compared WT controls (**Figure 2**). Cav-1 down-regulation in LXR-null macrophages possibly suggests that LXR might be controlling Cav-1 expression through transcriptional regulation. However, our previously reported LXR ChIP-Seq and RNA profiling studies (57), have not demonstrated direct LXR binding in the vicinity of the Cav-1 locus nor direct up-regulation of Cav-1 expression in response to LXR ligands, indicating that impaired Cav-1 expression in LXR-null macrophages could be the result of an indirect regulation. Our observations indicate that ABCA1 and ABCG1 are concomitantly recruited with Cav-1 to lipid-raft microdomains (**Figure 3**). Our results are consistent with previous studies that showed a role for Cav-1 in ABCA1 and ABCG1 function in other cell types (58, 59). Thus, the occurrence of Cav-1 in lipid-rafts of the plasma membrane augmented in response to LXR activation in macrophages and we hypothesize that the critical cholesterol efflux transporters ABCA1 and ABCG1 are important for this regulation. However, it is also possible that the combined induction of several other direct LXR-regulated targets in response to elevated cholesterol levels assist in Cav-1 recruitment to the plasma membrane in macrophages. Recently, elegant studies from Tontonoz and colleagues identified a family of proteins, called Aster, that facilitate the transport of excess accessible cholesterol in the plasma membrane to the endoplasmic reticulum in response to LXR activation (42, 44). Thus, although the factors that are responsible for Cav-1 down-regulation in LXR-deficient macrophages need further investigation, our observations indicate that an intact LXR signaling is crucial for Cav-1 subcellular localization within the plasma membrane in macrophages.

Previous reports have described that overexpression of ABCA1, ABCG1 and ABCG4 in HEK293 and CHO cells influences cholesterol efflux through their activity in non-raft domains (60), based on experiments using detergents that solubilize lipid membrane domains. Our experiments in primary macrophages, however, show that the majority of ABCA1 co-fractioned mainly with Cav-1 in light buoyant or caveolar fractions. Furthermore, we demonstrate that ABCA1 and ABCG1 induction promoted by LXR activation takes place mainly on caveolar fractions along with other lipid-rafts markers such as flotillin-1 (61). Our ultrastructural analyses (**Figure 4**) also indicate that ABCA1 and Cav-1 do not appear to interact physically but consistently co-localize within common membrane microdomains. Importantly, our observations demonstrate that loss of LXR in macrophages, that greatly increases the intracellular cholesterol content (62), leads to reduced Cav-1 and ABCA1 recruitment to lipid-rafts possibly by disrupting caveolae microdomains. Our conclusions are in agreement with previous results by Parks and colleagues that showed increased membrane lipid raft content with reduced Cav-1 in raft fractions and hyper-inflammatory responses in ABCA1-deficient macrophages (63). Our studies demonstrate that LXR deficiency in macrophages directs a reduction of Cav-1 within the lipid-raft membrane domains that would promote delocalization of ABCA1 from raft/caveolar to non-raft membranes and other subcellular locations. These molecular changes would probably influence the normal ABCA1 functions in macrophages. ABCA1 activity is critical for cholesterol efflux and was also found to play anti-inflammatory tasks in macrophages (7, 50, 63). Our data indicate that some of the LXR-dependent functions that are critically mediated by ABCA1, appear to rely on Cav-1 expression. Using Cav-1 deficient macrophages, we demonstrate that the ability of LXR ligands to control the expression of inflammatory mediators depends greatly on Cav-1 expression. These results are congruent with the studies by Ito et al. (7), which clearly showed that induction of ABCA1 and subsequent uncoupling of TLR signaling from the plasma membrane mediate the anti-inflammatory actions of LXR. It seems plausible from these studies and our results that Cav-1 and ABCA1 participate in the regulation of inflammation by controlling the recruitment of adaptor molecules that mediate inflammation downstream of TLR. When the connection between ABCA1 and Cav-1 within lipid-rich microdomains is enhanced by pre-treatment with LXR ligands, the magnitude of inflammatory responses is attenuated. Furthermore, the importance of Cav-1 expression in LXR-ABCA1 functions in macrophages has also implications in cholesterol efflux. Cav-1 deficiency results in a marked reduction in LXR-dependent cholesterol efflux to ApoA-I and HDL in macrophages. The defect in cholesterol efflux is not due to defect in ABCA1 or ABCG1 total protein expression, nor defects in LXRα or LXRβ expression in Cav-1$^{-/-}$ macrophages. It is likely that the disruption of caveolar microdomains by the loss of Cav-1 (64) affects the membrane localization and function of ABCA1 and perhaps other proteins regulated by LXR involved in cholesterol efflux. Overall, our studies conclude that Cav-1 plays an important role in LXR-mediated functions, both in inflammation and in cholesterol efflux and that Cav-1 functions in macrophages could provide additional intervention mechanisms to the LXR transcriptional regulation of cholesterol efflux and inflammation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Etica y experimentación animal Consejo Superior de Investigaciones Científicas and Universidad Las Palmas de Gran Canaria, with reference numbers PROEX171/2018 and OEBA-ULPGC 02/2015.

AUTHOR CONTRIBUTIONS

CMR and AC conceived the project. CMR, LB and AC contributed to the conceptual design, figure preparation and acquired the funding for the project. CMR, MTP, VPM, MFF, APG, CT, JVR, PMR, MDS, UN, MCO, PGT and MC performed experiments and data analysis. CMR and AC prepared draft manuscript. All authors revised the draft manuscript and contributed to its editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.635923/full#supplementary-material

Supplementary Figure 1 | LXR deficiency in the liver results in decreased Cav-1 expression in Kupffer cells. Representative confocal images of Cav-1 expression (red) and F4/80 (green) in livers from WT and *LXRαβ*$^{-/-}$ mice. Higher magnification of the insets is shown in the right panels. Scalebar: 20μm.

