Modulation of monocyte inflammatory response through estrogen receptor α and progesterone receptor signaling

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Ovarian hormone fluctuation has been associated to clinical status of several autoimmune conditions and the influence on inflammatory disease is under investigation. The incidence of inflammatory bowel disease is high in the West and rising in other parts of the world. Immunomodulating treatments are available but there is a need for a cheap, readily available treatment with an enhanced risk-benefit ratio. The effect of estrogen and progesterone receptor mediated signaling on the immune response of myeloid cells, such as monocytes or macrophages have been studied to some extent with varying results. In addition to ovarian hormones, selective estrogen receptor modulators have also been suggested to modulate inflammatory response of several types of immune cells. In this master´s thesis project, small interfering RNA-based receptor silencing was used to investigate the immunomodulatory effect of ERα and PGR signaling as well as to illuminate the molecular mechanism of SERM2, a novel compound. Further, the aim was to gain information for the development of hormone receptor mediated immunomodulatory treatment of autoimmune or inflammatory disease. The results indicate suppression of monocyte NF-κB activation by PGR signaling, while the ERα mediated signaling had a very varied effect. It is possible that ERα upregulation increases the proinflammatory potential of monocytes. Cytokine expression analysis of both cell line and primary monocytes exhibited modulation of inflammatory cytokines by PGR mediated signaling, which possibly could reduce the activity of Th17 cells in the gut, while the opposite could be true regarding ERα upregulation.

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1. Introduction

Traditionally, estrogen receptors (ER) and progesterone receptors (PGR) have been considered to mediate signaling in reproductive and mammary tissues. There, indeed, the main regulatory effects, such as proliferation of the mucosa, secretion and follicle maturation are executed. However, these receptors are also expressed in other tissues such as skin, neural, vascular, bronchial and intestinal tissues as well as in several types of immune cells. Enzymes involved in the synthesis of estrogen are also expressed in non-reproductive tissues, such as brain, adipose tissue and the skin. In females, after puberty, the ovaries are the main producing tissue of the lipophilic hormones, secreting estrogens and progesterone systemically. Non-reproductive ER and PGR signaling vary and be tissue specific, due to differential expression patterns of receptor types and other regulatory proteins.

Pro- and anti-inflammatory mechanisms play part in physiological hormone regulated processes, such as ovulation (Bouman et al., 2005) menstruation, pregnancy and labor (Gomez-Lopez et al., 2010). These processes have also been associated to disease status in autoimmune diseases (Oertelt-Prigione, 2012). Gender influences the incidence of several diseases, with a higher rate of women suffering of autoimmune disease such as multiple sclerosis (MS) or systemic lupus erythematosus (SLE) (Angum et al., 2020). Additionally, the outcome of sepsis differs between sexes: the mortality rate is higher in men (Nasir et al., 2015). It has been suggested that the adaptive immune function is increased in women, with a possibility of differences also in innate responses (Ngo et al., 2014). Amongst other things, ovarian hormones are thought to be involved in this phenomenon (Rubtsova et al., 2015).

A famous example of the complexity of hormone immune regulation is the effects of estrogens in the disease course of MS, which has T cell mediated neuroinflammatory pathophysiology. High estrogen conditions, like pregnancy, have been observed to be protective. On the other hand, the post-partum period has been associated with increased risk of relapse, hence the investigation of estriol as a potential therapeutic of MS (Gold and Voskuhl, 2009). Interestingly, the disease modulating effects of physiological hormone fluctuation or hormone replacement therapy can vary significantly by disease pathophysiology: in SLE, which is a B cell mediated disease, pregnancy as well as

hormone replacement therapy are associated with aggravated disease (Kassi and Moutsatsou, 2010).

Gender and hormonal status may affect inflammatory conditions such as inflammatory bowel disease. Hormonal fluctuation has been observed, for instance, to influence the GI transit time even in healthy females. In inflammatory bowel disease (IBD) a cyclical pattern of symptoms has been reported, as reviewed by (Bharadwaj et al., 2015). As prevalence of these inflammatory diseases are on the rise especially in Third World countries (GBD 2017 Inflammatory Bowel, Disease Collaborators, 2020), there is a need for an affordable, readily available and well-tolerated treatment of these long-term diseases. Selective estrogen receptor modulators (SERM) could prove a convenient alternative. However, especially since the experimental reports of ER and PGR signaling in intestinal inflammation have not been consistent, this controversy needs additional attention in order to illuminate underlying mechanisms.

In this thesis project the possibility of downregulating the monocyte immune response by ovarian hormone receptor signaling is explored. Further, the aim is to illuminate the receptor mechanism of the anti-inflammatory effects of SERM2 previously reported by our research group in human CD14+ cells (Polari, Lauri et al., 2018) and in a mouse model of colitis (Polari, L. et al., 2019). The experimental focus is on the immune response of monocytes by analysis of a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation model as well as by cytokine expression in both THP1 cell line monocytes and adult human primary monocytes. This can be valuable information in developing novel therapeutics. A specific interest in this project was the selective hormone receptor modulators (SHRMs). While searching for new ways to alleviate inflammatory disease through the modulation of ovarian hormone receptor signaling, one must realize steroid hormone therapy, including natural or synthetic estrogen of progesterone analogues, may not be the best option. Especially estrogen therapy increases the risk of deep vein thrombosis and cancer in estrogen sensitive tissues. SHRMs induce partly similar effects as hormones but have a more specific way of binding and can function as both agonists and antagonists for these receptors in a tissue specific manner.

1.1. Ovarian hormones

Estradiol (E2) is the predominant estrogen in circulation, while estriol (E3) and estretrol (E4) are associated with pregnancy and estrone with the menopause. Ovarian hormones are regulated by the hypothalamus-pituitary-gonadal (HPG) axis, in which the kisspeptin–neurokinin B–dynorphin and gonadotropin release hormone (GnRH) producing neurons in the hypothalamic infundibular nucleus stimulate the anterior pituitary to produce luteinizing hormone (LH) and follicle stimulating hormone (FSH). GnRH, LH and FSH can be released in pulses or surges, and pulse frequency regulate the cycle. Rapid GnRH pulses induce LH, while slower pulses induce FSH. The HPG axis and ovarian hormones regulates development, induce female traits and reproductive processes but also influence metabolism, olfaction, mood and cardiac function. Several feedback mechanisms regulate the axis.

During ovarian steroidogenesis, estrogen inducing LH and progesterone inducing FSH stimulate enzyme expression in follicular theca and granulosa cells, the final products being estrogens, progesterone and small quantities of androgens. The final reaction of androstenedione to estrone (E1) and estrone to estradiol, catalysed by aromatase and 17β-HSD, respectively, takes place in granulosa cells. E2 peaks at and induces ovulation. Progesterone is released by the corpus luteum and peaks after ovulation, during the luteal phase of the cycle. Chorionic gonadotropin induced progesterone also has an important role in maintaining pregnancy.

1.2. Estrogen receptor signaling

This thesis will be focusing mainly on ERα, transcribed by *ESR1* and second, on PGR, transcribed by *PGR*. The estrogen hormones, estradiol, estrone, estriol (E3) and estretrol (E4) have unique affinities to estrogen receptors. The nuclear ERs are divided into subtypes ERα and ERβ. E2 binds to ERα at a picomolar to low nanomolar range and to ERβ at almost similar affinity (Blair et al., 2000) (Yoo and Jeung, 2009). Apart from nuclear receptors, GPER1 is located at either the cell membrane (Martínez-Traverso and Pearl, 2015) the endoplasmic reticulum (Revankar et al., 2005) or in some cancers, the nucleus (Samartzis et al., 2014). Thus, estrogen receptor signaling can exert both rapid and long-lasting responses. Slow, long-lasting estrogen response is mediated by nuclear ER effect on gene expression while, in addition to the G Protein-Coupled Estrogen

Receptor 1 (GPER), rapid, nongenomic responses are mediated through the plasma membrane bound ERα isoforms.

ERα is known to be alternatively spliced in at least five ways, producing the full-length nuclear receptor ERα66 and amongst others, the cytosolic splice variants ERα46 (Flouriot et al., 2000) and ERα36 (Wang et al., 2006) (table 1). The homo- or heterodimer receptor is activated by serine phosphorylation at ligand binding, followed by disengaging corepressor proteins, which allows nuclear translocation and complexing with coactivators. Besides the genomic and non-genomic effects, ligand-independent signaling has been seen in cancer, for instance as crosstalk to insulin-like growth factor or epidermal growth factor signaling (Hewitt, S. C. et al., 2017). Physiological and immunological effects of these hormones and their receptors are a result of interplay.

Table 1. Structural features of ERα splice variants.

Variant	Shared domains	Unique domains	AF-1	AF-2	Localization
ERα66	A, B, C, D, E, F		Yes	Yes	Cytosol, nucleus
ERα46	C, D, E, F		No	Yes	Beneath plasma membrane, mitochondria
ERα36	C, D, E	F	No	No	Beneath plasma membrane, mitochondria

1.3. Structure and function of ERα

The full length nuclear ERα (or NR3A1) is large protein with six domains (A-F). The N-terminal A/B domain is very flexible, allowing it to interact with several coactivator proteins, due to intrinsically disordered regions. The DNA binding domain function in estrogen response element (ERE) palindrome recognizing and dimerization (C) and ligand binding (E) (Hewitt, Sylvia C. and Korach, 2018), represented in figure 1. In the inactive conformation or in the absence of coactivators, α-helix 12 (H12) of activation function (AF) -2 binds to a LXXLL motif in the ligand-binding domain. H12 changes conformation upon agonist binding, being competitively displaced by coactivators (Farooq, 2015) (Yaşar et al., 2016). N-terminal AF-1 and FF-2 transactivate and together form an interface for coactivator binding (Tora et al., 1989). Transcriptional intermediary factor 2 (TIF2) and members of steroid receptor coactivator-1/p160 family have been recognised as binders to the AF motifs (Benecke et al., 2000), as have p300-CREB binding protein coactivator family members (Kobayashi et al., 2000) and various heat shock proteins (Dhamad et al., 2016).

In the classical estrogen receptor signaling pathway the transcription complex binds to DNA at specific consensus estrogen-response elements. A second, tethered pathway exists, where ER participates in complexes also at non-consensus estrogen response elements. ERα binds EREs with greater affinity compared to ERβ, and ERαβ heterodimers induce a different response compared to ERα homodimers (Powell et al., 2012). The transcription initiation functions of EREs feature not only reproduction and proliferation associated genes, but also genes with, for instance, metabolic and cardiovascular function (O'Lone et al., 2004) (table 2).

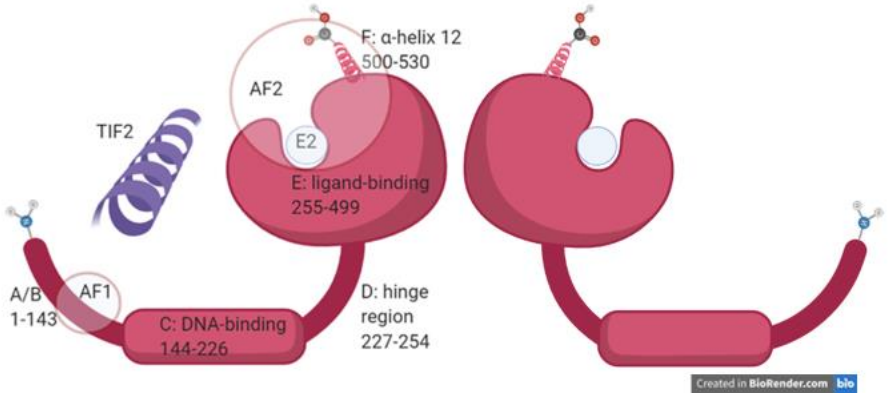


Figure 1. Schematic representation of ERα dimer. The full length nuclear ERα has six domains (A-F). The terminal A/B as well as E/F domains functions in forming interface for protein interactions while the C domain binds DNA. Ligand binding changes the conformation of α-helix 12, enabling the forming of an interaction surface for coactivator, such as TIF2, binding. Picture created with BioRender.com

Table 2. Examples of genes transcribed under estrogen-response elements. Adapted from O´Lone et al (2004).

Gene	Protein	Function
PGR	Progesterone receptor	Reproduction
ESR1	Estrogen receptor α	Reproduction
EGFR	Epithelial growth factor receptor	Growth, survival
BCL2	Bcl-2	Survival
CCND1	Cyclin D1	Proliferation
KRT19	Keratin19	Epithelial structural
LDLR	Low density lipoprotein receptor	Metabolism
AGER	Receptor For Advanced Glycosylation End Products	Chronic inflammation
ACE	Angiotensin	Cardiovascular
GRIN2D	NMDA glutaminergic receptor (subunit 2D)	Neurology

Structural differences accounts for differences in the response. There is low homology between ERα and ERβ in the protein-interaction interface forming A/B (18%) and F domains (17%) (Yaşar et al., 2016). The F domain is also less conserved between species

and has been suggested to play a part in the species-specific differences in SERM 4-hydroxytamoxifen induced activity (Arao, Y. and Korach, 2018).

Cytosolic localization for the truncated variants of ERα are complexed beneath the plasma membrane and in the mitochondria. Mitochondrial localization is seen especially in endothelial cells, where ERα isoforms seem to regulate some mitochondrial genes and play a part in preventing apoptosis and ligand-independently in the rescue after mitochondrial inter-membrane space stress (Yaşar et al., 2016). Membrane localization is enabled by specific serine residue interaction either directly or indirectly with scaffold protein caveolin-1 at caveolae rafts and more importantly by palmitoylation of a specific cysteine residue is known to be necessary for membrane localization (Acconcia et al., 2005). Membrane ERα can signal rapidly by activating protein kinase signaling pathways and may colocalize with G-protein coupled receptors. E2 downregulates palmitoylation of ERα (Adlanmerini et al., 2014), one would assume as a negative feedback mechanism to inhibit overactivation of the pathway.

Nongenomic ER signaling is associated to endothelial and cardiovascular protective effects (Chambliss et al., 2010) and this comprises also innate immunity ER response, which will be discussed in greater detail in sections 1.8-1.10. In short, reports have been made of the effects of specifically nongenomic ER signaling in immune cells. These include Ca^{2+} oscillations and both Akt and ERK phosphorylation in murine splenic T cells (Ádori et al., 2010), inhibitory crosstalk of ERα36/GPER affecting interleukin (IL)-6 production in human monocytes (Pelekanou et al., 2016) and that human monocytes and macrophages express ERα46 in an estradiol dependent manner (Murphy et al., 2009). Some indications of SERMs inducing non-genomic response can also be found. For instance, raloxifene (RAL) was seen to induce the rapid vasodilation response in endothelial cells via the phosphoinositide 3-kinase (PI3K) pathway and the effect was blocked by fulvestrant (ICI) (Simoncini et al., 2002).

The genomic signaling has been suggested to be dominant in the proliferative response in the uterus, and that non-genomic effects are associated only to cell-types not utilizing the nuclear pathway and that non-genomic response is not able to modulate the genomic response (Hewitt and Korach, 2018). However, differing information can be found regarding the tissue specificity of nongenomic ER effects. For instance, a palmitoylation site mutant mouse was generated to distinguish between nuclear and cytosolic ERα

signaling in vivo. These mice were found to have intact uteri, but aberrant ovaries and decreased progesterone hormone levels and a lack of E2 induced rapid vasodilation by eNOS activation (Adlanmerini et al., 2014). In other pre-clinical models, specific pharmacological activation of cytosolic ERα signaling showed no proliferative effects in the uterus of mice, nor in hormone sensitive breast cancer xenografts (Chambliss et al., 2010). However, recent research has implicated a role of cytosolic ERα36 in breast cancer progression, and in resistance to anti-estrogen therapy (Pagano et al., 2020).

1.4. Regulation of estrogen receptor activity

The cellular and tissue response to estrogen induced activation is regulated in a complex manner; the expression of the receptor is influenced by chromatin related mechanisms, post-transcriptional mechanisms, post-translational modifications, localization, hormones and growth factors as well as by diverse protein-protein interactions. There are several ER promoters in the genome, and the methylation pattern induce ER transcription in a tissue and situation dependent manner (Yaşar et al., 2016). ER dimerizes, transactivates and forms a heterogenous population in the cell. ERα/ERβ expression ratio and homo- vs heterodimerization alters the estrogen response, as αβ heterodimers share some of the transcriptional effects as either homodimer, but not all. The αβ heterodimer have been indicated to have antiproliferative effects, and the ERβ homodimer might be subjected to increased turnover (Chakraborty et al., 2012).

Dimer type and ligand influence the type of recruited interaction partners, and further, ligand affinity to the ER do not always correlate with the level of transcriptional response (Routledge et al., 2000). PTMs, such as, but not limited to, phosphorylation by several kinases can further induce variation on the protein-protein interaction networks leading to gene expression (Treviño and Weigel, 2013). Corepressors can bind the inactivated receptor, but the proteins can also inhibit transcriptional function by binding directly to DNA or by binding to activated ER complexes and for instance, interacting with nucleosome structure and histone altering enzymes (Fuentes and Silveyra, 2019). The genomic response element sequence also influences the composition of the transcription complex (Hall et al., 2002).

At least in mammary and reproductive tissues, in a normal setting, negative feedback inhibits overactivation of the pathway: ERα activation induces rapid ubiquitylation and proteasomal degradation of the receptor proteins, after which transcriptional upregulation

restores the cell to basal level. Binding of antagonist have been seen to increase turnover of ER protein but fail to increase the transcriptional response. Cells can also regulate ER responses by altering the levels of the co-activating or co-inhibiting molecules, and downregulation of ER corepressors has been associated with tamoxifen resistance in breast cancer (Légaré and Basik, 2016). Activation of ERβ isoform 2 regulates the expression of ERα trough proteasomal degradation (Zhao et al., 2008). Also, ERα induces PGR expression (Xu et al., 2004) and PGR and hCG (possibly also androgen and vitamin D receptor) activation downregulate ER expression, while GnRH regulate ER expression in a tissue dependent manner (Pinzone et al., 2004).