Supplementary Figure 2 | Cellular cholesterol redistribution upon LXR activation correlates with Cav-1 localization in peritoneal macrophages. Representative images showing the subcellular distribution of Cav-1 (green) and Fillipin (blue) in peritoneal macrophages isolated from WT and *LXRαβ*$^{-/-}$ mice and treated for 24 h with 1 μM GW3965 and 100 nM LG268 (G+L). Scalebar: 10μm.

Supplementary Figure 3 | Cav-1 and ABCA1 localization in caveolae-like and intracellular vesicles. Representative electron microscopy images showing individual immunogold staining of Cav-1 and ABCA1 in peritoneal macrophages isolated from WT and *LXRαβ*$^{-/-}$ mice. Scalebar: 200nm.

Supplementary Figure 4 | Cav-1 influences subcellular distribution of ABCA1 Representative images showing immunostaining of ABCA1 (red) by confocal microscopy in peritoneal macrophages from WT and *Cav-1*$^{-/-}$ mice treated for 24 h with 1 μM GW3965 and 100 nM LG268 (G+L). Nuclei were stained with DAPI. Experiment was performed 3 independent times. Scalebar: 20μm.

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Expression of Sex Hormone Receptor and Immune Response Genes in Peripheral Blood Mononuclear Cells During the Menstrual Cycle

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Sex hormones are known to interact with the immune system on multiple levels but information on the types of sex hormone receptors (SHR) and their expression levels in immune cells is scarce. Estrogen, testosterone and progesterone are all considered to interact with the immune system through their respective cell receptors (ERα and ERβ including the splice variant ERβ2, AR and PGR). In this study expression levels of SHR genes in peripheral blood mononuclear cells (PBMCs) and cell subsets ($CD4^+$ and $CD8^+$ T-cells, $CD56^+$ NK-cells, $CD14^+$ monocytes and $CD19^+$ B-cells) were analyzed using standard manual qPCR or a qPCR array (TLDA). Nine healthy individuals including men (n = 2), premenopausal (Pre-MP, n = 5) and postmenopausal (post-MP, n = 2) women were sampled for PBMCs which were separated to cell subsets using FACS. Ten Pre-MP women were longitudinally sampled for total PBMCs at different phases of the menstrual cycle. We found that ERα was most abundant and, unexpectedly, that ERβ2 was the dominant ERβ variant in several FACS sorted cell subsets. In total PBMCs, SHR (ERα, ERβ1, ERβ2, and AR) expression did not fluctuate according to the phase of the menstrual cycle and PGR was not expressed. However, several immune response genes (*GATA3, IFNG, IL1B, LTA, NFKB1, PDCD1, STAT3, STAT5A, TBX21, TGFB1, TNFA*) were more expressed during the ovulatory and mid-luteal phases. Sex hormone levels did not correlate significantly with gene expression of SHR or immune response genes, but sex hormone-binding globulin (SHBG), a steroid hormone transporting protein, was positively correlated to expression of ERβ1 gene. This study provides new insights in the distribution of ERs in immune cells. Furthermore, expression patterns of several immune response genes differ significantly between phases of the menstrual cycle, supporting a role for sex hormones in the immune response.

Keywords: menstrual cycle, estrogen receptor, progesterone, sex hormone, immune response, estrogen

INTRODUCTION

Men and women are affected differently by infectious diseases, with higher male mortality and morbidity from infectious diseases (1). One major reason to this may be that the immune response differs between men and women (2). In general, women mount a stronger response than men towards pathogens and/or seem to clear the pathogen more effectively (2). Men are more prone to contract certain infectious diseases related to differences in behavior (3), but even when controlling for exposure, women seem to have a benefit (4). For specific infectious diseases (e.g., severe dengue fever) a strong immune response could be detrimental, and therefore be a disadvantage for women (3).

A growing body of evidence suggests that sex hormones may both augment and dampen the immune response (5). The female advantage in mortality to infectious diseases decreases from the 5^{th} decade of life (1). As this coincides with the female menopause and decreasing levels of female sex hormones, it is plausible that estradiol (E_2) and progesterone (P_4) have roles in shaping the immune response (1, 2). Furthermore, autoimmune diseases are more common in women, a phenomenon also partly attributed to sex hormones (6).

The menstrual cycle involves fluctuation of P_4 and E_2 levels as well as follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates the ovarian follicles to produce E_2, which is necessary for the mid-cycle sharp surge of LH that initiates the ovulation. If fertilization does not occur, the corpus luteum breaks down and P_4 levels drop. As the levels of hormones shift, so might the immune response, affecting the temporal severity of autoimmune and infectious diseases (7, 8).

Sex hormone receptors (SHRs) have been found in several non-reproductive tissues, and sex hormones may affect e.g., bone density, muscular growth and blood coagulation. Not surprisingly, sex hormone receptors have also been found in several types of immune cells (9). SHRs include estrogen receptor (ER)α, ERβ, androgen receptor (AR) and progesterone receptor (PGR), and belong to the steroid activated nuclear receptor family of transcription factors (10). These receptors are intracellular and may upon ligand stimulation bind directly to DNA sequences, or tether with transcription factors (e.g., NFκB, AP-1 and SP1) to mediate gene transcription of among other immune related genes, such as type I interferons (IFN-α and IFN-β) (5). The two subtypes ERα and ERβ are found in several splice variants (isoforms), the latter including ERβ1-5, of which several have been associated to disease development by e.g., antagonizing full-length ERα or ERβ (10–12).

In the present study, we sampled pre-menopausal (pre-MP) women over four phases of the menstrual cycle to analyze variation in serum hormone levels, expression of SHRs, and several key immune response genes in peripheral blood mononuclear cells (PBMCs). Our data demonstrate that the expression of several immune response genes changes over the menstrual cycle and that the ERβ splice variant, ERβ2 that cannot bind E2, may be more prominent in this process than full length ERβ (ERβ1). This study adds new evidence to the sex differences in immune response.

MATERIALS AND METHODS

Participants

Healthy premenopausal women (pre-MP, n = 15), postmenopausal women (post-MP, n = 2) and men (n = 2) were included according to a protocol approved by the Central Ethical Review Board (Swedish Research Council, Stockholm, Dnr: Ö 24–2009), and body-mass index (BMI) and age were registered for all subjects. For women, parity, menstrual cycle length or years since initiation of menopause was noted. Exclusion criteria were (1) medication with hormonal replacement therapy, or contraceptives during the last three months (2), Regular medication with ASA, NSAIDs (e.g., ibuprofen and diclofenac), morphine, morphine-derivatives or cortisone compounds (3), pregnancy or childbirth within the last year and (4) irregular or perimenopausal bleeding.

Sampling Procedure

All the samples were collected at 8–10 a.m. The samples from menstruating females were collected during one menstrual cycle, early in the follicular phase (cycle day 1–3), during mid-follicular phase (day 8–10), and at the ovulatory phase day 12-15. The follicular size was measured by ultrasound. The day of the LH and FSH peaks was determined by using Ovustick (Monoclonal Antibodies, Mountain View, CA, USA) in urine from day 12 until the day after the LH peak and in the luteal phase (5–7 days after the day of the LH-Peak and FSH peak). Ovulation was confirmed when progesterone levels were above 22 nmol/mL. Similarly, the post-MP females and male participants were sampled once a week on the same weekday over four weeks. At every time-point, serum analysis was performed for hormones (S-estradiol, S-testosterone, S-progesterone, S-prolactin, S-FSH, S-LH, S-SHBG, S-TSH, S-T4) and blood cells complete blood count and differential count (lymphocytes, monocytes, neutrophils, basophils and eosinophils). Vacutainer CPT mononuclear cell preparation tubes (BD Biosciences, Franklin Lakes, NJ, USA) were used according to the manufacturer's description to separate PBMCs from whole blood. PBMCs were slowly frozen in 20% DMSO and Heparin solution and kept at -135°C. Serum samples were drawn, left to coagulate at room temperature for 30 min, and then centrifuged for 10 min at maximum speed before storing at -20°C. The blood samples were drawn at Kvinnohälsan (Karolinska University Hospital, Huddinge) and analyzed at the Karolinska University Laboratory (KUL, Huddinge, Sweden) and analyzed as previously described (13). Separate serum samples were also drawn to estimate 5-α dihydrotestosterone, (performed at HUS-lab, Helsinki, Finland), using a liquid chromatography-tandem mass spectrometry method (LC-MS/MS).

Fluorescence-Activated Cell Sorting

Frozen PBMC samples from pre-MP (n = 5), post-MP (n = 2) and males (n = 2), collected at 4 different time-points (as described above) were prepared for cell storing using FACS. The samples were thawed in a 37°C water bath and diluted with ice-cold PBS followed by 2 washing steps with ice-cold PBS by

centrifugation (300 x g, 5 min) at 4°C. The cell pellet was resuspended in 200 µL ice-cold PBS. The cell suspension was incubated in darkness with respective antibodies (CD3 PE-Cy 7, Cat No 341111; CD4 PerCP-Cy 5.5, Cat No 332772; CD8 APC-H7 RUO, Cat No 641400; CD56 PE (MY31), Cat No 345810; CD19 APC (SJ25C1), Cat No 345791, all from BD Biosciences (San Jose, CA, USA), and CD14 [DakoAgilent, Santa Clara, CA, USA)], for 15 minutes and diluted with 2 mL PBS before centrifugation (600 x g, 5 min) at 4°C. Unbound antibodies (supernatant) were discarded and the cell pellet was resuspended in 400 µL PBS followed by cell sorting using FACSAria (BD Biosciences). At least 30'000 cells were collected from each category before storing at -80°C.

RNA Extraction and cDNA Synthesis

RNA was extracted using the Qiagen RNeasy kit (Qiagen, Hilden, Germany) and cDNA synthesis performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Vilnius, Lithuania) with random hexamers, according to manufacturer's instructions.