1.5. The progesterone receptor

Progesterone signals mainly via the nuclear PGR, which is expressed at least in ovaries, uterus, mammary tissue and in neuroendocrine tissue. PGR protein is transcribed as two isoforms under two promoters: truncated PGR-A and full-length PGR-B. These are thought to be expressed in distinct tissues and can induce transcription as dimers and convey extranuclear signaling as monomers, at least in breast cancer cells (Dressing, G. E. et al., 2009). Much like the GPER, progesterone can also signal through membrane G-protein coupled receptors PGRMC1 and PGRMC2 which convey rapid signaling (Pru and Clark, 2013). Progesterone receptor signaling regulate cell growth and differentiation and the effect is tissue specific.

PGR, as ERα, has two flexible AF domains which are conformationally activated by ligand binding and dimerization. The AFs, N-terminal AF-1 and LBD located AF-2, including H12, are involved in the interaction surfaces for binding coregulator proteins, such as p300, and transcription factors. The availability of the transiently interacting proteins varies between developmental stage and tissue. The protein complexes bind to progesterone response elements in the genome and activates transcription. PGR is regulated by several post-translatory modifications, of which some are hormone influenced. MAPKs, CDKs and PKA are known to phosphorylate PGR and modifications of specific residues can modulate the function and influence the genes transcribed downstream PGR activation. Progesterone induced SUMOylation suppresses transcriptional effects and provides negative feedback. PGR A signaling suppresses PGR B. Additionally, PGR induced miRNAs target mRNA of proteins involved in cell cycle progression, thus counteracting proliferation. (Grimm et al., 2016)

1.6. Selective estrogen receptor modulators

SHRMs are pharmacological modulators of the ERs, PGRs or AR. SHRMs are usually large lipophilic molecules, which induce a mixed agonist/antagonist response at the target receptors. Selective estrogen receptor modulators are a group of compounds targeting the estrogen receptor E2 binding site, competitively inhibiting the agonist binding (described in figure 2). The large hydrophobic sidechain of SERM molecules (fig 2a) influence the conformation, most often inducing repression of transcription or transcription initiated under a different set of response elements. SERMs are used as prevention or treatment of for instance osteoporosis or ER-positive breast cancer (table 3).

Table 3. Commonly used SERMs.

SERM	Indication	Moxt common adverse effects
Raloxifene	Osteoporosis	Hot flashes, leg cramps
Tamoxifen	ER-positive breast cancer	Atrophic vaginitis
Fulvestrant	ER-positive metastatic or advanced breast cancer	Nausea, vomiting, constipation
Toremifene	ER-positive breast cancer	Vision problems, dizziness, headache
Clomiphene	Female infertility	Pelvic pain, abnormal vaginal bleeding
Ospemifene	Vaginal atrophy	

SERMs target ERs in a tissue selective manner as partial agonists or antagonists, except ICI, which is a full ER antagonist. RAL binds to ERα at levels similar to E2 (Rey et al., 2009). RAL has been suggested to have a weaker affinity to ERα46 compared to ERα66, while estradiol bound each with an equal affinity (Lin et al., 2013).

The displacement of H12, mentioned earlier to be crucial for ER forming the transcription complex, is either in part or fully inhibited from adopting agonist conformation when a SERM is bound, due to the large, hydrophobic side chain (fig 2b). After the binding of a mixed agonist/antagonist, a switch to agonist conformation can occur as tissue specific levels of coactivators increase (Farooq, 2015). Partial agonists might induce a flexible conformation, with a slight bias towards antagonism, allowing other protein-protein interactions to influence the conformation in tissues which favor activation (Chakraborty et al., 2013). Binding of 4-hydroxytamoxifen, for instance, unwinds helices 3 and 11 which causes the antagonist conformation of H12 to inhibit the binding of coactivators to the nearby ligand-binding cleft LXXLL motif, unless the tissue specific coactivators have

the ability to replace it (Farooq, 2015). Negative charge of the D351 residue located in AF-2 domain has been shown important for RAL and 4-hydroxytamoxifen agonist activity *in silico* (fig 2c). The shape of the aspartate sidechain along with a short 2.7Å hydrogen bond is suggested to be important for antagonist activity, since it would distort the piperidine ring to shield the negative charge which would be important to cofactor binding of AF-2 (Liu, H. et al., 2002) (MacGregor Schafer et al., 2000).

Besides coactivator expression, the predominance of either ERα or ERβ might be a factor determining the agonist/antagonist activity and binding to specific genomic RAL response elements (fig 2d) (Rey et al., 2009). The F domain-AF-1 interface is possibly responsible for ERα homodimerization (Arao, Yukitomo and Korach, 2019).

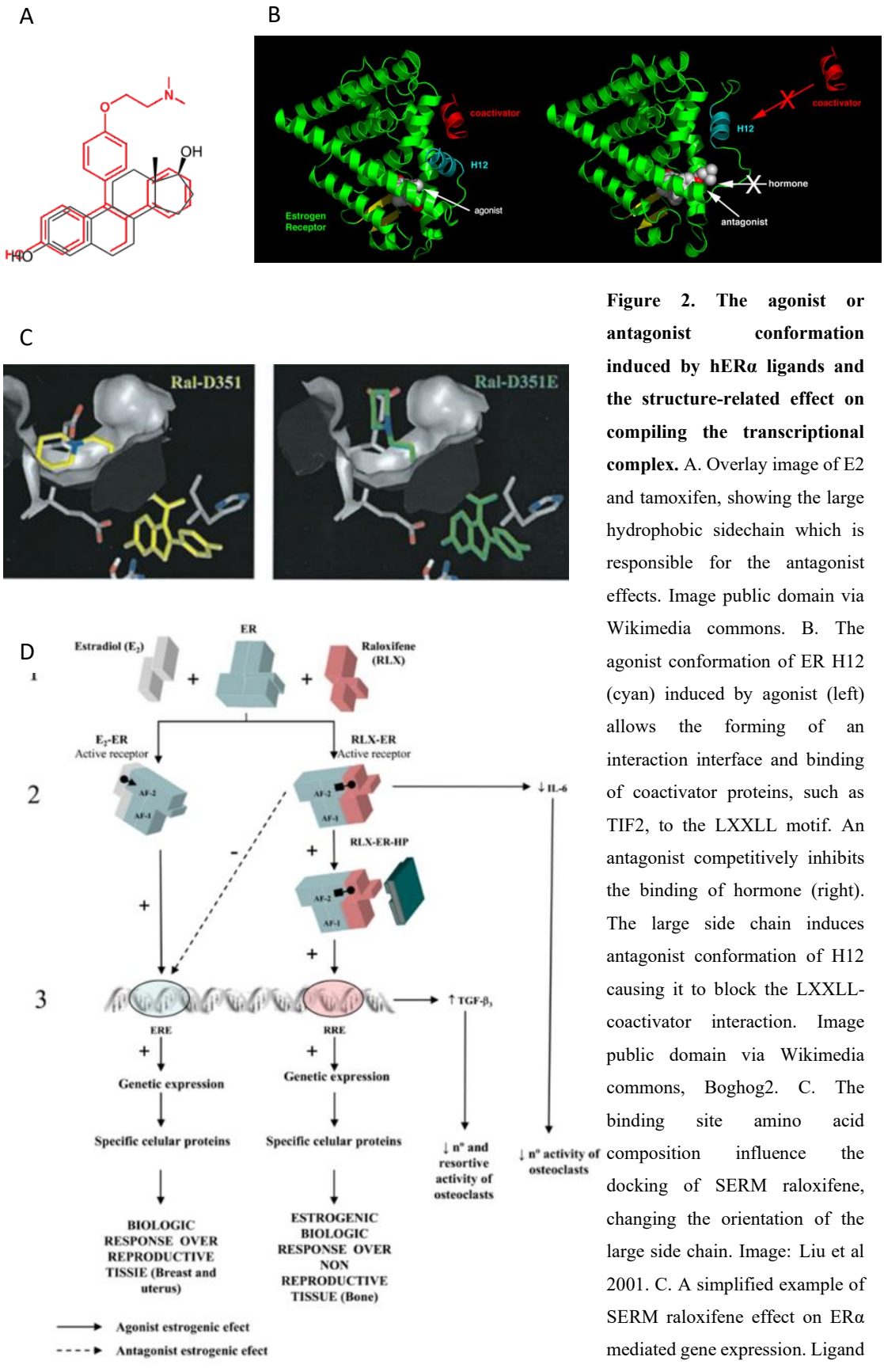


Figure 2. The agonist or antagonist conformation induced by hERα ligands and the structure-related effect on compiling the transcriptional complex. A. Overlay image of E2 and tamoxifen, showing the large hydrophobic sidechain which is responsible for the antagonist effects. Image public domain via Wikimedia commons. B. The agonist conformation of ER H12 (cyan) induced by agonist (left) allows the forming of an interaction interface and binding of coactivator proteins, such as TIF2, to the LXXLL motif. An antagonist competitively inhibits the binding of hormone (right). The large side chain induces antagonist conformation of H12 causing it to block the LXXLL-coactivator interaction. Image public domain via Wikimedia commons, Boghog2. C. The binding site amino acid composition influence the docking of SERM raloxifene, changing the orientation of the large side chain. Image: Liu et al 2001. C. A simplified example of SERM raloxifene effect on ERα mediated gene expression. Ligand differences induces changes in complex formation and response element binding. Image: Rey et al 2001.

The novel SERM2 compound used in this project also binds to ERβ and PGR, but neither the glucocorticoid receptor nor the androgen receptor. Affinity was measured using a radioligand binding assay, resulting in a 50% of maximum binding at <1nM for ERα, 13 nM for ERβ and 210 nM for PGR (Polari et al., 2019).

As the conformation of the ligand bound receptor is defined by the interacting proteins, the response can vary greatly. If the protein-interactions, thus the response could be induced with even greater specificity, this could possibly be utilized to develop more specific receptor modulators to the benefit of various patient groups. Illuminating the mechanisms of ER and PGR mediated immune effects could, albeit being an arduous task, in best case further the development of an ovarian hormone receptor specific immune supporting therapy. Tissue and receptor type specific ER signaling could benefit also cardiovascular and possibly cancer patients. Allosterically modulating the receptor conformation or the flexible protein-protein interfaces could also prove an interesting new treatment option.

1.7. The innate immune system and the monocyte/macrophage lineage

The innate immune system is the first-line, fast-acting and non-specific defense towards pathogens. It comprises of tissue mechanisms, such as the epithelial surfaces and mucus layers, antimicrobial proteins such as lysozyme or defensins, cytokine releasing and tissue modifying proteases, the complement and the coagulation systems. Endothelial and epithelial cells are also involved in pathogen recognition and defense, as well as the sequential signaling events. Additionally, highly specialized immune effector cells carry out several important functions. These include NK cells and innate lymphoid cells as well as cells of the myeloid lineage: granulocytes (neutrophils, basophils, eosinophils and MAST cells), monocytes, macrophages and dendritic cells. The function of the cells includes phagocytosis of both pathogens and damaged host tissue, releasing of reactive oxygen species and enzymes acting on either the pathogen or host-tissues and second messengers such as cytokines and chemokines.

The innate immune response is not specific to a single antigen epitope, as in the case of adaptive immunity. However, there is some categorization of the classes of pathogens recognized and in the functions of differentiated cells. Innate cells differentiate and adopt a phenotype according to the surrounding immune signals. Especially monocytes and macrophages can function in both a pro- and anti-inflammatory manner. Immune cells

are classified according to the level of expression of cell surface molecules. These include cluster of differentiation molecules (CD), which include pattern recognition receptors such as TLRs or antibody recognizing FcRs. (Aristizábal and González, 2013)

During the first hours and days of acute inflammation, neutrophils are the most abundant cells. Local expression of chemotactic and cell-adhesion molecules recruits increasing amounts of other immune cells, such as monocytes and T cells. Monocytes are short-lived circulating innate immune cells which carry out inflammatory responses after recognizing a broad range of antigens by secreting reactive oxygen species, phagocytosis and secretion of immune related signaling proteins. The monocyte/macrophage lineage cells also play an important role in the interplay of the immune response, by participating in the differentiation of adaptive immune cells by cytokine and chemokine contribution to the immune environment.

Monocytes in human and most other animals can be roughly divided into three subsets by the expression levels of lipopolysaccharide (LPS) coreceptor CD14 and FcγIII receptor, also known as CD16. $CD14^{hi}$ have been referred to as classical monocytes and $CD16^{hi}$ as non-classical. The $CD14^{+}/CD16^{+}$ population is seen as an intermediate in differentiation from the classical to non-classical maturation. In mice the classical monocyte population express high levels of lymphocyte antigen (Ly)6, and the chemotaxis to the gut is C-C chemokine receptor type 2 (CCR2) dependent in both mouse and human (Ziegler-Heitbrock, 2014). Non-classical $CD16^{hi}$ monocytes have been suggested to patrol endothelia and have no or only limited ability to extravasate (Auffray et al., 2007). In the classical monocyte population, there are precursors of some macrophages and dendritic cells which, when differentiated, serve as migratory antigen presenting cells (APCs).

However, not all macrophages are differentiated peripheral monocytes from the adult bone marrow. Most tissue-resident, like microglia, Langerhans and Kupfer cells have migrated to the tissues already at embryonic and fetal developmental stages (Kleer et al., 2014) and might renew independently, as differentiated cells (Sieweke and Allen, 2013).

Macrophages can function in both a pro- and anti-inflammatory manner. Differentiation is orchestrated by immune related molecules and signals from the stroma (Desalegn and Pabst, 2019). The outdated view of macrophage activation, with classical, IFNγ activated M1 and alternative IL-4 activated M2 phenotype extremes is now replaced by a spectrum,

within which macrophages can have intermediate traits depending on the tissue location and signals received (Murray et al., 2014).

Anti-inflammatory activation of macrophages is crucial for homeostasis, but also for wound healing and the remodeling of tissue. Resolution of inflammation is a complex tissue microenvironment-orchestrated feedback loop leading to cessation of proinflammatory processes. An appropriate inflammatory response results in the activation of anti-inflammatory processes, in which macrophages can participate either in an active or in a passive manner. Passive regulation by macrophages support the epithelia and ECM production, while the active regulation includes secretion of anti-inflammatory lipids and chemokine cleaving proteases, thus inhibiting further neutrophil and monocyte recruitment. Resolution associated macrophages switch to anaerobic glycolysis, resulting in lactate secretion, which also is an anti-inflammatory signal. Anti-inflammatory macrophages can be identified by increased expression of for instance IL-10, TGFβ (Watanabe et al., 2019), mannose receptor (MRC-1) (Gan et al., 2018) and arginine metabolizing enzyme ARG1. However, ARG1 is involved in NO production and can be induced at lower levels also in the proinflammatory response (El Kasmi et al., 2008). As in all physiological and pathophysiological processes, a plethora of cell and molecule mediators can be associated to each process.

1.7.1. Intestinal population of monocyte/macrophage lineage cells

Even in steady state, most of the mouse adult skin and intestinal macrophages are monocyte derived, CCR2-dependent, locally differentiating and influenced by the microbiota (Bain, Calum C. et al., 2014). The macrophages are thought to remain in the tissue where they have differentiated but may transfer antigens to the dendritic cells which in turn migrate to activate adaptive immune cells in the lymph node (Mazzini et al., 2014). Several surface markers can be used to identify macrophage subsets, but a lot of overlap is seen and often an expression level approach is useful. In the steady state human intestine $CD14^{hi}$ monocytes differentiate to $CD14^{lo}$ $MHCII^{hi}$ macrophages, while in inflammatory conditions $CD14^{hi}CD11c^{hi}$ cells are suggested to accumulate, as was seen in a set of intestinal resection samples from IBD patients (Bain, C. C. et al., 2013). Intestinal macrophages divide into different populations which localize to the lamina propria, submucosa and muscularis. In the muscularis, microbiota-influenced macrophages communicate with enteric neurons and issue a role in peristalsis (Muller et

al., 2014). Fetal-derived macrophages reside also in the intestine, and are important actors promoting tolerance through IL-10 (Lavin et al., 2015).

In mice the CX3C chemokine receptor 1 (CX3CR1) surface expression levels have been seen to relate to the responsiveness and function of macrophages. $Ly6^{hi}$ monocytes differentiate to $CX3CR1^{hi}$ $Ly6^{lo}$ lamina propria-resident macrophages, which are hyporesponsive to TLR stimulation but nevertheless highly phagocytic and clear apoptotic or senescent epithelial cells as well as any breaching microbiota. These steady-state $CX3CR1^{hi}$ cells secrete prostaglandin E2 which maintains the epithelial progenitors and play a part in intestinal tolerance by inducing the expansion of activated Treg cells in the mucosa through the secretion of IL10, while also constitutively secreting physiological levels of TNFα (Bain, Calum C. and Schridde, 2018).

It is suggested that differentiation into $CX3CR1^{hi}$ macrophages is disturbed in mouse colitis: Triggering receptor 1 (TREM-1) signaling in TLR responsive $CX3CR1^{int}$ macrophages decrease secretion of IL-10 and increase secretion of pro-inflammatory IL-6, IL-12, TNFα (Weber et al., 2011), IL-23, VEGFa, and iNOS (Zigmond, Ehud et al., 2012). In inflammation, CCR2-dependent recruitment is upregulated (Desalegn and Pabst, 2019). The intestinal macrophages have the ability to induce T helper (Th) 17 cells and innate lymphoid cells 3 (ILC3) (Bain and Schridde, 2018).

1.8.Inflammatory bowel disease

Inflammatory bowel diseases, mainly Crohn´s disease (CD) and ulcerative colitis (UC), are states of mucosal inflammation residing in the small or large intestine, respectively. The highest incidence is reported in North America, UK and northern Europe, while in southern Europe and Asia the incidence is rising. The global age-standardized prevalence is 84.3/100 000 with 6.8 million overall cases in 2017. The highest age-standardized prevalence, in the higher socioeconomic population of North America, is 422/100 000 (GBD 2017 Inflammatory Bowel, Disease Collaborators, 2020).

The etiology of intestinal inflammatory disease is not entirely elucidated. It is thought to be in part genetic and in part environmental. For instance, CD is associated with NOD2 loss-of-function variants, leading to dysfunction in microbial sensing. Other polymorphisms found in CD patients involve the autophagy and unfolded protein response as well as antimicrobial peptide response. The genetic component of CD is 50%,

while in UC it is thought to be lesser with only 20% genetic impact and a greater environmental role. IBD pathophysiology involves dysfunctional immune processes and loss of epithelial integrity, with some overlap in the CD and UC inflammatory processes, but also distinct features and differences of the localization of the lesions. Whereas CD lesions are patched and can reach through the intestinal wall, the UC lesions often affect the inner lining of the rectum and distal colon continuously. The symptoms include diarrhea and abdominal pain, sometimes bloody stools as well as weight loss and fatigue.

IBD treatment includes oral short-term and long-term immunosuppressive medications, which aim to induce and maintain remission (table 4).

Table 4. Current pharmacological treatment of IBD.

Medication	Mode of action	Special features	Risks and adverse effects
5-aminosalicylates	Suppression of mucosal inflammation	Local effect	Headache, nausea, abdominal issues, rash, fever
Corticosteroids	Systemic NF-κB inhibition	Effective as a short-term treatment	Long-term use: fragility fractures, infections, venothromboembolism
Tiopurines	Unknown, systemic immunosuppression	widely used. Slow, moderate immunosuppression	Infection, hepatotoxicity, leukopenia, pancreatitis
Metotrexate	Several pathways, systemic immunosuppression	Especially effective in CD	Genotoxicity, infections
Cyclosporine	Inhibition of T cell activation (IL-2 inhibition), systemic	Fast, strong immunosuppression	Nephrotoxicity, shaking, headache, excessive hair growth, GI issues
Tofasitinib	JAK tyrosine kinase inhibitor	UC treatment	Infections, elevated cholesterol
TNFα-inhibitors	mAbs targeting either TNF or TNFR	Administered by infusions and injections	Infections, headache, GI issues, allergic reaction

The pharmaceuticals can be used in different combinations, if the previous treatment has lost efficacy or if the patient does not respond. Also, probiotic products may help, and sometimes CD can be treated using antibiotics. Interestingly, but not unexpectedly, there is a female predominance of hypersensitivity reactions induced by monoclonal TNFα inhibiting antibodies (Zelinkova et al., 2012). Future treatment options could include targeting leukocyte trafficking, IL-12, IL-23 or inhibiting cytokine production by antisense oligonucleotides (Wilhelm and Love, 2017).

1.8.1. Innate immunity and intestinal inflammation

The macrophages and dendritic cells contribute to intestinal inflammation and are suggested play a role in the pathogenesis of IBD. Antigen presenting cell secreted IL-12 is involved in Th1 induction and macrophage secreted TNFα plays a part in the

proinflammatory crosstalk with Th1 cells. Adding to the vicious cycle of inflammation, TNFα induces monocytes to secrete IL-6 and IL-1β. These proinflammatory cytokines have been identified to partake in increased Th17 differentiation (Zheng et al., 2014). Th17 cells are implicated to be involved in IBD pathophysiology (Gálvez, 2014) and IL-17 is also increased in patient samples of inflamed gut tissue (Fujino et al., 2003).

IL-6 combined with TGF-β induces Th17 secretion of IL-21 and IL-23 as a positive feedback loop (Zhou et al., 2007) and IL-17 which indirectly induces neutrophil infiltration, inflicting tissue damage (Pelletier et al., 2010) and augment further monocyte recruitment (Prame Kumar et al., 2018). TNFα and IL-21 induce tissue protease secretion, which further exacerbates the forming of intestinal lesions and induce anoikis in enterocytes (Geremia et al., 2014). Notably, ablation of Ly6^{hi} monocytes alleviate intestinal inflammation in mice (Zigmond et al., 2012).

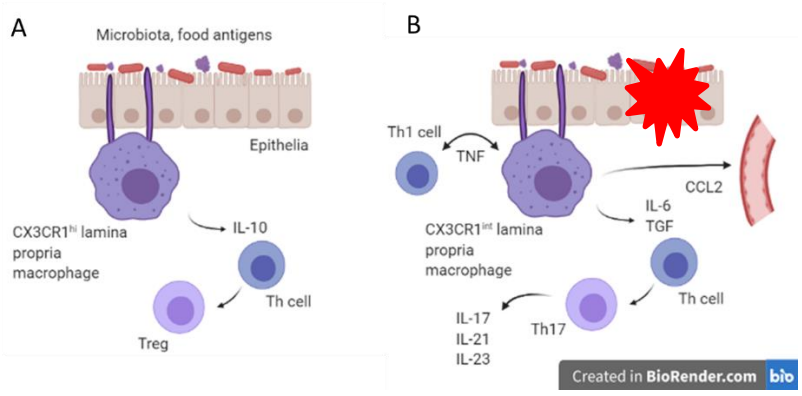


Figure 3. The normal and inflammatory function of lamina propria macrophages, simplified. A. In a normal situation, CX3CR1hi macrophages sample luminal antigens and promote tolerance through, for instance IL-10 secretion. IL-10 induces T helper cells to differentiate to regulatory T cells. B. Immune activation disturb macrophage differentiation and creates proinflammatory signaling and leukocyte influx, which in turn induces Th17 cells and activates Th1 cells ultimately leading to tissue destruction. Created with BioRender.com

TGF-β is produced by several cell types, including immune cells, stromal cells and enterocytes (Jiang et al., 2016). It has a dual role in intestinal inflammation, as it is involved in Th17 cytokine induction, but it also has an important function in sensitizing T cells to regulatory signaling (Fahlén et al., 2005). Smad7, a protein which inhibits TGF signaling has been observed to be upregulated in inflamed intestinal samples from Crohn´s patients, while the inhibition of Smad7 restored TGF-β1 - Smad3 signaling and downregulation of the inflammation (Monteleone et al., 2001). Stimulated upregulation

of TGFβ has been observed in isolated lamina propria macrophages of UC patients, but not in those of CD patients (Del Zotto et al., 2003).

1.8.2. Hormonal impact in the intestine and in IBD

Estradiol might have a direct effect on intestinal permeability and have been reported to rescue the expression of mucin 2 gene (*MUC2*) and tight junction related genes sequential to induced colitis induced downregulation (Song et al., 2018). Loss of estrogen receptor signaling is also reported to influence epithelial stem cells and promote crypt expansion via TGFβ, Wnt and Ihh mediated stromal crosstalk in transgenic mice (Hasson et al., 2014). Other studies have proposed an ERβ specific mechanism for tight junction upregulation in the colon (Braniste et al., 2009) as well as for inhibiting shedding of the epithelial cells (Wada-Hiraike et al., 2006).

Straub (2007) discussed an environmental influence on estrogen modulatory abilities and proposed that inflammatory context stromal cells induces proinflammatory signaling in macrophages. ERβ is expressed in the epithelium (Rudolph et al., 2012) and GPER in the epithelial as well as smooth muscle cells (Jacenik et al., 2019). Low amounts of cytoplasmic PGR are found in the adult male and female colon, more precisely in the epithelial cells of the crypts (Asavasupreechar et al., 2020). The rapid acting progesterone membrane receptors have been found in neuroendocrine tissues, as well as in both cell line and primary murine macrophages (Dressing, Gwen E. et al., 2011).

Epidemiological studies of Western populations have showed higher prevalence of CD in females and UC in males, but with a female predominance in some specific forms of UC and an observation of a divergent pattern in Asian populations, where both IBD forms seem to be more common in males (Goodman et al., 2020). Ovarian hormone influenced changes in disease status have been reported in IBD patients and IBD can sometimes influence ovarian hormone regulated processes. For instance, premenstrual and menstrual (i.e. low estrogen status) exacerbation of CD symptoms has been reported, but also menstrual abnormalities (Bharadwaj et al., 2015) and infertility in CD, but not UC patients.

Interestingly, flares are increased in pregnancies conceived during a period of aggravated disease, but not in those conceived at remission, although the sequential cessation of

medication can produce some of the flaring. High estrogen status induced by endometriosis or oral contraceptives, but not hormone replacement therapy has been associated with increased IBD risk in susceptible individuals (Goodman et al., 2020).

1.9. Hormone receptor mediated signaling and inflammatory response

ERs have previously been reported to be expressed in several kinds of immune cells, both adaptive and innate (Khan and Ansar Ahmed, 2016). Some variation between cell type and species have been observed (Kovats, 2015). The nature of both the immune system and of the ER and PGR signaling is incredibly complex. Both processes are strongly influenced by the environment and by the age and health of the individual. Additionally, the ovarian hormone induced effects are tissue specific. Coming together, experimental results of ovarian hormone modulatory effect on the immune response are to a great extent contradictory.

1.9.1. Estrogen receptor signaling and inflammation

In THP1, the leukemic monocyte cell line originating from a 3-year old child, expression of ERα36, ERα66, ERβ and GPER1 have been reported (Pelekanou et al., 2016). According to our previous research, both ERα and ERβ mRNA is found in THP1 and human male CD14+ monocytes *ex vivo*. Further, the expression of GPER was substantially higher in THP1 than in CD14+ monocytes, while ERα was predominant in the CD14+ cells disregarding activation status (Polari et al., 2018).

Both full length ERα66 and AF-1 truncated ERα46 expression have been observed in human female CD14+ monocytes and macrophages, along with a macrophage differentiation associated increase in ERα46 simultaneously with a decrease in ERβ expression. Macrophages, but not monocytes, responded to E2 stimulation by upregulating ERα expression through promotor F activity (Murphy et al., 2009). However, in a more recent study the ERα36 isoform as well as GPER1 are suggested to be the only ERs expressed in human monocytes of both adult males and females, and that the previous findings of ERα66 and ERα46 expression would be accounted to differences in cell populations, antibody cross-reactivity and translatory issues (Pelekanou et al., 2016). The expression was observed to be stable throughout the menstrual cycle and monocyte differentiation.

A significant number of survival-, immune- and metabolic process related genes, as well as genes related to stress response and protein folding was differentially expressed after short E2 treatment in murine macrophages *ex vivo* (figure 4) (Pepe et al., 2017)

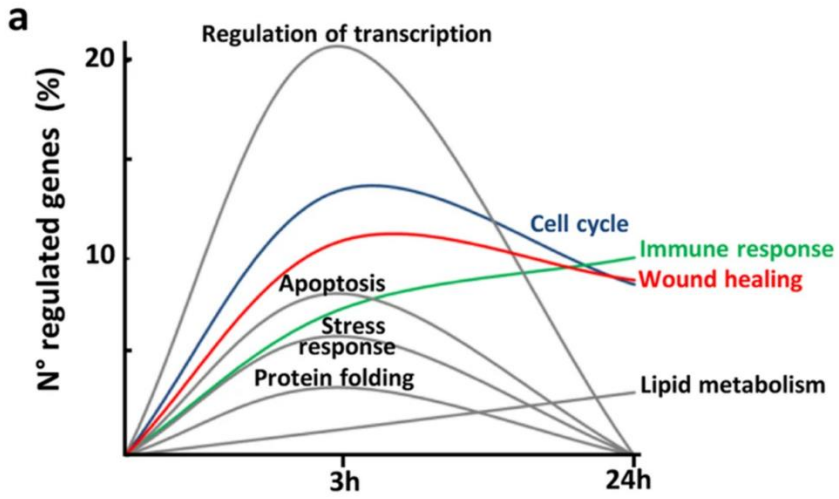


Figure 4. Differentially expressed genes after short E2 treatment of murine peritoneal monocytes in metestrus phase. E2 regulated transcription during 24h shows differentially regulated genes related to the immune response, metabolism and cell survival, as well as stress response and protein folding. Image from Pepe et al (2017).

There are varied gender differences in the pro- and anti-inflammatory response pattern of human monocytes and macrophages. A large meta-analysis of the *ex vivo* monocyte inflammatory response from 15 study populations, conducted by Beenakker et al (2020), showed a higher monocyte count in men and a slight increase in LPS stimulated IL-10 per *ex vivo* monocyte from adult females, independent of age, health status and geographical location. The study also presented slight but persistently elevated TNFα, IL-1β, IL-6 and IL-12 in stimulated male monocytes compared to increased GM-CSF and IFNγ levels in female monocytes, although the difference diminished after normalization to cell counts (Beenakker et al., 2020).

Hormonal changes in menstrual cycle are associated with increased monocyte secretion of IL-1β, IL-6 and TNFα in healthy females, most pronounced during menstruation but already seen in the luteal phase of the ovulatory cycle (Willis et al., 2003). Cycle phases and associated hormone levels described in figure 5 (Hong and Choi, 2018). An increase in serum E2 was found to associate with increased neutrophil and monocyte count in subfertile women in treatment to induce ovulation. With the increase in numbers, a decrease in proinflammatory as well as an increase in anti-inflammatory surface markers was observed (Habib et al., 2018). In female PBMC derived macrophages TNFα, IL-1β

and IL-10 intracellular levels were reported to decrease by E2 modulation of LPS response. Further, markers of alternative activation in menopausal female PBMC derived macrophages *ex vivo* were found to decrease after IL-4/IL-13 stimulation in comparison with those from premenopausal females, while IFNγ/LPS treated macrophage responses were comparable (Toniolo et al., 2015).

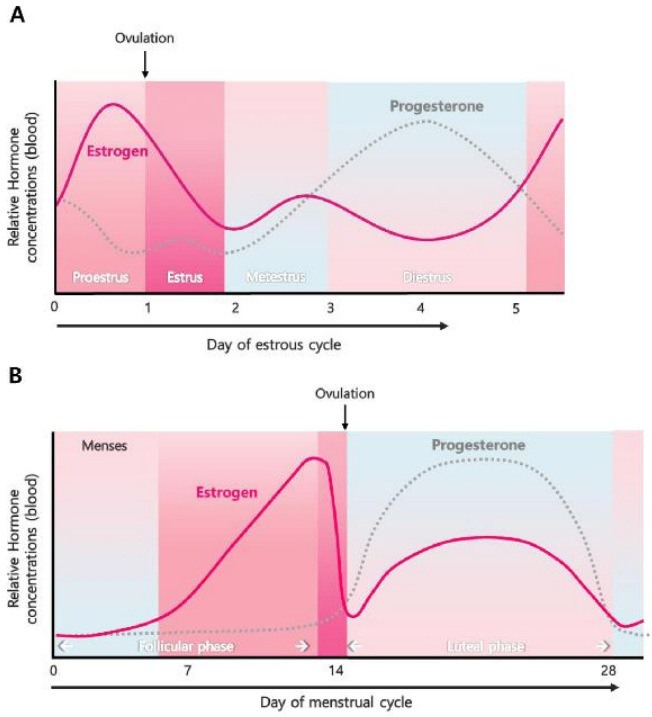


Figure 5. Cycle hormone concentrations in blood. The relative estrogen (red line) and progesterone (dashed line) concentrations in blood during A. estrus cycle of female mice and B. menstrual cycle of human females. The concentration of estrogen increases before ovulation after which progesterone concentration in turn increases. Picture from Hong et al (2018).

Without the impact of the surrounding tissue, i.e. in *in vitro* or *ex vivo* experiments, monocytes transiently treated with ovarian hormones often respond in an anti-inflammatory manner. Estradiol (100nM, 24 h) lowered both IL-6 and TNFα expression after LPS stimulation (110 ng/ml, 3 h) in human *ex vivo* $CD14^+$ monocytes, while fulvestrant inhibited the E2 effect (Pelekanou et al., 2016). In another experiment, E2 (100 nM, 24 h) modulated LPS (10 ng/ml, 12 h) response and inhibited IL-8 secretion to media after 72 h, with a paralleled ERα46 increase (Murphy et al., 2009). In RAW 264.7 murine macrophage cell line ERα activation have been suggested to amplify the IL-4 induced resolution of the inflammatory response through regulation of STAT3 and SOCS3 pathways, leading to increased *Arg1* and *Il10* expression (Villa et al., 2015). E2 also prevented LPS induced morphologic changes associated to activation in RAW 264.7 macrophages (Vegeto et al., 2004). In a review on estrogen effects on immune processes, low level E2 was concluded to induce an increase in IL-1β secretion of monocyte/macrophage lineage cells, while high E2 inhibited it (Straub, 2007).

1.9.2. Rodent studies of ERα modulated inflammation

Animal studies on modulating inflammation via ERα signaling are very varied in setting and display an unfortunately broad range of results. The complex nature of both the immune system as well as ER signaling and regulation, together with species specific differences, may also contribute to some of the variation. Except the E2 dose, length of administration may affect the results. In some cases, E2 seems to have an ability to aggravate innate inflammation, but it does not cause inflammation in healthy tissue. ERα signaling might be stronger associated with aggravated colitis.

A modest decrease of IL-1β and iNOS mRNA induced by short-time E2 treatment, while long-term administration led to proinflammatory response in *ex vivo* stimulated peritoneal macrophages collected without eliciting. The authors suggested that the seemingly contradicting, beneficial effects of higher long-term estrogen concentrations seen in MS and experimental autoimmune encephalomyelitis were related to surrounding tissue responding to E2 in an anti-inflammatory manner (Calippe et al., 2008).