Manual qPCR on Sorted Cells and PBMCs

cDNA from both unsorted and FACS sorted PBMCs ($CD4^+$ T-cells, $CD8^+$ T-cells, $CD56^+$ NK-cells, $CD14^+$ monocytes and $CD19^+$ B-cells) were used analyzed by qPCR using 0.5 µl cDNA, 300 nM forward and reverse primers (ERα: forward, 5'-GAATCTGCCAAGGAGACTCGC -3'; reverse, 5'-ACTGGTTG GTGGCTGGACAC-3'; ERβ1, forward, 5'- TCCATGCGC CTGGCTAAC -3'; reverse, 5'- CAGATGTTCCATGCCCT TGTTA -3'; ERβ2, forward, 5'- TCCATGCG

CCTGGCTAAC -3'; reverse, 5'- CCATCGTTGCTTCAGG CAA -3'; GR forward 5'-GAGCAGTGGAAGGACAGCA-3'; reverse,

5'-TTTCTTCGAATTTTATCGATGATGC-3'; GPER1, forward, 5'- TCACGGGCCACATTGTCAAC; reverse 5'- GTC TCCCCGAGAAAGCTGTAG-3'; and GAPDH: forward, 5'-CCCATCACCATCTTCCAG-3'; reverse, 5'-ATGACCTTGC CCACAGCC-3'), and SYBR green FAST PCR master mix according to manufacturer's instructions (Applied Biosystems, Foster City CA, USA). The qPCRs were setup and run on a 7500 FAST real-time PCR system (Applied Biosystems, Foster City, CA, USA) and relative mRNA expression was analyzed using the ΔCt method relative to GAPDH expression.

Taqman Low Density PCR-Array Analysis

PBMC cDNA from Pre-MP women ($n = 10$) with complete set of samples representative for different phases of the menstrual cycle was mixed with TaqMan Fast Advanced master mix (Applied Biosystems) and RNase-free water. cDNA mix was loaded into each of the 8 loading ports of a Taqman low density Array (TLDA, Applied Biosystems). The array was sealed, centrifuged 2 minutes at 1800 x g, and the following qPCR performed on a 7900HT qPCR system (Applied Biosystems) with ABI software SDS v2.4 installed using standard TLDA array cycling. GAPDH was used as reference gene for ΔCT calculations using the ABI software RQmgr 1.2.1 followed by DataAssist v3.0 (Applied Biosystems). Each sample was analyzed in triplicates for each of the 30 genes assayed, including sex hormone receptors, proinflammatory markers as well as T_H1-, T_H2-, Treg- and T_H17-related immunological markers (**Supplemental Table 1**). The qPCR results are presented as ΔCT values to allow linear model analyses on normal distributed values.

Statistical Analyses

Student's t-test with Welch's correction was used to compare the amount of ERα, ERβ1 and ERβ2 in the different PBMC cell subsets. Linear mixed modeling (LMM) was used to estimate the effect of sampling timepoints and gene expression similarly as described by us before (14). In brief, the R-package *nlme* was used for LMM analysis where timepoint was tested as fixed effect, and the expression of the various genes for each pre-MP individual was set as random effects, and *p*-values were calculated. Bonferroni correction was used to adjust the significance level of *p*-values relative to the number of repeated LMMs for the different genes studied. The repeated measures correlation test (15) was used to determine associations between gene-gene expressions and between gene expression and hormone levels.

RESULTS

Clinical Characteristics

Brief characteristics of 19 unique individuals that donated blood samples for analysis by manual qPCR on FACS-sorted cells or a Taqman low density PCR array (TLDA) on total PBMCs are shown in **Table 1**.

Distribution of ERs in PBMCs

To investigate the presence of ERs in $CD4^+$ T-cells, $CD8^+$ T-cells, $CD56^+$ NK-cells, $CD14^+$ monocytes and $CD19^+$ B-cells, we sorted PBMCs from healthy pre-MP, post-MP, and males by FACS ($n = 9$). ERα (*ESR1*) expression was found in all cell types (**Figures 1A–E**). The ERβ1 (*ESR2_ERb1*, RefSeq NM_001437) transcript was found in very small amounts in all cell types

TABLE 1 | Clinical characteristics of participants, range (median).

Analysis	Subject group	Age	Parity	Menstrual cycle length in days	Years since last menses	BMI
qPCR on FACS-sorted cells and PBMCs	Pre-MP ($n = 5$)	25–32 (31)	0–2 (0)	28–31 (28)	–	21.5–27.5 (22.0)
	Post-MP ($n = 2$)	60 and 61	0 and 2	–	10 and 14	25 and 23.7
	Males ($n = 2$)	21 and 68	–	–	–	21.9 and 23.8
Taqman PCR array on total PBMCs	Pre-MP ($n = 10$)	24–36 (31,5)	0–2 (0)	21–31 (28)	–	17.9–27.5 (22.4)