Lambert et al (2004) had previously reported that the TGC-elicited peritoneal macrophages of ERα deficient mice secreted higher amounts of TNFα and that E2 had no effect on TNFα secretion. Similarly, bone marrow derived macrophages had not exhibited any differences, neither had ERβ deficient peritoneal macrophages (Lambert et al., 2004). A more recent study by Pepe et al (2017) added to the evidence of short-term, endogenous surge-mimicking E2 immunomodulatory effects in *ex vivo* murine bone-marrow and peritoneal macrophages. This was observed also *in vivo* after repeated E2 5 µg/kg s.c. injections during 36 or 60h. Both the *in* and *ex vivo* models exhibited an increase in anti-inflammatory markers, such as Arg1 (Pepe et al., 2017).

In later research, Calippe et al (2010) combined *in vivo* treatment using estrus or early pregnancy levels of estrogen in ovariectomized mice with *ex vivo* LPS stimulation of thioglycolate (TGC)-elicited peritoneal macrophages and reported inflammatory effects. E2 decreased PI3K-Akt mediated inhibition of Toll-like receptor (TLR) 4 downstream signaling, which increased induced nitrogen oxide synthase (iNOS) and proinflammatory cytokines IL-1β, IL-6 and TNFα. The same proinflammatory cytokines were downregulated in OVX animals without E2 treatment when compared to intact animal cells *ex vivo*, at both basal and LPS stimulated conditions. Further results from mice with a conditional ERα knockout (KO) were presented, confirming that the effect was indeed

ERα mediated. The authors speculated that the inflammatory effect of ERα was due to chronic administration and referred to their earlier research where only one dose of E2 was used, leading to an anti-inflammatory result. They did not offer any extensive discussion on the inflammatory context here, although eliciting macrophages with TGC causes sterile peritonitis. However, in this model, E2 treatment reduced the number of elicited macrophages (Calippe et al., 2010).

In *in vivo* experiments conducted in inflammatory disease models, researchers have reported that the disease activity as well as proinflammatory signaling can be both exaggerated or alleviated by estradiol or ERα activation. In intact female mice, induced disease models of adaptive and innate mediated colitis were observed to respond differently to E2. Th1 driven dinitrobenzene sulfonic acid induced colitis was alleviated, but not abolished, by supraphysiological estrogen administration, while in dextran sulphate sodium (DSS)-induced colitis which induces an innate response, E2 was seen to aggravate tissue destruction and increase TNFα secretion both in M-CSF ablated and in wildtype mice. This introduced the question whether the effect was macrophage mediated at all. However, monocytes and tissue resident macrophages should still be present in the colon of M-CSF-KO mice. The effects of E2 were eliminated by simultaneous administration of tamoxifen. In mice without induced intestinal inflammation, E2 did not cause any inflammatory changes. Based on the results, the author suggested a genetic component in the varied hormonal response of IBD patients, involving for instance E2 sensitization to bacterial products (Verdú et al., 2002).

Another Th1-driven colitis model exhibited alleviated disease in E2 treated OVX mice after a 90-day release of supraphysiological levels. However, specific ERα agonist treated OVX mice exhibited slightly aggravated disease after ERα stimulation, while ERβ stimulated group had less animals with severe disease. Intact, untreated ERα-KO mice had a lower incidence of severe disease, while ERβ-KO mice did not differ from control animals. Further ER-KO/adoptive transfer-experiments indicated ERα deficiency in non-$CD4^+$ cells was responsible for aggravated disease (Cook et al., 2014).

Administering a lower, but still supra-physiological dose of E2 to OVX animals and a reduced DSS-challenge had a beneficial effect on histological markers of inflammation as well as disease activity. This was seen in both intact and OVX E2 treated female mice while male mice were more susceptible to inflammation. The mice were allowed to

recover before sacrifice, and female mice showed improved recovery measured by regaining of body weight (Bábíčková et al., 2015). induced colitis, which resemble CD by histopathological features, was observed to be exacerbated if first administered during the luteal phase, compared to follicular phase in rats. In the same set of experiments, a DSS-induced colitis model in OVX rats E2, but not progesterone, produced suppression of inflammatory infiltration and downregulation of proinflammatory macrophage inhibitory factor (MIF), myeloperoxidase and IL-1β. Anti-MIF treatment sequentially decreased TNFα (Houdeau et al., 2007). The previously described studies administered E2 as a *subcutaneous* pellet enabling constant release for an extended time.

An HLA-B27 transgenic rat model has shown beneficial effects of E2 (10 μg/kg) on MAST cell and neutrophil inflammatory markers as well as histological and disease activity features, using only 5 days *p.o.* administration. Simultaneous administration of fulvestrant reversed the effect, which the authors interpreted as indication of an ERα specific activity (Harnish et al., 2004).

1.9.3. SERM effect on inflammation

SERMs have been suggested to modulate inflammatory response of several types of immune cells. For instance, RAL and tamoxifen affected murine dendritic cells, altering the antigen presenting cells towards a less inflammatory phenotype (Nalbandian et al., 2005). In another study, SERMs induced an anti-inflammatory effect on rat microglia (Smith et al., 2011). RAL has been reported to dose-dependently induce the anti-inflammatory enzyme hemeoxygenase-1, through an ER independent mechanism in RAW264.7 cells. Hemeoxygenase-1 product CO is suggested to inhibit iNOS. RAL was seen to directly increase xanthine oxidase activity, which was associated to an increase in intracellular ROS. ROS increase induced phosphorylation of p38 and CREB, resulting in hemeoxygenase-1 expression (Lee et al., 2011).

In our previously published work, SERM2 and RAL downregulated the TNFα induced activity of NF-κB in THP1 cells but upregulated the LPS induced response in contrast to E2. E2 had a similar effect on both LPS and TNFα stimulated cells. SERM2 increased surface marker expression of MRC-1 and CD163 scavenger receptor in a $CD14^+$ human male *ex vivo* monocyte population differentiated by IFNγ and activated by LPS. Significant changes in cytokine expression were not found, except for a slight increasing effect on IL-10 by E2, SERM2 and RAL. However, SERM2 and RAL reduced the

proliferation rate of T cells in co-culture with donor matched IFNγ and LPS stimulated $CD14^+$ monocytes (Polari et al., 2018).

In another study by Polari et al (2019) SERM2 alleviated DSS induced colitis in male mice, measured by histological analysis of erosion, edema, crypt loss and hyperproliferation as well as the staining of anti-inflammatory $Mrc\text{-}1^+$ monocyte/macrophages matched to observed disease activity scoring. *Il10* expression was seen to increase in the colon of SERM2 treated healthy mice, but interestingly not in those with induced colitis (Polari et al., 2019) possibly reflecting the environment effect of ovarian hormones on immune processes. However, the precise molecular mechanisms of SERM2 colitis alleviating effect is still unclear, and this thesis project is carried out in hope to further illuminate the role of ERα and PGR. Polari speculates, based on increased *Pgr* expression after exposure to SERM2, that the novel compound would function as an ERα agonist in this experiment but also addresses the possibility of PGR activation.

RAL (*p.o.*, 5 mg/kg) has been indicated to alleviate inflammation in DSS-induced colitis using female Balb/c mice, measured by disease activity, histological features and serum IL-6 and TNFα. Styrene maleic acid encapsulated micellar RAL was observed to further improve the effect, due to increased bioavailability of the lipophilic drug. The author also suggested the limited distribution of the micellar drug could be used as a means to limit adverse effects (Greish et al., 2017).

1.9.4. Progesterone receptor signaling and inflammation

As with estrogens, the immunomodulating effect of progesterone is not well understood. As there are many factors which influence effects, the results in the literature presented here are in part contradictory. It is clear, however, that both innate and adaptive immune cells respond to progesterone receptor signaling and immunological processes play a part in pregnancy. Monocyte inflammatory responses *ex vivo* have been reported to increase in the first trimester and then to decrease compared to non-pregnant females and was associated to higher hCG levels (Ziegler et al., 2018). Progesterone binds to the glucocorticoid receptor and can exert some anti-inflammatory effect through that pathway (Attardi et al., 2007). Levonorgestrel (LVN), used in the experiments here is PGR specific and has greater affinity to PGR than progesterone (Sitruk-Ware, 2006).

In mice, progesterone reduced matrix-metalloproteinase 9 (MMP-9) in cervical neutrophils and monocytes as well as decreased systemic IL-1β (Furcron et al., 2015). MMP-9 is increased in the inflamed tissue of IBD patients and animal models of colitis, and it induces intestinal permeability through tight junction loss (Al-Sadi et al., 2019) and can facilitate the release of TNFα (Yabluchanskiy et al., 2013). Besides the direct effects on immune cells, progesterone signaling to surrounding cells can influence inflammatory status. Specific knockout of osteoprogenitor PGR aggravated synovial inflammation in both male and female mice (Liu, L. et al., 2020). Progesterone decreased LPS stimulated NO production and arginase along with *Mrc1* and *Il23* mRNA expression activity induced murine bone marrow derived macrophages (Menzies et al., 2011).

Progesterone has been attributed anti-inflammatory effects, but also proinflammatory features, possibly depending on the type of signaling. PGR A to B signaling ratio may upregulate proinflammatory proteins, such as cyclooxygenase (COX) 2 and NF-κB in labor (Shah et al., 2019). Progesterone has been reported to increase survival and augment clearance of intestinal parasite infection in mice (Escobedo et al., 2011). In OVX rats progesterone increased inflammation induced by trinitrobenzene sulfonic acid, measured by myeloperoxidase activity (Houdeau et al., 2007). In human U937 monocyte cell line progesterone treatment was observed to moderately decrease IL-6, while it clearly increased TNFα secretion (Jain et al., 2004).

1.10. Integration of the hormone induced inflammation modulating pathways

Already established therapeutics of IBD, such as glucocorticoids, anti-TNFα recombinant proteins and aminosalicylates, function at least partially by downregulation of NF-κB activity. This chapter presents the NF-κB inhibitory potential of ovarian hormone receptor mediated signaling. Crosstalk between ER and NF-κB was been reported by Boyce et al already in the 1990´s, when the loss of estrogen mediated NF-κB inhibition in osteoclasts was presented to play a part in the menopausal exacerbation of bone resorption, via the secretion of IL-6 (Boyce et al., 1999).

NF-κB is a widely expressed protein complex involved in the development and support of especially immune cells, but its activation induces proinflammatory response also in for instance epithelial cells. Persistent activity of these regulatory proteins is linked to many diseases, including inflammatory and autoimmune conditions as well as cancer.

The NF-κB response can be initiated through several pathways, such as pathogen-associated molecular pattern activated TLR through MyD88 signaling or through different cytokine receptors, such as TNFR and as such collects the signals from various different immunogenic pathways. The κB sites regulate the expression of chemokines, adhesion molecules and various cytokines such as IL-1β, TNFα and IL-6. NF-κB also orchestrates the expression of genes with products contributing to proliferation and cell survival (Kalaitzidis and Gilmore, 2005). NF-κB activation is described in figure 6.

The inhibitory effect of ovarian hormones on NF-κB can be executed both in the cytoplasm and the nucleus. It is feasible, that the interactions vary between cell types and tissues. The reported cytoplasmic mechanisms include inhibitory κB kinase (IKK) downregulation (Lasarte et al., 2013a), inhibitory κB (IκB) upregulation (Xing et al., 2012) or a microtubular effect (Ghisletti et al., 2005). In the nucleus, ER activation is suggested to block DNA binding (Galien and Garcia, 1997a), compete for co-activator proteins (Edith et al., 2000), displace coactivators in the proinflammatory transcriptional complex (Nettles et al., 2008), downregulate expression of *Nemo* at estrus levels (10 nM) (Lasarte et al., 2013b) and repress the transcription function of bound NF-κB (Galien and Garcia, 1997b). The ligand-binding domain, the hinge region (Ray et al., 1997) and the DNA-binding domain of ER have been associated with this interaction and in NF-κB the Rel homology domain is involved in forming the interaction (Stein and Yang, 1995).

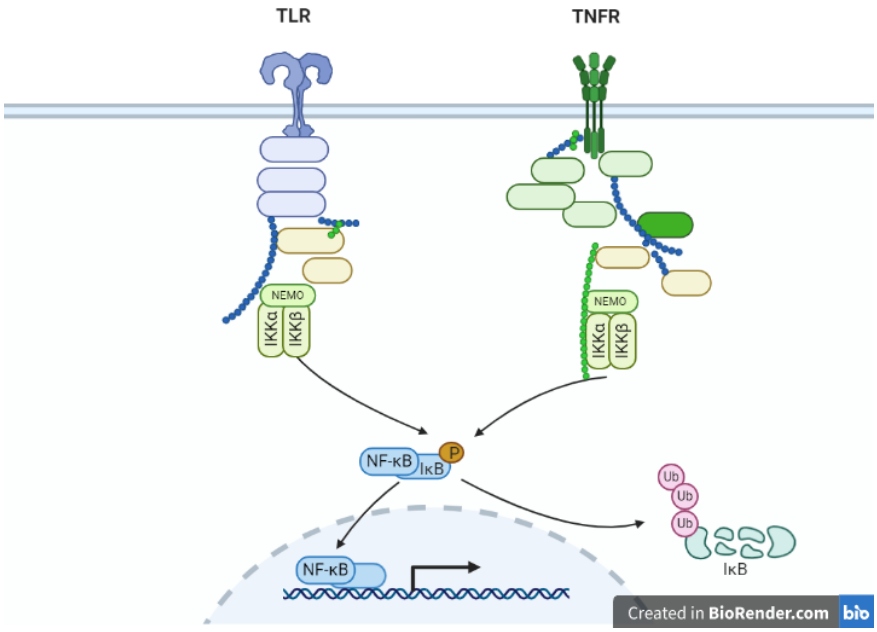


Figure 6. NF-κB activation upon immunogenic stimuli. NF-κB consists of dimerizing proteins, RelA (p65), c-Rel and RelB, p100/p52 and p105/p50 which function as transcription factors. These Rel homology domain containing proteins are regulated by IκB family of proteins, which inhibit the DNA-binding activity of the Rel homology domain. The response can progress through the canonical and non-canonical pathways involving varied sources of stimulation and different compositions of IKK´s and NF-κB dimers. In the canonical pathway of activation, a broad range of immunogenic stimuli can activate the IκB kinase complex(IKK) including, amongst other proteins, IKKα, IKKβ and regulatory subunit NEMO. This leads to phosphorylation and degradation of binding IκB, releasing the cytoplasmic NF-κB dimers and enables nucleic translocation and binding to genomic κB sites (Kalaitzidis and Gilmore, 2005). Created with BioRender.com.

In an *ex vivo* experiment performed with human peripheral monocytes, activated ERα36 was suggested to interact with RelA after LPS stimulation and inhibit NF-κB induced transcription in the nucleus, and GPER1 to coregulate the process via direct interaction with the ERα36-RelA complex before nuclear translocation (Pelekanou et al., 2016). In human peripheral macrophages estradiol has been proposed to regulate NF-κB activation through inhibiting miRNA inhibition of κB-Ras2 in a hormone stripped environment (Murphy et al., 2010). Estradiol have been suggested to suppress JNK1/2 induced IKK activity and IκB degradation in cardiomyocytes (Vegeto et al., 2004) or to prevent nuclear translocation of RelA via ERα-PI3K pathway without modifying IκB degradation in TNFα or LPS stimulated RAW 264.7 macrophages and microglia, but not in MCF-7 or SK-ER3 cells.

PGR signaling is also suggested to play part in the inhibition of the NF-κB pathway. Pregnancy level progesterone treatment (4h) inhibited NF-κB activity and decreased iNOS and IL-6 expression in RAW 264.7 macrophages. Additionally, progesterone upregulated suppressor of cytokine signaling proteins (Su et al., 2009).

SERMs tamoxifen, RAL and fulvestrant have been observed to antagonize the effect of E2 on nuclear NF-κB translocation in RAW264.7 macrophages (Ghisletti et al., 2005). However, inhibition of the PI3K pathway and sequential increase of NF-κB transcriptional activity have also been observed, sequential to chronic administration of estrogen *in vivo* in elicited intraperitoneal macrophages, stimulated *ex vivo* with LPS (Calippe et al., 2008). The interaction can also regulate the ER, as observations of NF-κB – ERα crosstalk have been made in, for instance, breast cancer (Frasor et al., 2015). In non-immune cells, unliganded ERα, along with NF-κB dimers have been suggested to function in the TNFα autoinducing transcription complex, and that E2-bound receptor would recruit transcriptional repressors, inhibiting TNFα expression (Cvoro et al., 2006).

1.11. Summary

In conclusion, inflammatory disease affects an increasing number of people. While there are many current forms of treatment, a well-tolerated immune modulatory treatment could benefit IBD patients. The hormonal influence on inflammatory disease is probable, but the mechanism is unclear because of contradictory results. Most *in vitro* experiments indicate an anti-inflammatory effect of E2 and RAL, while the results of *ex vitro* and *in vivo* studies are conflicting. Negative NF-κB crosstalk have by many been suggested to mediate the anti-inflammatory effects of receptor signaling *in vitro*, although proinflammatory, positive crosstalk could also be the case. Signals from the surrounding tissue as well as intracellular receptor type or coactivator levels are possible determining factors when defining the inflammatory capacity of monocytes and macrophages *in vivo*. In this thesis project, monocyte and macrophage inflammatory responses were modelled by THP1 NF-κB reporter monocytes and CD14+ primary monocytes *in vitro*. Monocyte cell lines, such as THP1, can be used as an *in vitro* model for monocyte and macrophage function, especially when paralleled by *in vivo* studies to draw more definite conclusions (Chanput et al., 2014). THP1-Lucia stably express a NF-κB-inducible Luc construct and the product luciferase is secreted into cell culture supernatant (figure 7).

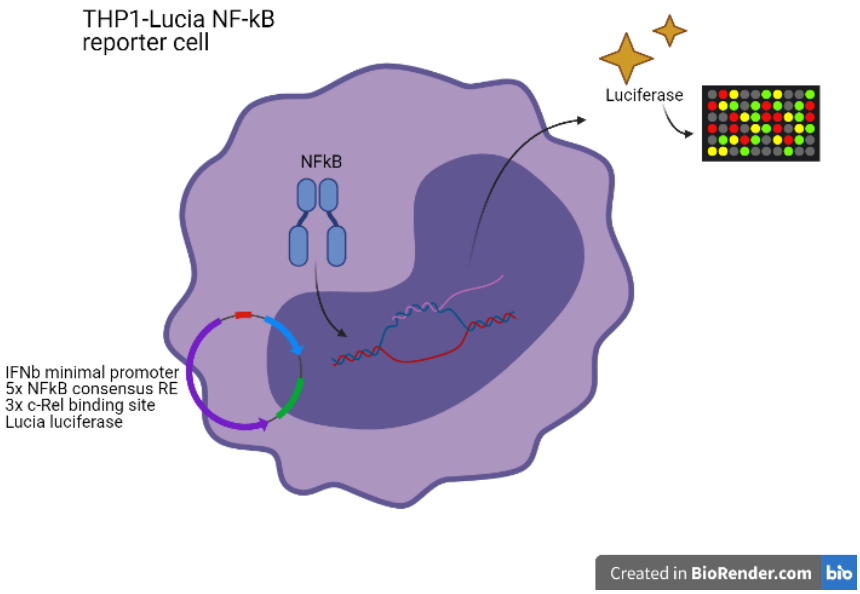


Figure 7. The THP1-Lucia NF-κB reporter cell line. The THP1-Lucia cells are stably transfected with a Lucia luciferase, INFβ minimal promoter together with several copies of both the c-Rel binding site and NF-κB response elements. NF-κB activity induces secretion of luciferase to culture media, which is the sampled and provides a quantifiable luminescent signal after the appropriate substrate is added. Image created with Biorender.com.

2. Results

2.1. No ligand induced cytotoxicity in THP1 cells

Neither SERM2, LVN or mifepristone (MFP) exhibited cytotoxic effect at 1 nM to 3 µM concentrations when measured using fluorescence-based viability assay (figure 8).

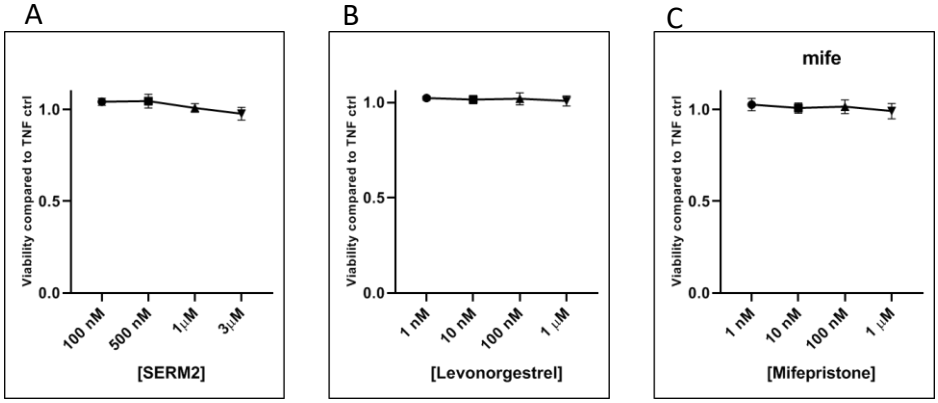


Figure 8. No cytotoxic effects of ER and PGR ligands. Viability assay of A. SERM2, B. LVN and C. MFP treated cells, exhibiting no toxic effect in TNFα stimulated THP1-Lucia cells (10^6 cells/ml). Viability was analysed using a fluorometric assay and results were normalised to the viability of TNFα

2.2. PGR ligands modulated the NF-κB activity of THP1 cells

NF-κB activity assay performed using THP1-Lucia reporter cells showed a statistically significant reduction of activation in LVN treated cells (P=0.0008), compared to TNFα control. SERM2 together with LVN altered NF-κB activation slightly less, but still significantly (P=0.0135) (fig 3a). E2, MFP or SERMs SERM2, ICI and RAL did not alter NF-κB activation in a statistically significant manner in this assay. (fig. 9b-c). In this system, SERM2 did not function as an agonist at PGR.

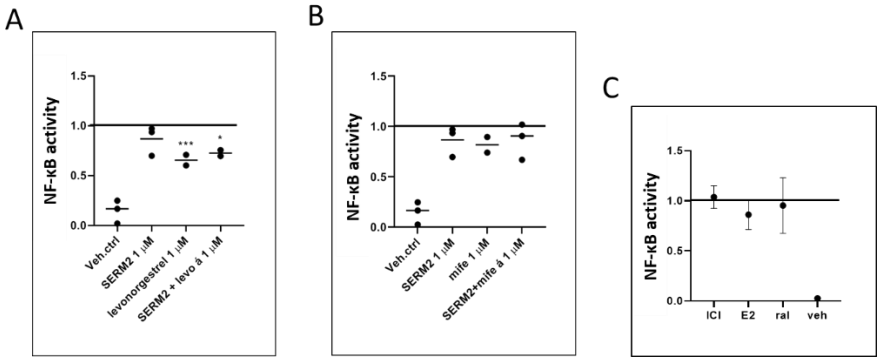


Figure 9. LVN induced modulation of NF-κB activity in THP1-Lucia cells. A. LVN downward modulated NF-κB activity in THP1 cells (10^6 cells/ml) after 48h ligand incubation followed by immune activation by TNFα, while B. SERM2 and MFP or C. ICI, E2 or RAL did not induce statistically significant changes. NF-κB activity was measured by reporter-system from culture media and luminescence was related to viability and subsequently normalized to TNF control. Analysis of variance was performed using Kruskal-Wallis test followed by Dunn´s test for multiple comparisons.

2.3. Transfection upregulated receptor levels, but a siRNA silencing effect was observed on both mRNA and protein levels

The siRNA silencing of ERα and PGR was verified by RT-qPCR and Western blots. The transfection induced an increase in the mRNA levels of *ESR1*. Nonetheless, expression was decreased compared to negative control siRNA treated cells at 48h after transfection, but not after 72h. However, residual receptor mRNA was present in silenced cells at similar levels of the untreated cells (fig 10a). Treating cells with *PGR* siRNA did not alter the expression of *ESR1* (fig 10b). *PGR* expression was also upregulated by the transfection, seen also in the transfection reagent control (HiP24, fig 10c) and at both negative siRNA timepoints. *PGR* mRNA was downregulated compared to negative control cells at both 24h and 48h post-transfection and again, siRNA knockdown rendered the mRNA levels to a similar to that of the untreated cells (fig 10c). The protein level differences of ERα (fig 10d) and PGR (fig 10e) were depicted by Western blot and sequential image-based analysis of bands. Modest differences of ERα (fig 10f) and PGR (fig 10g) protein levels could be seen 24h after transfection. The siRNA verification by qPCR and WB indicated unexpected effects and these results indicated that the response of the transfected cells could not be interpreted as that of a knockdown model. Instead

only negative siRNA treated, receptor upregulated cells could be compared to receptor siRNA treated, especially in the case of ERα. Still, as the results were normalized to the negative control siRNA transfected THP1 monocytes it was probable that the data could provide some indications on the nature of the ligand induced modulation of the inflammatory response.

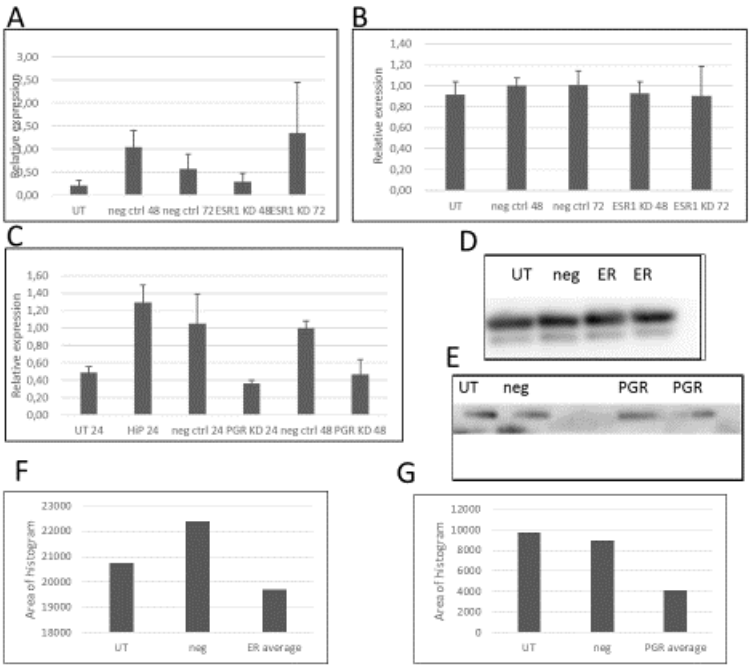


Figure 10. RT-qPCR and WB verification of siRNA silencing of ERα and PGR after 24, 48 or 72 hrs. Relative expression of A. *ESR1* 48 and 72 h after *ESR1* siRNA treatment, showing an increase of ESR1 mRNA levels in negative control siRNA at both 48 and 72 h, compared to untreated THP1 cells B. unchanged *ESR1* relative expression 48 and 72 h after *PGR* siRNA treatment and C. knockdown of *PGR* 24 and 48 h after *PGR* siRNA treatment, with controls for transfection reagent (HiP) and a mock transfection of a scramble siRNA (neg ctrl). D. ERα and E. PGR protein Western blots (24h after transfection) with F. quantification of ERα and G. PGR bands, showing a silencing effect on receptor protein levels compared to negative siRNA treated cells. All lanes contain equal amounts total protein (50 µg). Bands quantified using histogram areal measurements in ImageJ software.

2.4 Decreased basal NF-κB activity by ligand modulation of receptor upregulated cells

Measurements indicated statistically significant decrease in basal NF-κB activation induced by receptor ligands in the receptor upregulated cells. Without any immunogenic activation, both *ESR1* and *PGR* siRNA treated cells exhibited significant elevation in baseline NF-κB activity by ligand modulation. In the *ESR1* siRNA treated cells SERM2

and LVN increased NF-κB activity (P<0.0001 and P=0.0004 respectively). Further, in cells with increased receptor capacity SERM2 and E2 treatment differed in response (P=0.0049) while in the knockdown cells SERM2 induced more NF-κB activity compared to RAL (P=0.0124) (fig 11a), possibly reflecting SERM2 partial agonism at ERα and its activity at PGR.

In the *PGR* siRNA treated THP1 cells additional baseline elevation of NF-κB activity could be seen in SERM2 (P=0.0322), RAL (P=0.0007) and LVN (P=0.0008) treatment. Further, the change in activity was greater when compared to control, than in the *ESR1* siRNA treated cells. SERM2 and RAL response was not identical here either (P=0.0326) as RAL and SERM2 both function as a partial agonist at ERα, but only SERM2 at PGR (fig 11b).

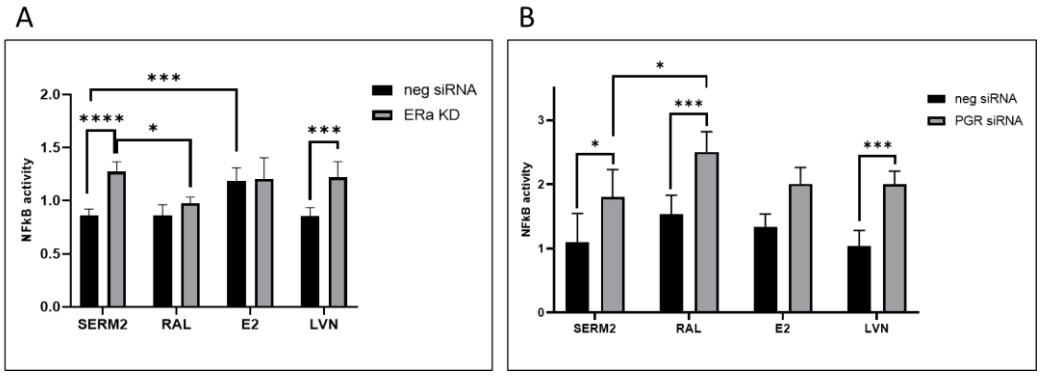


Figure 11. Baseline measurements of NF-κB activation in *ESR1* and *PGR* siRNA treated cells: no TNFα stimulation. With 24 h ligand treatment, but without immunogenic stimulation, A. ERα reduced, SERM2 or LVN treated cells showed an increase in NF-κB activation compared to receptor upregulated negative control, while B. PGR deficiency resulted in a significant increase of NF-κB in SERM2, RAL and LVN treated groups. Statistical analysis by ordinary two-way ANOVA followed by Sidak´s test for

Then immunogenic stimulation was added to the experiment. The level of NF-κB activation of untreated THP1 cells with, or without TNFα stimulation was compared to the level of the negative control siRNA treated cells and found to be slightly lower (fig 12a). The negative siRNA+TNFα treated control is indicated by horizontal line in the graphs.

NF-κB activity was then measured in siRNA treated cells to observe the modulatory effects of receptor ligands on TNFα immunogenic stimulus. In the negative siRNA treated cells, i.e. cells with increased receptor capacity, a downward trend could be seen by all modulatory treatment, compared to negative siRNA TNF control (fig 12b). In the *ESR1*

siRNA treated cells E2, LVN and MFP modulation decreased NF-κB activity significantly (P=0.0449, P=0.0012, P=0.0212, respectively) (fig 12c), indicating a pro-inflammatory effect of upregulated ERα in this system.

PGR siRNA treatment seemed to induce a trend of increasing NF-κB activation, seen by elevated TNFα control activity and which was strengthened by SERM2 treatment (fig 12d). SERM2 and RAL exerted similar effects both in the negative control siRNA treated cells (fig 12b) as in the *ESR1* silenced cells (fig 12c). As expected, and providing a hint that the silencing effect was sufficient, LVN did not affect the response of the *PGR* silenced cells statistically significantly. Also, SERM2 was not comparable to neither full agonists in this system and markedly, but not statistically significantly increased NF-κB activity in *PGR* siRNA treated cells (fig 12d).

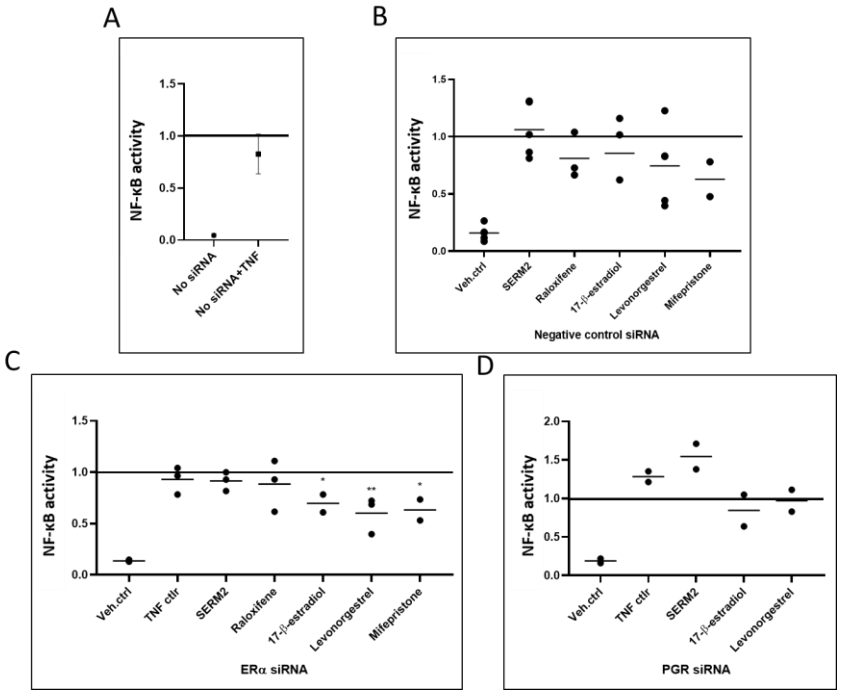


Figure 12. Pooled data of NF-κB activity assays, showing individual experiments and mean. A. siRNA treatment slightly increased the response to TNFα stimulation. B. No significant change in TNFα induced NF-κB activation in SERM, E2 or LVN treated (24 h), negative control siRNA transfected cells, but a downward trend by RAL, E2, LVN and MFP treatment. C. ERα silenced cells showing a slight but significant reduction by E2 as well as PGR ligands, but not by SERM treatment. D. PGR silenced cells with no statistically significant changes, although an average 50% increase in NF-κB activity was seen in SERM2 treated cells. All results were normalized to negative control siRNA treated TNFα control (black line). Analysis of variance was performed using Kruskal-Wallis test followed by Dunn´s test for multiple comparisons.

When the NF-κB activity data from receptor knockdown cells (fig 12) was analyzed for ligand induced differences in NF-κB activation no significant effect could be found in the *ESR1* silenced cells (fig 13a). In the PGR knockdown experiment, however, SERM2 and LVN modulated cells responded with elevated NF-κB activation compared to receptor increased negative control, when stimulated with TNFα (fig 13b), although only SERM2 significantly (P=0.0009).