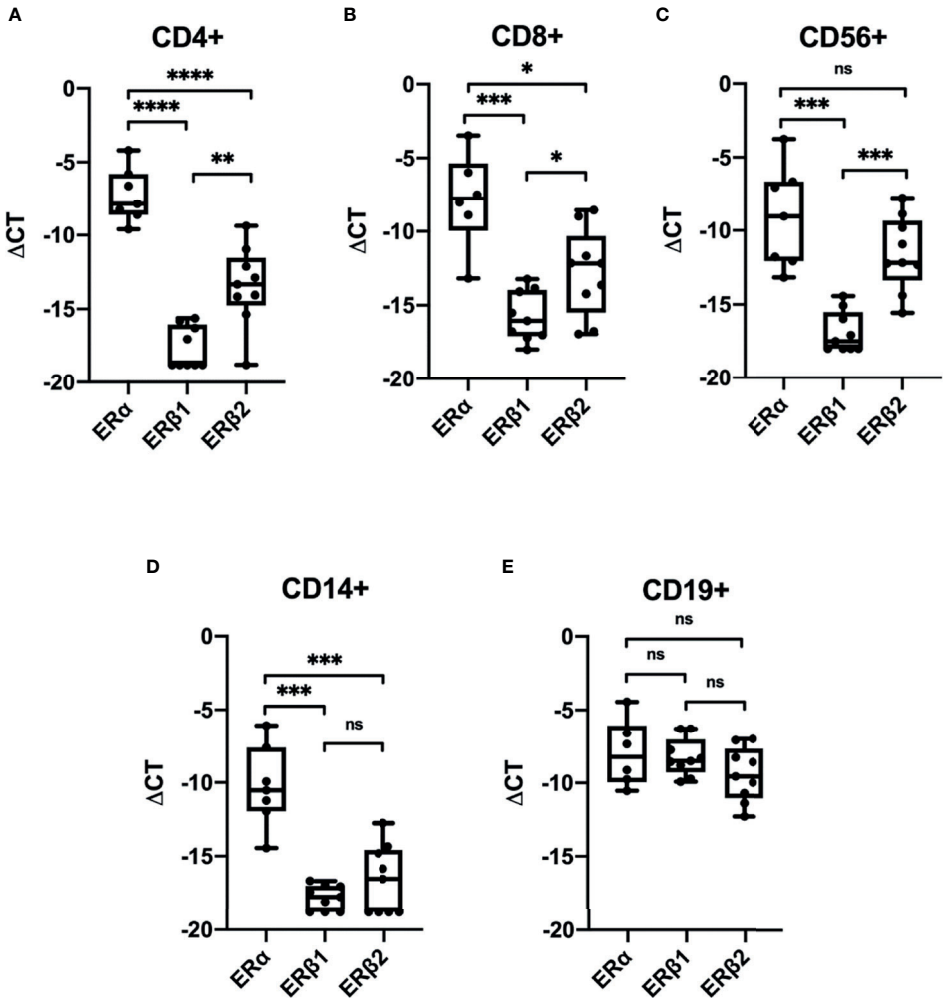

FIGURE 1 | Expression of estrogen receptors in sorted PBMCs. Relative expression (ΔCT relative to GAPDH) of ERα (*ESR1*), ERβ1 (*ESR2_ERb1*) and ERβ2 (*ESR2_ERb2*) in $CD4^+$ T-cells **(A)**, $CD8^+$ T-cells **(B)**, $CD56^+$ NK-cells **(C)**, $CD14^+$ monocytes **(D)**, and $CD19^+$ B-cells **(E)** from men, pre-MP and post-MP women (n = 6–9). P values were obtained using Welch's t-test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, ns, not significant.

except in B-cells. Similarly, the ERβ splice variant ERβ2 (*ESR2_ERb2*, RefSeq NM_001291712) was also most abundant in B-cells but was also found in higher amounts in ($CD4^+$ and $CD8^+$) T-cells and NK-cells compared to ERβ1 (**Figures 1A–E**). In monocytes *ESR2_ERB1* and *ESR2_ERB2* was either very low or not detected (**Figure 1D**). The membrane-associated ER, GPER1, was only expressed in $CD8^+$ T-cells, $CD14^+$ monocytes, and $CD19^+$ B-cells in relatively high amount (**Supplemental Figure 1A**). For comparison, the expression of the glucocorticoid receptor (GR) was found highly expressed in all cell types (**Supplemental Figure 1B**). Although underpowered, we could not detect any significant differences in ER distribution between cell types and between pre-MP and post-MP/men (**Supplemental Figure 2**). Our data demonstrate that the ERβ splice variant ERβ2 (*ESR2_ERb2*) is present in higher abundance than the full-length ERβ1 in most PBMC cell types.

Effect of Menstrual Cycle on Sex Hormone Receptor and Inflammatory Gene Expression

We next analyzed the expression of SHRs, and selected genes associated with immune response (**Supplemental Table 1**) in PBMCs during the menstrual cycle. To this end, we longitudinally sampled PBMCs from healthy Pre-MP women (n = 10) at 4 time-points representing early follicular (EF), mid-follicular (MF), ovulatory (OV) and mid luteal (ML) phases during the menstrual cycle and used a TLDA for gene expression analysis. We chose to use PBMCs rather than sorted cells to better illustrate the pooled expression profile of effector cells in the blood. A generalized linear mixed model (GLMM) based on ΔCT was performed to analyze the expression levels (**Supplemental Table 2**). Serum hormone levels were measured to confirm the hormone phases (**Supplemental Figure 3**). We could not detect any difference in SHR gene expression during the menstrual cycle

for *AR, ESR1, ESR2_ERb1 or ESR2_ERb2* (*CYP19A1*, *IL17* and *PGR* were not expressed and omitted from the GLMM). However, several immune related genes (*GATA3, IFNG, IL1B, LTA, NFKB1, PDCD1, STAT3, STAT5A, TBX21, TGFB1, TNFA*) varied in their expression patterns during the menstrual cycle with significant differences comparing MF with ML phases and MF with OV phases (**Figure 2** and **Supplemental Table 2**). Interestingly, expression of both pro-inflammatory/T_H1 response genes (*IL1B, TNF, LTA, IFNG, NFKB1, TBX21*, and *PDCD1*) and genes associated with T_H2 response (*STAT3, STAT5A, TGFB1*, and *GATA3*) were significantly upregulated during OV and ML phases compared to the MF phase (**Figure 2**). No difference could be observed for GPER1 expression between the phases (**Supplemental Figure 4A**).