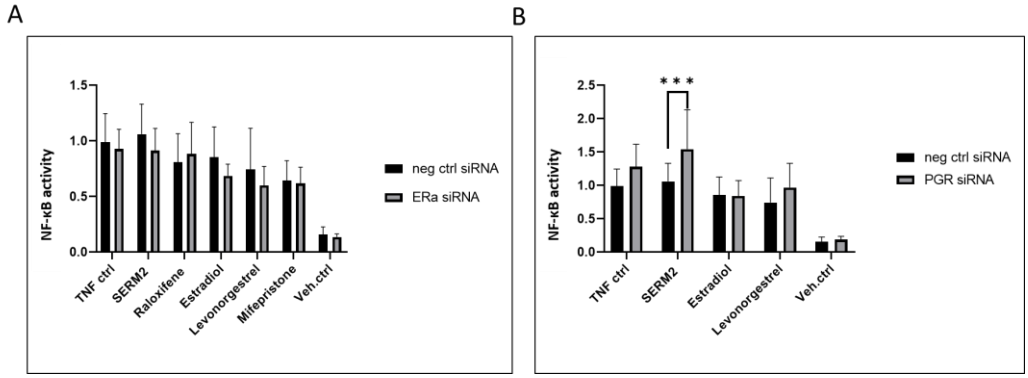


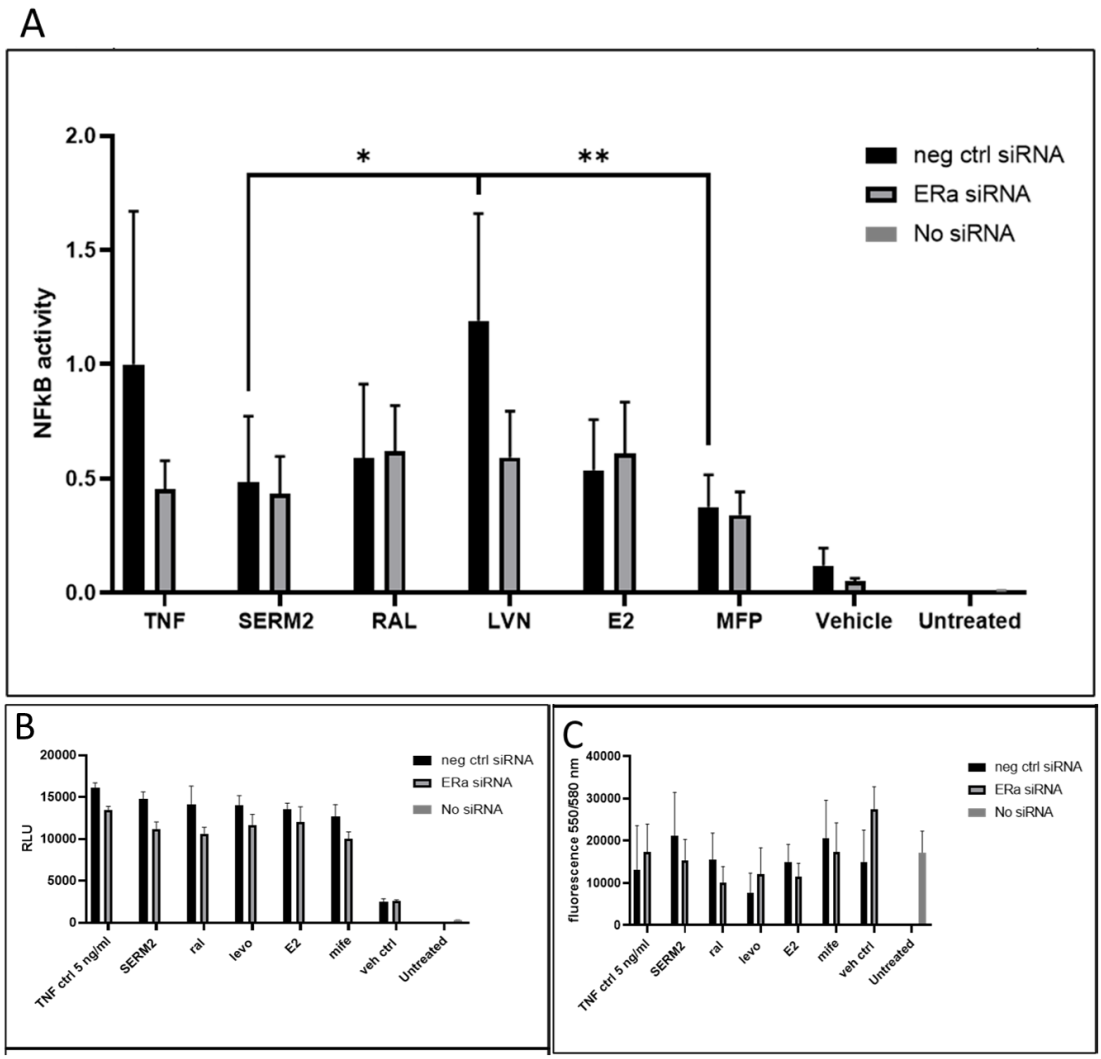
Figure 13. The pooled NF-κB data analysed by differences in ligand sensitivity. The picture represents the same data as depicted in fig 12, but statistical analysis differs. Here, it was conducted by by ordinary two-way ANOVA and Tukey´s test for multiple comparisons.

2.5 PGR modulation induces a strong proinflammatory response in a hormone depleted environment

One experiment was conducted using hormone stripped media to distinguish whether possibly iFBS originated hormones could interfere with the results of previous experiments. In a hormone-depleted environment a trend towards higher NF-κB activation was observed by both PGR mediated signaling in the *ESR1* siRNA treated cells and by PGR transfection induced upregulation. Reduced PGR receptor capacity decreased NF-κB activity as in the *ESR1* silenced TNFα control and in the LVN treated cells. In the *ESR1* negative control SERM2 differed from LVN treated cells (P=0.0259), but not as much as LVN differed from MFP (P=0.0052) (fig 14a). The luciferase assay exhibited the trend clearer (fig 14b), while the fluorescence-based viability assay exhibited high variability (fig 14c).

The PGR silencing experiment exhibits the results in a more consistent manner, and E2 treatment increased NF-κB activity in receptor upregulated cells (P=0.0056) (fig 14d).

SERM2 and LVN differed in NF-κB modulation (P=0.0056). Here, receptor upregulation was also seen to increase NF-κB activity in the raw luminescence data (fig 14e), and the high variability in viability measurements did not hide the effect (fig 14f).



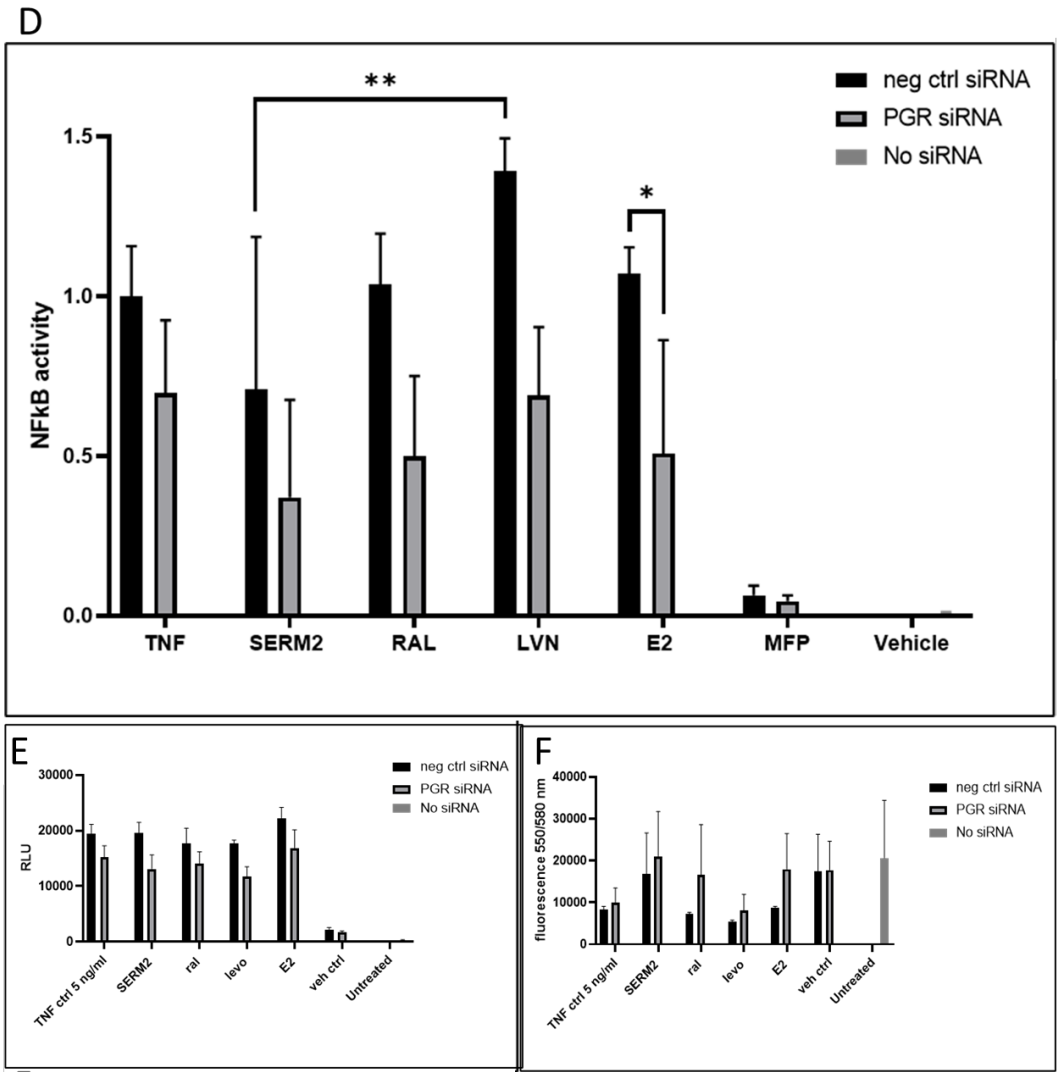


Figure 14. NF-κB activity of THP1 cells in hormone depleted culture. The DCC depletion of iFBS derived hormones from the culture media induced proinflammatory signaling in the receptor increased control cells. A. Receptor increased LVN treated cells exhibited significantly higher NF-κB activity compared to SERM2 or MFP treated cells. B. luciferase measurements were higher in all negative siRNA treated cells compared to ESR1 siRNA, but C. variances in viability abolished the effect. D. In *PGR* siRNA treated cells the proinflammatory features of receptor upregulated cells were more evident, seen also in E. luciferase measurement and after being normalize to F. viability. THP1-Lucia cells were cultured in a hormone depleted media, and similarly assayed for NF-κB activity as in previous experiments, using receptor ligands and TNFα for immunogenic activation. Statistical analysis by two-way ANOVA followed by Tukey´s test for multiple comparison.

2.6 PGR antagonist increased *TGFβ, IL1β* and *IL6* expression in THP1 cells

The cytokine expression modulating effects of ovarian hormone receptor signaling was measured in THP1 cells in order to compare the line cell and primary cell responses and to verify the previously presented NF-κB activity modulating model. The measured effect of ER and PGR ligands on LPS induced cytokine gene expression in THP1-Lucia cells was LPS dependent for *MRC1* (fig 15a) and *ARG1* (fig 15b) and neither ER nor PGR ligands had any effect. *IL10* (fig 15c) expression by LVN was similar to vehicle treated cells but did not differ significantly from LPS control.

Both LVN (P=0.0061) and MFP (P=0.0127) treatment produced a significant increase in the expression of *TGFβ* (fig 15d). *IL1β* expression (fig 15e), however, was upregulated by MFP, and likewise downregulated by LVN, hinting at an agonist-antagonist effect of PGR which unfortunately was not statistically relevant. *IL6* expression (fig 15f) was significantly upregulated by MFP (P=0.0215), while LVN did not seem to have a strong effect. *IFNγ* was very weakly expressed, or not expressed at measurable levels (fig 15g). SERM2 and RAL effects on LPS induced THP1 cytokine expression were similar, except for IFNγ expression (fig 15g), which was not measurable in the SERM2, but was present in RAL treated cells at levels similar to LPS control. LPS stimulation did not affect the expression of *TLR4* (fig 15h), but possibly a hint of PGR agonist-downregulation and antagonist-upregulation could be seen in the expression levels.

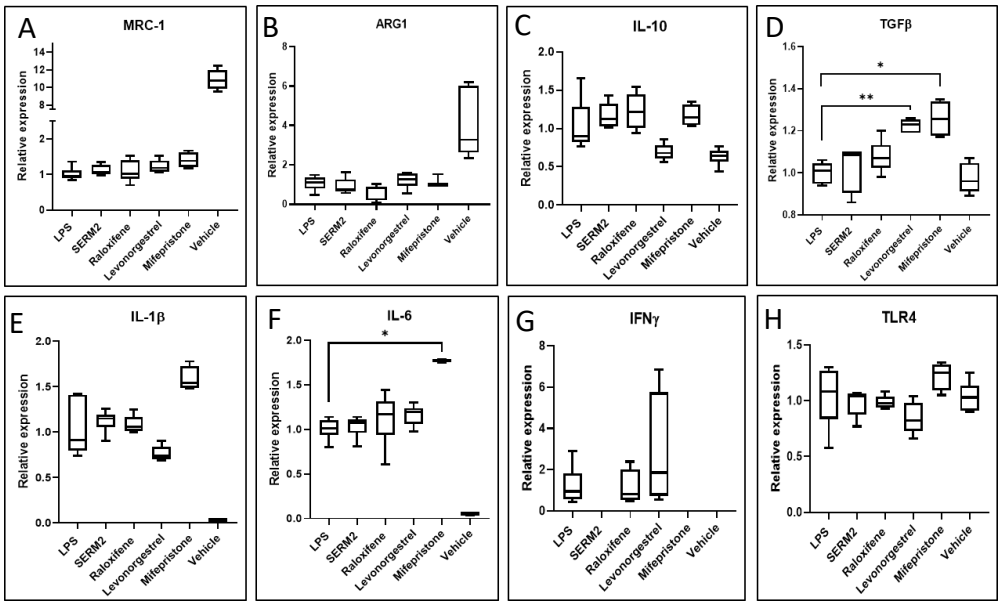


Figure 15. Receptor ligand effect on the cytokine expression of LPS treated THP1 monocytes. The expression of a) *MRC1* and b) *ARG1* in the ligand and LPS treated cells did not differ from LPS control. c) *IL10* was downregulated by LVN, but not significantly and d) *TGFβ* was increased by both LVN and MFP. e) *IL1β* was decreased by LVN, while both *IL1β* and f) *IL6* were increased by MFP. THP1-Lucia cells were cultured together with SERM2, raloxifene, PGR agonist or PGR antagonist, all at 1 uM concentration for 24 hrs before stimulating with LPS (10 ng/ml) and additional 24 h incubation before NF-κB activity was assayed by bioluminescence. Analysis of variance was performed using the Kruskal-Wallis non-parametric method and Dunn´s test for multiple comparison.

2.7 PGR agonist LVN downregulated MRC-1, IL-10, TGFβ as well as IL1β and IL6 in hCD14+ monocytes ex vivo

The ovarian hormone mediated signaling modulatory effect on LPS treated hCD14+ monocyte cytokine expression was measured to verify the line cell results and to bridge the previously presented results to the upcoming siRNA silenced hCD14+ monocytes (sections 2.9 and 2.10) The effect of ER and PGR ligands on the LPS induced cytokine expression was modest and mostly reflected the strong proinflammatory stimuli in human primary CD14+ monocytes.

PGR agonist LVN statistically significantly downregulated *MRC1* expression (P=0.0493) (fig 16a). *ARG1* was upregulated by RAL and to a lesser extent E2 (fig 16b). *IL10* (fig 16c) and *TGFβ* (fig 16d) were both significantly downregulated by LVN (P= 0.0015 and P=0.0181, respectively). Proinflammatory cytokine expression was decreased by LVN treatment, both *IL1β* (fig 16e) and *IL6* (fig 16f) significantly (P=0.0268 and P=0.0027,

respectively). IFNγ expression was stable in the primary monocytes, but the downward trend induced by RAL, E2 and LVN was not statistically significant (fig 16g). The effects of SERM2, RAL and E2 on cytokine expression were similar, except for *ARG1* of which the expression by SERM2 was decreased compared to RAL (fig 16b), and *IFNγ* of which SERM2 increased expression compared to RAL (fig 16g).

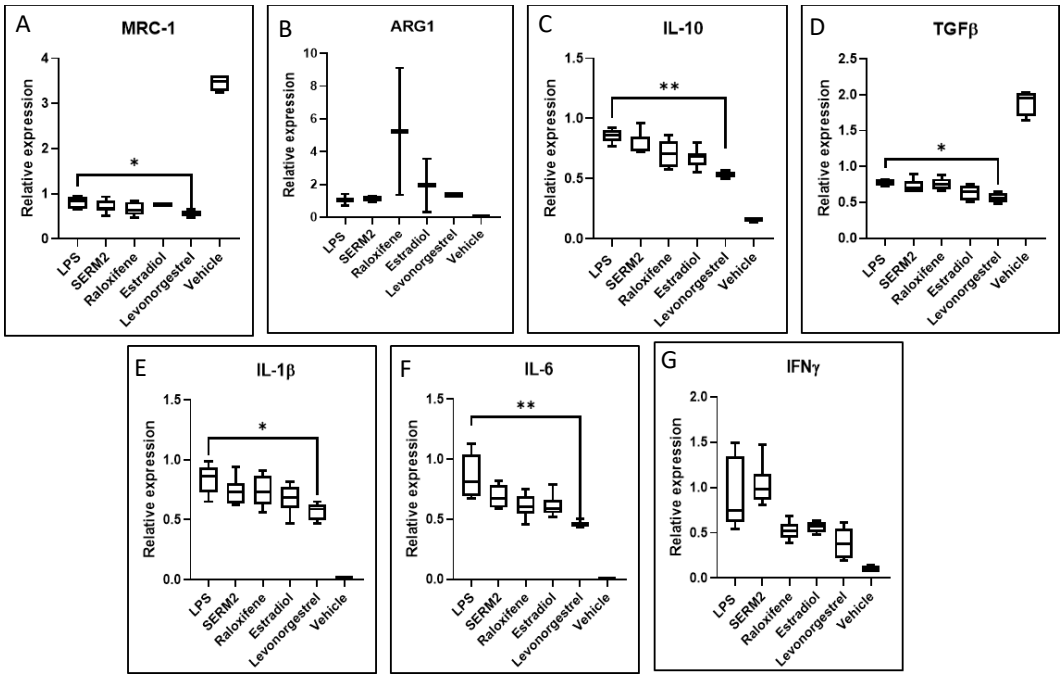


Figure 16. Receptor ligand effect on the cytokine expression of LPS treated human primary CD14+ monocytes. The expression of a) *MRC1* was significantly reduced from LPS control by LVN. b) *ARG1* was upregulated by RAL and to a lesser extent E2, but it did not reach significance. c) *IL10* mRNA was significantly downregulated by LVN as well as d) *TGFβ*. Expression of e) *IL1β* and f) *IL6* were significantly decreased by LVN. *IFNγ* activated CD14+ monocytes were cultured together with receptor ligands, all at 1 uM concentration for 24 h before stimulating with LPS (10 ng/ml) and additional 24 h incubation before RNA extraction and RT-qPCR. Analysis of variance measured by Kruskal-Wallis test followed by Dunn´s test for multiple comparisons.

The cytokine expression of THP1 was not identical to that in primary CD14+ monocytes. THP1 had a stronger *MRC1* response to LPS, with a 10-fold reduction in the expression compared to vehicle treated cells (fig 15a). hCD14+ cells responded with a 3-fold reduction after LPS treatment (fig 16a). *ARG1* seems to be more strongly expressed in the line cells with an LPS induced decrease (fig 15b), as LPS treatment of hCD14+ cells increased expression of *ARG1*. Also, the RAL and E2 induced *ARG1* response was present only in the primary cells (fig 16b). *IL10* was stronger induced by LPS in the

primary cells (fig 16c), and LVN downregulation was comparable, although it did not reach statistical significance in THP1 cells.

The hCD14+ *TGFβ* expression was more extensively downregulated by LPS (fig 16d), and interestingly the ligand induced modulation of LPS response was upregulation in THP1 cells (fig 15d), while the trend was opposite in primary cells. However, the expression of proinflammatory *IL1β* and *IL6* seemed comparable between the THP1 line cells and the adult primary monocytes (fig 15e-f, fig 16e-f), although the LVN response was clearer and statistically significant in the mature primary cells. *IFNγ* was more stably expressed in the primary cells with a trend towards downregulation by RAL, E2 and LVN (fig 16g), while in *IFNγ* was not measured in the SERM2 treated THP1 cells at all and LVN modulation augmented *IFNγ* expression (fig 15g), in opposite of the primary cells.

2.8 ERα upregulation increased proinflammatory cytokine expression in hCD14+ monocytes ex vivo

An *ESR1* siRNA silencing experiment was conducted to compare the modulation of LPS induced cytokine response by ERα mediated signaling in the receptor upregulated versus reduced cells. Downregulation of the receptor could be seen in the vehicle treated, *ESR1* reduced cells, compared to upregulated negative siRNA treated cells. LPS reduced the overall expression of the receptor but the knockdown effect was still evident in RAL treated cells (P=0.0146) (fig 17a). *MRC1* was slightly downregulated in *ESR1* silenced, vehicle treated cells compared to the NEG counterpart, and an approximately 10-fold reductive LPS effect could be observed (fig 17b). *ARG1* expression was not influenced by the receptor silencing or to any greater extent by LPS (fig 17c). *IL10* was significantly increased in the NEG+RAL cells, compared to LPS NEG control but otherwise any consistent silencing effect could not be seen (fig 17d).

TGFβ was downregulated in the *ESR1* silenced, vehicle treated cells, but the effect was indistinguishable after LPS stimulation (fig 17e). The expression of *IL1β* was increased in the receptor upregulated NEG cells, compared to *ESR1* silenced counterparts, and LPS control as well as RAL treated cells (P=0.0005) (fig 17f) . *IL6* was likewise upregulated in the NEG cells, with a statistically significant decrease in all groups (LPS P=0.0002, SERM2 P<0.0001, RAL P=0.0007), except vehicle treated cells where, without immunogenic stimulation *IL6* was not expressed (fig 17g). IFNγ mRNA increased consistently, although not significantly, in the *ESR1* reduced cells (fig 17h). *REL A*

expression levels were significantly affected by silencing in the LPS control (P=0.0452) as well as RAL (P=0.0057) treated cells, but not in SERM2 or vehicle treated cells (fig 17i). The expression of *MRC1* (fig 17b) and cytokine genes *IL1β* (fig 17f), *IL6* (fig 17g) and *IFNγ* (fig 17h) were consistently different in the silenced cells. Less clear, but still detectable difference in *ERα* (fig 17a), *TGFβ* (fig 17e) and *RELA* (fig 17i) expression was seen, hinting at an effect of reduced receptor capacity. The cytokine relative expression analysis in LPS and ER and PGR ligand treated human primary monocytes was normalized to the cytokine expression of a negative control siRNA and LPS treated control.

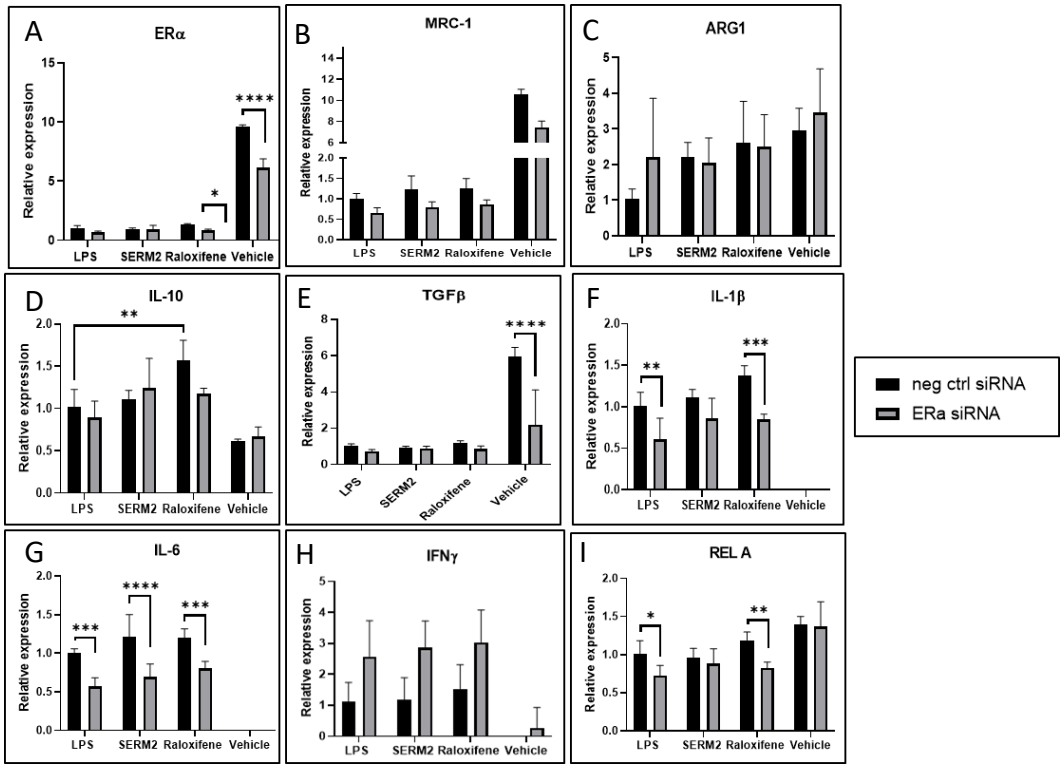


Figure 17. Receptor ligand effect on the cytokine expression of LPS treated human primary CD14+ monocytes. The expression of A. *ESR1* B. *MRC1* was strongly reduced by LPS and the expression was consistently but not significantly upregulated in the receptor increased cells. Please note the two-part y-axis. C. *ARG1* was not consistently affected by *ESR1* silencing. D. *IL10* was not consistently affected by silencing, while E. *TGFβ* was significantly downregulated in vehicle control but not after LPS treatment. Expression of F. *IL1β* and G. *IL6* mRNA was significantly and consistently downregulated in the silenced cells. H. *IFNγ* was consistently upregulated in the silenced cells. I. *REL A* was downregulated in the LPS control as well as RAL modulated cells. Negative control siRNA treated or ERα silenced, IFNγ activated CD14+ monocytes were cultured together with receptor ligands, all at 1 uM concentration for 24 hrs before stimulating with LPS (10 ng/ml) and additional 24 h incubation before RNA extraction and RT-qPCR. Statistical analysis by ordinary two-way ANOVA followed by Tukey´s test for multiple comparisons.

2.9 PGR upregulation increased proinflammatory cytokines in hCD14+ monocytes ex vivo

A similar, but *PGR* silencing experiment was also performed in the human primary monocytes but here the siRNA effect was not fully articulated. In the LPS control samples PGR mRNA is present in the NEG cells while no *PGR* expression could be measured in the *PGR* siRNA treated. However, the effect in vehicle control is opposite, and neither SERM2 nor RAL treated cells showed reduced expression in the cells which should have reduced receptor capacity (fig 18a). *MRC-1* was downregulated by LPS, but a decrease could be observed in the PGR silenced cells only in the vehicle control ($P<0.0001$) (fig 18b). *ARG1* was expressed rather evenly in all cells, except of a marked downregulation in the SERM2 treated PGR silenced cells (fig 18c).

IL-10 was consistently downregulated in the PGR silenced cells compared to NEG counterparts, significantly so in the SERM2 modulated LPS response ($P=0.0284$) (fig 18d). *TGFβ* mRNA levels were markedly reduced by LPS compared to vehicle, with only a weak hint of downregulation in the PGR reduced cells (fig 18e). *IL-1β* and *IL-6* were slightly but consistently downregulated in the PGR reduced cells with a clear LPS effect (fig 18f). LPS induced *IFNγ* expression varied, with a trend towards upregulation in the NEG cells except for in the RAL modulated response (fig 18h). *REL A* was expressed evenly, with downregulation in PGR silenced SERM2 treated monocytes , and a consistent trend in all the LPS treated cells, while without immunogenic stimuli the PGR reduced cells expressed *REL A* to a greater extent (fig 18i).

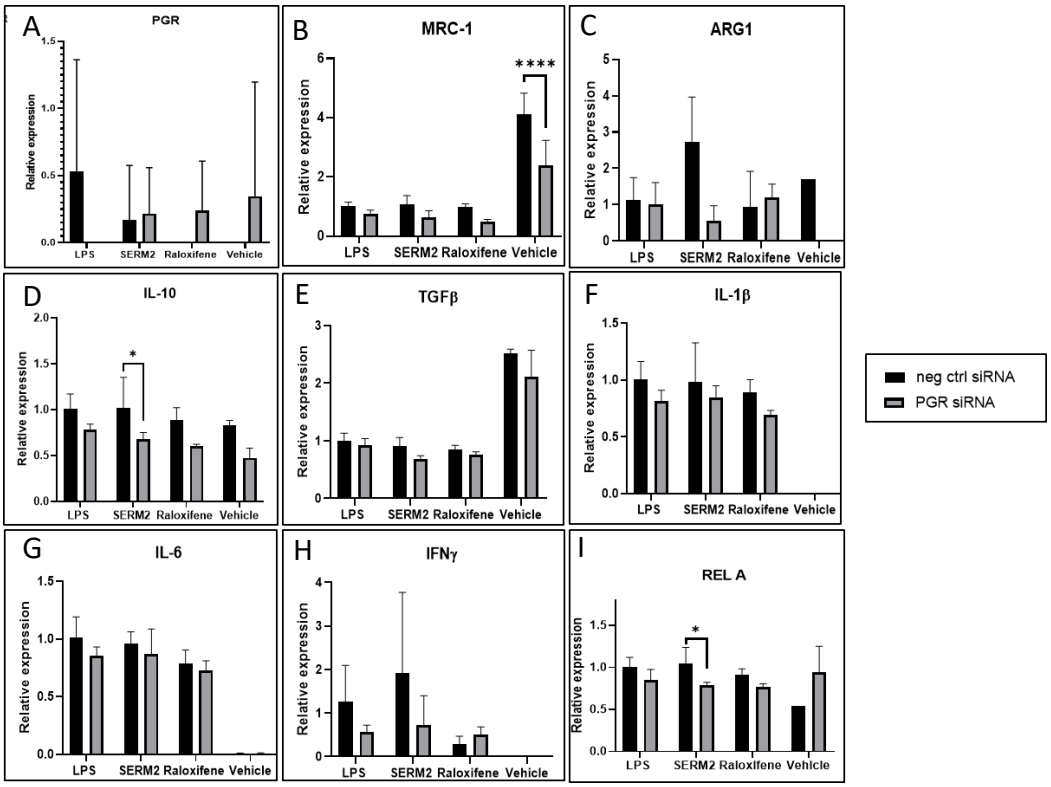


Figure 18. Receptor ligand effect on the cytokine expression of LPS treated human primary CD14+ monocytes. The expression of A. *PGR* was unstable with no consistent differences. B. *MRC-1* was reduced by LPS, and significantly downregulated by PGR in vehicle treated cells. C. no consistent differences were observed in *ARG1* expression. D. *IL10* was consistently downregulated by PGR silencing, reaching significance in SERM2 modulated cells. E. Only an LPS effect could be seen on *TGFβ* mRNA. Expression of F. *IL1β* and G. *IL6* were slightly but consistently downregulated by PGR silencing. H. *IFNγ* was downregulated in the silenced LPS control cells as well in SERM2 modulated. I. *REL A* was downregulated in the silenced SERM2 modulated cells. Negative control treated or PGR silenced, IFNγ activated CD14+ monocytes were cultured together with receptor ligands, all at 1 uM concentration for 24 hrs before stimulating with LPS (10 ng/ml) and additional 24 h incubation before RNA extraction and RT-qPCR. Analysis of variance by ordinary two-way ANOVA followed by Tukey´s test for multiple comparison.

Cytokine expression of *ESR1* or *PGR* reduced human primary CD14+ monocytes was not identical to that of non-transfected CD14+ human primary monocytes. First, regarding receptor silencing effect, *PGR* expression was less affected compared to *ESR1*, but the latter was clearly affected by LPS (fig 17a). *MRC1* was upregulated by the *ESR1* siRNA treatment, with a 10-fold reduction compared to vehicle after LPS treatment (fig 17b) while non-transfected as well as PGR silenced CD14+ cells decreased by approximately 4-fold (fig 16a, fig 18b). Receptor upregulated NEG siRNA treated cells expressed higher levels of *MRC-1* compared to both ERα and PGR reduced cells (fig 16a, 17b, 18b). *ARG1*

expression was increased by RAL and E2, but not SERM2 or LVN modulation of LPS response in non-transfected cells (fig 16b), while it was rather evenly expressed in both silencing experiments, except for an decrease by SERM2 modulation in PGR silenced cells which was not observed in the ERα reduced cells (fig 17c, 18c).

Statistically significant upregulation of *IL10* by RAL was observed in the ERα reduced cells (fig 17e), but not in the non-transfected cells (fig 16c). The receptor reduction effect was clearer in PGR silenced cells (fig 18d), compared to ERα silencing (fig 17d), possibly indicating a role for unliganded PGR in *IL10* expression since LVN reduced *IL10* in the non-transfected cells (fig 16c). The LPS effect on *TGFβ* expression was increased in the ERα reduced cells (fig 17e) compared to PGR reduced (fig 18e) and non-transfected (fig 16d). RAL did not significantly alter *IL1β* nor *IL6* expression in the non-transfected cells but induced a downward trend (fig 16e-f) which was reversed in ERα reduced cells (fig 17f-g) but not in the PGR reduced cells (fig 18f-g).

Reducing ERα upregulated *IFNγ* in disregard of the ligand treatments (fig 17h), while reducing PGR downregulated it (fig 18h) compared to receptor upregulated NEG cells. SERM2 modulation of *IFNγ* expression was similar in both non-transfected and ERα silenced cells. Downward modulation by RAL was seen in non-transfected cells (fig 16g) and PGR silenced cells (fig 18h) while not in the ERα reduced cells (fig 17h). *REL A* was possibly affected by both ERα and PGR reduction.

SERM2 might have modulated responses via PGR activation, since the decrease was significant in PGR silenced cells (fig 18i), but no effect was seen in the ERα (fig 17i) silencing experiment. RAL, which in non-transfected cells imitated the response of E2, did not induce any change in *REL A* expression in PGR reduced cells (fig 18i) but in ERα reduced the difference was significant. Overall SERM2 and RAL had similar effects on the cytokine expression profile of hCD14+ monocytes *ex vivo*, except differences in *ARG1* and *IFNγ* in both non-transfected (fig 16 b and g) and PGR siRNA treated monocytes (fig 18 c and h), probably because of the affinity of SERM2 but not RAL, to PGR.

3. Discussion

According to the results presented here, ERα signaling alone has little immunomodulating effect on monocytes. However, upregulation of ERα protein levels results in proinflammatory signaling via the activation of NF-κB and increased expression of proinflammatory cytokine IL-1β and IL-6. The results also indicate that PGR mediated signaling decreases the NF-κB activity of THP1 monocytes and reduces the hCD14+ monocyte expression of proinflammatory cytokines IL-1β and IL-6. However, PGR signaling also decreased MRC-1, IL-10 and TGFβ, which are thought of as anti-inflammatory markers. However, upregulation of PGR strongly induces inflammation in hormone depleted culture, while without immunogenic stimuli PGR upregulation and signaling consistently decreased NF-κB activity. Since receptor silencing leads to an increase in the activation of NF-κB even without immunogenic activation, one could speculate on a baseline inhibitory effect of PGR signaling.

3.1 PGR but not ERα signaling modulates inflammation in non-transfected THP1 and CD14+ cells

A greater proportion of the *in vitro* research conducted on the effects of SERMs, E2 and ERα signaling indicates an anti-inflammatory effect, especially in experiments using short treatment periods (Ghisletti et al., 2005) (Polari et al., 2018). These kinds of results have been reproduced in e*x vivo* experiments using monocytes or macrophages from E2 or SERM treated animals (Pepe et al., 2017) (Greish et al., 2017). Also, some *in vivo* experiments suggest that estrogen receptor mediated signaling alleviates inflammation and decreases disease activity scores in innate colitis (Harnish et al., 2004) (Bábíčková et al., 2015).