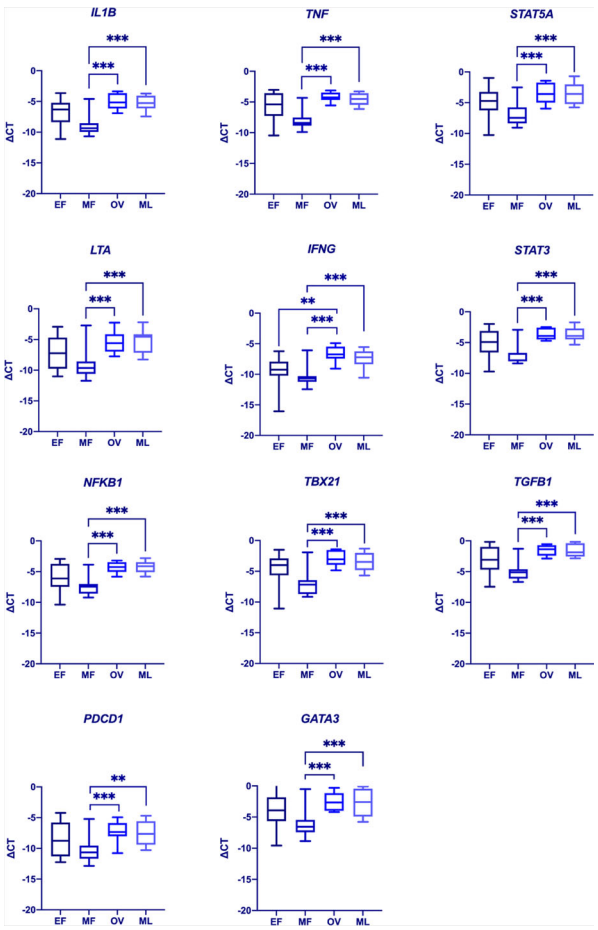

FIGURE 2 | Differences in immune gene expression during the menstrual cycle. A generalized mixed model (GLMM, $\alpha = 0.002$) was used to determine differences in gene expression between the various menstrual cycle phases (EF, early follicular phase; MF, mid follicular phase; Ov, ovulatory phase; ML, mid luteal phase). Significant differences could be observed for *IL1B*, *TNF*, *STAT5A*, *LTA*, *IFNG*, *STAT3*, *NFKB1*, *TBX21*, *TGFB1*, *PDCD1*, and *GATA3*. Data represent medians ±0.975 quartiles at df=9 in a t-distribution. Whiskers represent min and max values. $^{**}p = 0.001$. $^{***}p < 0.001$. Complete list of *p*-values is included in **Supplemental Table 2**.

Correlation Between SHR and Inflammatory Response Gene Expression

To analyze if SHR expression was associated with specific sets of inflammatory response genes, we performed gene correlation analysis. *AR* and *ESR2_ERb1* correlated poorly with most genes studied (**Figure 3** and **Supplemental Table 3**). In contrast, *ESR1* and *ESR2_ERb2* had more similar correlation to each other and to most other genes studied. Most pronounced, *ESR1* had significant correlation with both proinflammatory T_H1 and T_H2-response genes (**Figure 3** and **Supplemental Table 3**).

Correlation Between Hormone Levels and Immune Gene Expression in Pre-MP Women

Correlation of serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), estrogen (E_2), progesterone (P_4), and testosterone (T), as well as sex hormone binding globulin (SHBG), with the expression of inflammatory markers was analyzed in pre-MP women ($n = 10$) (**Figure 4** and **Supplemental Table 4**). Although we could not detect a significant correlation between any gene and hormone (at $\alpha = 0.00032$), it is noteworthy that progesterone stood out with high r-numbers and/or low p-values to several genes (*NFKB1* [r = 0.509, $p = 0.00342$], *LTA* [r = 0.504, $p = 0.00381$], *STAT5A* [r = 0.474, $p = 0.00700$], *TGFB1* [r = 0.474, $p = 0.00708$], *STAT3* [r = 0.472, $p = 0.00737$], *GATA3* [r = 0.457, $p = 0.00978$], *IL1B* [r = 0.434, $p = 0.0147$], *TNFA* [r = 0.426, $p = 0.0169$], *TBX21* [r = 0.400, $p = 0.0257$], *IFNG* [r = 0.387 $p = 0.0315$]). This is potentially interesting for further investigations since the progesterone receptor (*PGR*) expression in PBMCs could not be detected, as mentioned above. Additionally, our data indicate

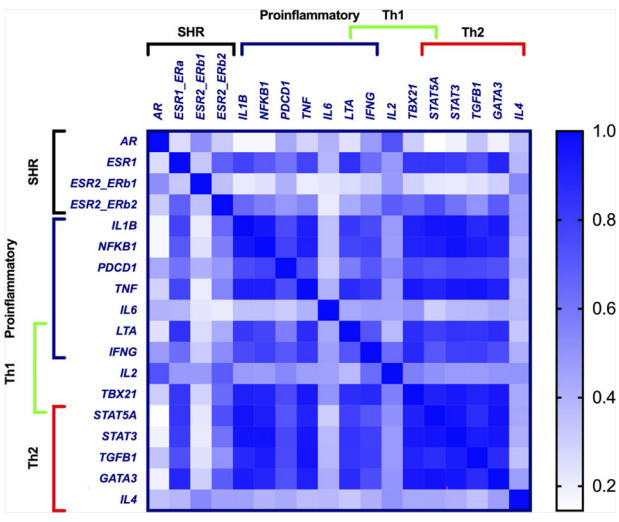

FIGURE 3 | A heatmap of repeated measures correlation coefficients between gene expressions in PBMCs from pre-MP women. Increased blue color represent increased correlation (r-value → 1) ($n = 10$, each sampled 4 times). Proinflammatory genes, SHR genes, and genes associated with T_H1, and T_H2 response are indicated. *P*-values are listed in **Supplemental Table 3**.

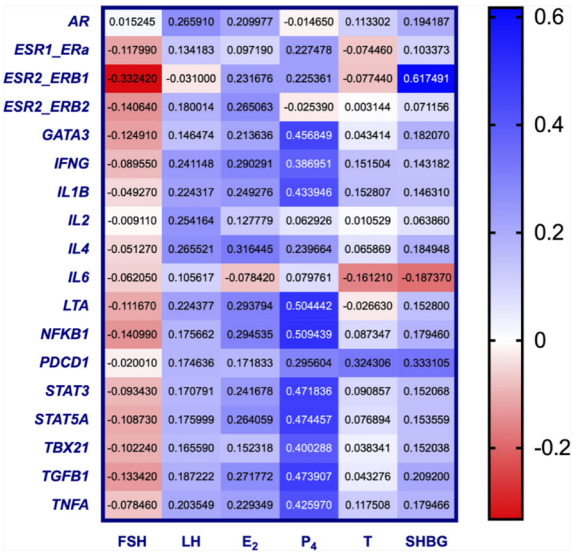

FIGURE 4 | Heat map of repeated measures correlation coefficients between serum hormone levels and selected gene expression (-ΔCT) in PBMCs from pre-MP women. Increased blue color represent increased correlation (r-value → 1), increased red color represent increased anti-correlation (r-value → -1), and white represent no correlation (r = 0) (n = 10, each sampled 4 times). P-values are listed in **Supplemental Table 4** (α = 0.00032).

that the levels of SHBG correlates positively with *ESR2_ERb1* (r = 0.617, p = 0.000215) (**Figure 4** and **Supplemental Table 4**). No significant correlation could be observed for GPER1 expression and hormone levels (**Supplemental Figure 4B**).

DISCUSSION

In this study we identify that ERα is the predominant estrogen receptor in PBMCs and that the expression of the ERβ alternative splice variant ERβ2 generally is more abundant than the full-length ERβ1 variant in PBMCs. Further, we demonstrate that the expression of several immune-related genes fluctuates in relation to the menstrual cycle. Using FACS to sort out $CD4^+$ T-cells, $CD8^+$ T-cells, $CD56^+$ NK-cells, $CD14^+$ monocytes, and $CD19^+$ B-cells from PBMCs, we could identify that $CD19^+$ B-cells have high expression of all ERs studied (ERα, ERβ1, ERβ2, and GPER1). In contrast, $CD14^+$ monocytes have very low expression of ERβ1 and ERβ2, but high ERα and GPER1 expression. In addition, GPER1 was only found in $CD8^+$ T-cells, $CD14^+$ monocytes, and $CD19^+$ B-cells with an overall high expression in these cell types. Taken together, the findings provide new information to better understand the interplay between sex hormones and immune responses.

We show that ERβ2 is significantly more abundant than ERβ1 (full length) in most immune cell subsets. Phiel *et al.* did previously report presence of both ERα and ERβ in PBMC (9), but they did not discriminate between ERβ isoforms. Importantly, ERβ2 does not bind E_2 but can dimerize with both ERα and ERβ1, to inhibit their transcriptional activity. Oppositely to the present study, it was earlier described that patients with chronic lymphocytic leukemia (CLL) had higher levels of ERβ2 in PBMCs compared to healthy donors where ERβ1 dominated (16). However, that study was performed by assessing ERβ2-protein staining (using immunocytochemistry, ICC), rather than quantifying absolute expression. In addition, the median age among CLL patients (68 years) and healthy donors (43 years) differed, so an age difference in ERβ1/ERβ2 distribution cannot be excluded. Although we did not analyze ERα splice variants, the study by Stygar and colleagues (17) detected some expression of ERα splice variants in PBMCs and that this expression could vary with the menstrual cycle. However, the samples used in that study were derived from 6 pre-MP women in the follicular phase, and 3 in the secretory phase (i.e., the individuals were not sampled repeatedly) so an inter-individual difference cannot be excluded. Clearly, more studies are needed to determine ERβ2's role in relation to other ER variants, sex hormone levels, and age.

Furthermore, we show that the expression of several immune genes in bulk PBMCs (*GATA3, IFNG, IL1B, LTA, NFKB1, PDCD1, STAT3, STAT5A, TBX21, TGFB1, TNFA*) differed between phases of the menstrual cycle. We did not observe differences in SHR expression patterns between the phases, possibly this is linked to an important limitation of our study which is the low number of participants. In addition, the low amount of sample material prevented sorting out the cell populations for TLDA analysis by FACS. Nevertheless, differences in immune gene expression patterns were significant and we speculate that even more immune related genes could potentially be found by increasing the participant number. *IFNG, TNFA* and *IL1B* are all genes that encode proinflammatory responses. NFκB is an inducible transcription factor that can be regulated by steroid hormone signaling (18), and controls expression of several stress response genes and genes associated with development of innate immunity. Among NFκB target genes are regulators of inflammatory cytokines, cell survival, proliferation and cell surface proteins (18–20). NFκB activity has also been suggested to play a significant role for female fertility by participating in angiogenesis during corpus luteum formation, endometrial implantation and indeed also for the T_H1-T_H2 immune response shift seen during the menstrual cycle (important for the tolerance of the semi-allogenic blastocyst implantation) (21).

Additionally, we demonstrate that the expression of *GATA3* and *TBX21* are both fluctuating during the menstrual cycle. *TBX21* (encoding for T-bet) and *GATA3* are both key transcription factors for T_H1 and T_H2 immune response respectively. It should be noted that the distinction of T_H-cells into T_H1- and T_H2-cells, although still widely in use, have been questioned since the discovery of further T_H-subsets (as T_H17 and Treg-cells) (22–24). Prior studies suggest that the immune response shifts from a T_H1 to T_H2 response over the menstrual cycle (7). Although the expression of *GATA3* and *TBX21* differs over the menstrual cycle, our data do not support a T_H1-T_H2 shift, as both *GATA3* and *TBX21* are highly expressed during the

latter part of the cycle (OV and ML phases). LTA (TNF-β) is also related to T_H1 response, as it is secreted from T_H1 but not T_H2 cells. A different experimental design including more participants (and more frequent sampling during the menstrual cycle) may reveal a more fine-tuned regulation of *GATA3* and *TBX21*. The same expression pattern is seen with *PDCD1* and *TGFB1* which are significantly more expressed during OV and ML phases. *PDCD1* and *TGFB1* are both related to immune tolerance. PD-1 may have implications for development of autoimmunity, chronic infectious diseases and several types of cancer, and expression of its gene *PDCD1* is related to sex hormones, particularly E_2 (25). TGF-β, stimulates differentiation of $CD4^+$ T-cells to Treg-cells and has an inhibitory effect on B-cell proliferation. Previous studies have showed a positive correlation between E_2 and Treg numbers during the menstrual cycle (26).

Like the genes mentioned above, *STAT3* and *STAT5A* are also significantly higher expressed during OV and ML phases. STAT5 has previously been associated with sex differences in liver metabolism (27) and pulmonary hypertension (28), both with a proposed neuroendocrine regulation through hypothalamus-growth hormone-STAT5 axis. In addition, STAT5 has an important role in the priming of $CD4^+$ T-cells for T_H1, T_H2 and T_H9 development (29). STAT3 has on the other hand been pointed out as factor of major importance in the pathogenesis of gastrointestinal bacterial infections and cancer development along with viral infectious diseases (HBV, HCV and HPV) which in turn may drive cancer development (30).

We further found that ERβ1 (*ESR_ERB1*) stood out as significantly positively correlated to SHBG levels. SHBG is a circulating glycoprotein synthesized and secreted by the liver, with a main function of transporting sex steroids, mainly testosterone, in the circulation, thereby modulating sex hormone bioavailability. In a study by Maggio *et al.* (31) on postmenopausal women, SHBG was negatively correlated to inflammatory markers such as C-reactive protein (CRP), IL-6 and soluble IL-6 receptor (sIL-6r). In the same study E_2 was positively correlated to CRP and IL-6 (but not sIL-6r). It is possible that the opposite correlation between E_2 and SHBG on inflammation might be due to increased expression of ERβ1 which oppose the action of ERα.

In this study, we could neither observe a general immunostimulatory nor an immunosuppressive signature that could be linked to the different phases of the menstrual cycle. Rather, both immunostimulatory and immunosuppressive response genes were upregulated during ovulation and the mid luteal phase. Generally, E_2 is immunostimulatory while progesterone (P_4) and testosterone have immunosuppressive properties (described in detail in e.g (2).,). Testosterone will e.g., decrease humoral immunity (increase B-cell apoptosis of immature B-cells). P_4 will decrease hypermutation and class-switch of B-cells and E_2 will decrease B-cell apoptosis, promote class-switching and hypermutation and increase the number of autoreactive antibodies (32).

An increase in P_4 in the luteal phase is attributed a general suppressive effect on the innate immune response by e.g., decreasing the production of proinflammatory cytokines (33). In the present study, gene-hormone correlations were not clear-cut, the P_4-levels might be involved in the regulation of several immune response genes, but our statistical evaluations did not provide a significant signal (**Figure 4** and **Supplemental Table 4**). PGR is reportedly present in immune cells (34). Recent findings by Hierweger and coworkers (35), however, question its presence in T-cells suggesting that P_4 may signal through the glucocorticoid receptor (GR) in these cells. The suggestion by Hierweger is in line with our data since PGR was not expressed in our material. Although GR was not part of our qPCR array, we could detect high GR levels in all sorted PBMCs (**Supplemental Figure 1**). Therefore, we hypothesize that any correlation of P_4 with gene expression in PBMC is indirect, e.g., through GR. Future studies including GR could help answering these questions.

In conclusion, we demonstrate that several key immune related genes in PBMCs fluctuate in their expression according to the phase of the menstrual cycle. This includes both proinflammatory, T_H1- and T_H2-response genes. In addition, this paper illustrates that mRNA for ERβ2 is more abundant than ERβ1 in PBMCs, which suggests that ERβ2 may play a more prominent role than previously thought in the immune response. Our study provides evidence that the menstrual cycle can influence the immune response. Larger studies enrolling pre-MP women sampled over more timepoints of the menstrual cycle and including more ER splice variants and inflammatory genes in sorted PBMCs are warranted. In the end, such studies may provide information that allows for the development of personalized immune treatments to the benefit of both pre-MP women, post-MP women and men.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Swedish Research Council (Dnr: Ö 24-2009). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PB: Conceived the study, performed the experiments, analyzed data, and wrote the manuscript. B-ML: Conceived the study, collected the patient material, analyzed data, and wrote the manuscript. PF: Analyzed the data and wrote the manuscript. MS: Performed the experiments. J-AG: Financed the experiments and contributed with laboratory equipment, and wrote the manuscript. AJ: Conceived the study, analyzed data, and wrote the manuscript. IN: Conceived the study, performed the experiments, analyzed data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.721813/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a shared affiliation with several of the authors, PB, B-ML, MS, J-AG, and IN, at time of review.

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