However, contradictory results have been published. Long-term administration of E2 to OVX mice before eliciting monocytes have induced proinflammatory signaling when challenged *ex vivo* (Calippe et al., 2010). DSS-induced colitis has also been reported to exacerbate during E2 treatment of intact mice (Verdú et al., 2002) (Houdeau et al., 2007). Consistently with the flaring of pregnant IBD patients (Goodman et al., 2020), E2 does not induce an increase in inflammation in the intestines without an induced inflammatory foundation (Verdú et al., 2002).

E2 induces a downward trend in THP1 NF-κB activity (fig 9c). Since there are indications that the E2 rapid response mediates the anti-inflammatory effects, a shorter ligand incubation could have stronger accentuated the immunomodulating features. For instance, previously a 10-minute E2 incubation have been successful in inhibiting NF-κB translocation *in vitro* (Ghisletti et al., 2005) and the approximately 22 h activity period induced by LPS was shortened to 8 hrs in E2 modulated cells (Villa et al., 2015). This indicates a possibility of anti-inflammatory properties in short, but not in longer experiments. Besides timing, concentration of E2 may be crucial to the results, as miRNA-mediated inhibition of NF-κB, for instance, was seen using higher, 100nM concentrations of estradiol (24 h incubation) in hormone stripped media (Murphy et al., 2010).

In our setting PGR seems to downward modulate NF-κB activity (figures 9, 11b, 12c, 13) and induce an anti-inflammatory cytokine expression pattern. PGR antagonist increases *IL1β* and *IL6* in THP1 cells (fig 15) while PGR agonist decreases them in CD14+ monocytes (fig 16). A downward modulating effect of PGR signaling on IL-1β have been reported in choriodecidual tissue, while IL-6 downregulation has been reported in cell line monocytes (Jain et al., 2004). The cytokine expression experiments reveal comparable IL-1β and IL-6 responses in line and primary cells while for instance IFNγ is substantially more stably expressed in the primary monocytes. Any upregulating effect of ERα signaling on the markers of anti-inflammatory phenotype, as MRC-1 or ARG1, could not be observed. However, hCD14+ cells downregulate *MRC1, IL10* and *TGFβ* expression sequential to PGR signaling (fig 16). MRC-1 downregulation by progesterone treatment have previously been reported in *ex vivo* IL-4 stimulated macrophages from male mice (Menzies et al., 2011).

Previously, inflammation induced decrease of IL-10 have been reported *in vivo* or in extracted intestinal macrophages (Bain et al., 2013) (Weber et al., 2011). In our experiments, *IL10* is upregulated sequential to the pro-inflammatory response, and this is seen here especially in the LVN treated CD14+ cells. Less pro-inflammatory activity decrease *IL10* expression and it consistently follows the level of *IL1β* and *IL6* mRNA (fig 16). Similar results are reported also in female IFNγ/LPS induced PBMC derived macrophages (Toniolo et al., 2015). IL-10 provides negative feedback in inflammation (Chang et al., 2007), and in this monocyte monoculture it might be reactive. Anti-

inflammatory cytokine IL-10 have been associated with intestinal barrier sparing effect in DSS-inflamed colon, through increased macrophage oxidative species mediated tissue damage in knockout mice (Li et al., 2014) possibly suggesting a higher role in macrophage IL-10 response than IL-10 secretion. Although macrophage secretion is thought to induce tolerance in the gut, CX3CR1 macrophage-specific IL-10R ablated mice developed spontaneous colitis, while inhibition of IL-10 secretion had no effect in that model (Zigmond, E. et al., 2014).

None of the effects could be attributed to changes in TLR4 expression, indicating changes in signaling downstream to the receptor. In other research, LPS have been observed to induce TLR4 expression in RAW 264.7 cells (Vegeto et al., 2004) but not in the elicited murine peritoneal macrophages, stimulated *ex vivo* (Calippe et al., 2010).

3.2 Strong transfection effect brought challenges to interpreting ligand effect on the inflammatory response

The human primary monocyte cytokine expression analysis of siRNA treated cells was initially designed to enable verification and to provide a tool to examine the compatibility of cell line experiments. The strong transfection effect on monocytes definitely brings challenge to interpreting results. It is noteworthy, that the transfection actually increases the levels of receptor protein and thus the results are interpreted as the effect of receptor upregulation (fig 10). As the liposomal transfection reagent induced morphological changes, such as membrane blebbing in both cell line and primary monocytes as well as increased cell-cell adhesion and decreased viability (data not shown). It is known that cells under various stresses upregulate mitogenic and survival enhancing pathways in order to escape apoptosis (Portt et al., 2011) and could speculate on the possibility that the ERα upregulation could induce anti-apoptotic signaling in the cells.

Baseline measurements of NF-κB activation in transfected cells indicates an anti-inflammatory effect by especially PGR upregulation without immunogenic stimuli, as it rather consistently reduced the NF-κB response. It is possible, that an apoptotic effect of TNFα affected the measurements in the other experiments (Dreschers et al., 2013). As the progesterone effect on innate immune cells varies, perhaps one could speculate that PGR upregulation would, in a temporary manner, downregulate the inflammatory response but by longer exposure upregulate it and thus increase the ability of the monocytes or macrophages to clear pathogens.

In normal culture conditions, upregulation of ERα can induce a proinflammatory effect on NF-κB activity (fig 12b) and an increase in *TGFβ*, *IL-1β* and *IL-6* mRNA (fig 17 e-g). This might be comparable with specific ERα signaling in vivo, which was attributed inflammatory features in a Th1-driven colitis model where E2 alleviated the colitis in intact animals but ERα specific signaling did not, and a decreased incidence of severe disease was observed in ERαKO animals (Cook et al., 2014).

In the primary hCD14+ monocytes LPS decreased ERα mRNA levels in primary CD14+ monocytes. Many research groups have noted pro- or anti-inflammatory effects of estradiol and ERα on the response to LPS in different cell types. Downregulation of ERα by LPS have been reported in microglia (Smith et al., 2011), endothelial cells (Holm et al., 2010) and female PBMCs (Toniolo et al., 2015). Opposite results, i.e. an LPS induced increase in *ESR1* mRNA levels have also been reported (Vegeto et al., 2004) and ERα at protein levels in adherent human PBMCs, with a male predominance in upregulation (Campesi et al., 2017). LPS did not exert the same effect on PGR mRNA (fig 18a).

ERα signaling induced a trend towards downregulating IFNγ in the hCD14+ monocytes (fig 16g and 17h), opposed to what have been reported in innate (Siracusa et al., 2008) and NK cells (Gourdy et al., 2005). The IFNγ modulating effect is suggested to be more pronounced in females (Kovats, 2015), which might explain why our male primary monocytes responded differently.

3.3 Is SERM2 an agonist or antagonist in this system?

SERM2 (1μM) treated cells did not decrease NF-κB activity in these experiments, interestingly even increasing it in the PGR silenced cells (fig 12d). This could be explained by SERM2 partially inhibiting PGR. According to Polari et al (2019), SERM2 binds 50% efficiently to ERα at below nanomolar, to ERβ at 13 nanomolar and to PGR at higher, 210 nanomolar concentrations. The agonist-antagonist effects change at different concentration of SERM2. According to Polari et al (2018) SERM2 has antagonist properties at lesser, 2-100 nM concentrations in MCF-7 and Ishikawa cancer line cells while agonism was observed at higher, 100-1000 nM concentration but only in the Ishikawa cells. One could speculate that 1 μM SERM2 functions as a partial agonist at ERα in THP1 and CD14+ cells, while its effects are mostly not similar to LVN at PGR and the adding of both LVN and SERM2 to THP1-Lucia cells indicated slight antagonism (figure 9a). There is no data available on the nature of the SERM2-PGR response.

As the unliganded ERα was seen to participate in the transcription of TNF and receptor activation recruited corepressors of the cytokine production (Cvoro et al., 2006), a question remains whether ERα upregulation could leave enough unliganded receptor to override the inhibition? Another possibility would be the predominance of ERα homodimers, as specific ERα mediated signaling induce and increase proinflammatory signaling (Cook et al., 2014). Since the response to the same E2 ligand can vary so greatly even *in vivo*, it could indicate that the reason lies in processes below the receptor activation. As E2 activation negatively regulates the receptor capacity, this might be a possible explanation of high concentration anti-inflammatory effect. One could speculate on the possibility whether receptor saturation would have an effect on dimer formation, and if so, would it result in increased or decreased ERαβ heterodimers?

Since the ligand-based, consistent pharmacological modulation of ERα and possibly also PGR is difficult, a possibility of allosteric activators or co-agonists (Leitman et al., 2010) stabilizing a certain conformation could be an attractive idea. What if it was possible, not just to enhance or inhibit the effects of endogenous ovarian hormones, but to find a part in the pathway where the endogenous response can be modulated towards or even stabilized as a more beneficial one in specific tissues? As the coactivator expression varies by cell type and is a defining factor for the receptor signaling, protein-interaction studies conducted as close to *in vivo* conditions as possible, could shed further light on the possibilities of ovarian hormone receptor induced immune modulation.

3.4 Confounding factors

It is clear, that without the signals from the surrounding tissues as well as other types of immune cells, *in vitro* and *ex vivo* experiments do not necessarily replicate the animal studies. The effects of estrogen on the immune response seem to be highly context and dose-time dependent. There are still many open questions regarding this field of research, and choosing the relevant models is of utmost importance as the setup has such great influence on the results. As the experiments done by different groups are not standardized, they are hard to compare, but possibly a meta-analysis of the experiment setups including time and concentration of treatment as well as immune stimulation and model could verify some patterns which could be anticipated here.

E2 signaling through other pathways can evidently not be ruled out in these experiments. Besides ERα, the remaining estrogen responsive receptors ERβ and GPER could be

confounding factors regarding the E2 and SERM induced modulation of inflammatory response. GPER promotes rapid anti-inflammatory signaling by estrogen in murine macrophages (Rettew et al., 2010). ERβ is a possible interactor in these experiments but investigating this was ruled out from this project. Another thing not considered is ER and PGR complexing. Although short silencing experiments are not suitable to investigate these effects, full knockout immune cells could be generated to improve the chances of gaining reliable results.

As previously discussed, the immune system is very complex and highly influenced by both the environment and the physiological state of the individual. The reports of ER mediated direct effects on the immune response, such as cytokine release, are contradictory and several other mechanisms need to be considered. ER and PGR have important neuroendocrine function (Dressing et al., 2011) and their signaling can affect neuroendocrine immune regulators such as substance P (Sarajari and Oblinger, 2010). Another possible direction of research could be tissue inflammatory processes, such as prostaglandin signaling (Blesson et al., 2012).

When using THP1 line cells in experiments, the cancerous origin must be considered. Cancer cells might use aberrant signaling pathways and leukemic cells are intrinsically immature. The expression profile and function may differ from circulating monocytes *in vivo*, and to even a greater extent from the monocytes homing and differentiating in the gut. THP1 cells have been observed to be a good model for monocytes in tuberculosis (Madhvi et al., 2019) as well as in atherosclerosis, although validation in either *ex* or *in vivo* experiments is stressed (Qin, 2012). Still, the THP1 used here were a mixed population and thus the response varies to a greater extent compared to extensively characterized monocyte populations.

Circulatory CD14+ monocytes are not macrophages either. Villa et al (2015) measured an anti-inflammatory response induced by IL-4 and found an E2 induced increase in *Il10* and *Arg1* expression, which was not seen in these results (fig 16), but the experiments also differed in angle and set up. However, IL-4 activated macrophages may resemble the homeostatic residential CX3CR1^{hi} intestinal macrophages to a greater extent than the inflammatory CX3CR1 intermediate (fig 3) and thus monocytes may be an appropriate model for the only partly differentiated inflammatory cells recruited to the gut.

Other confounding factors can be culture conditions, transfection efficacy and inconsistencies in viability after immunogenic stimulation. The notably consistent proinflammatory response to PGR upregulation (fig 10) in the hormone depleted negative transfected cells seen both in the *ESR1* siRNA experiment LVN treated cells as in the *PGR* siRNA experiment was unexpected. Nonetheless there are reports of ERα and PGR signaling inducing a strong proinflammatory response in ovariectomized mice, which could represent a similar, hormone depleted situation (Calippe et al., 2010) (Houdeau et al., 2007).

Furthermore, a genomic analysis in breast cancer cells showed positive crosstalk between NF-κB and ER to be significantly more probable than negative (Frasor et al., 2009). As the use of DCC medium increased the inflammatory response of THP1 cells and produced notable variance in viability (fig 14), a hormone depleted cell culture was not seen as a good base for these experiments.

3.5 Anti-inflammatory PGR signaling in monocytes could alleviate intestinal inflammation by reducing IL-6, IL-1β and TGFβ

In conclusion, the silencing experiments possibly indicate a mixed inflammatory effect of both receptors, with PGR being a stronger anti-inflammatory influence while ERα might induce a weak proinflammatory effect. Several different directions of immunomodulating properties of ovarian hormones can be found in the literature, and it is not clear what causes this discrepancy. All animal experiments should verify the serum hormone levels and receptor expression levels when investigating immune modulation by ovarian hormones and ideally the experiments would be as far standardized as possible in order to untangle the underlying cause of all the variance in the results. When conducting *in vitro* research, origin of the cells should be considered carefully. Cell line monocyte immune response is not identical to primary CD14+ monocytes, as seen in figures 15 and 16.

The results presented here may indicate that upregulation of ERα may induce proinflammatory signaling and LPS decreases ERα expression, possible as a negative feedback mechanism. OVX has been observed to upregulate ERα protein in neuronal tissue and similarly high dose estrogen to decrease receptor expression (Liu, X. and Shi, 2015). Although many studies have addressed the effect of ERα mediated signaling, there are no reports on the association of high ERα levels with increased immune responses.

PGR agonist LVN has showed the most consistent anti-inflammatory effects in this project. It might be possible that PGR signaling in monocytes could beneficially modulate gut inflammation through a tissue damage reducing effect, as it reduces TGFβ, IL-1β and IL-6 expression in primary human CD14+ monocytes *ex vivo*. These cytokines are involved in Th1 and Th17 differentiation and secretion of Th17 secretion of IL-17 and IL-21 in the gut (Zhou et al., 2007) (Pelletier et al., 2010). Further, according to the results presented here ERα upregulation is proinflammatory and induces an increase in the mentioned cytokines which, in turn could exacerbate tissue damage in colitis.

4. Materials and methods

To study the role of ERα, ERβ, and PGR on myeloid cell signaling, cell culture experiments were performed using THP1-Lucia (InVivogen) cell line as well as human primary monocytes. Small interfering RNA-based silencing was utilized to temporarily knock-down ERα and PGR. Cells were then cultured with SERMs or other steroid hormone receptor ligands, to distinguish the effect of ERα and PGR receptor signaling on immune response of monocytes. NF-κB activity assay was accompanied by a fluorometric viability assay, performed on THP1-Lucia cells. THP1-Lucia and primary monocyte gene expression of pro- or anti-inflammatory cytokines was analyzed using RT-qPCR. All experiments include a vehicle control including consistent concentration of diluent of the lipophilic pharmaceuticals, as well as a control for immunogenic stimuli. These cells have been treated with 0,1% DMSO or 0,1% DMSO together with consistent levels of LPS or TNFα.

4.1 Pharmaceuticals

To study the effects on ER and PGR ligands estradiol (Sigma), levonorgestrel and mifepristone (Tocris) were used, as well as selective estrogen receptor modulating drugs RAL and fulvestrant (Sigma) and a novel compound produced by Forendo Pharma Ltd., referred to here as SERM2 (table 5). If not stated otherwise, SERM, RAL, LVN and MFP were used in 1 μM concentrations, and E2 in 10 nM.

Table 5. Target, function and used abbreviations of the compounds used.

Pharmaceutical	Target	Function	Abbreviation
SERM2	ERα, ERβ, PGR	Antagonist or partial agonist	SERM2
Estradiol	ERα, ERβ	Agonist	E2
Raloxifene	ERα	Antagonist or partial agonist	RAL
Fulvestrant	ERα	Antagonist	ICI
Levonorgestrel	PGR	Agonist	LVN
Mifepristone	PGR	Antagonist or partial agonist	MFP

4.2 Line cell culture

All cell culture reagents were of the Gibco® product line (ThermoFisher, USA), unless mentioned otherwise. THP1-Lucia^{TM} NF-κB reporter monocytes(InVivogen, USA) were cultured at 37˚C and 5% CO_2 in RPMI-1640 media without phenol red and supplemented

with 1x GlutaMAX™, 4.5 g/l glucose, 10mM HEPES and 1mM sodium pyruvate, 10% iFBS-EU and 50 µg/ml Pen-Strep for up to 20 passages. The cells were subcultured every 3-7 days and to every other passage Zeocin™ (InVivogen, USA) was added for reporter selection. MCF-7 cells were cultured to 90 % confluency at 37˚C and 5% CO_2 in αMEM without phenol red, with 10% iFBS-EU, 1x GlutaMAX™, 25 µg/ml Pen-Strep and 10 nM estradiol.

4.3 Dextran-coated charcoal treated iFBS

iFBS was stripped of hormones by dextran coated active charcoal, including 0.25% charcoal (Merck), 0.0025% dextran 170 (Sigma), 0.25 M sucrose (SigmaUltra, Sigma), 1,5 mM $MgCl_2$ hexahydrate (J.T Baker) and 10 mM HEPES (Gibco). The dextran-charcoal solution was incubated at 4°C overnight and then pelleted (500 x g, 10 min) and washed with sterile H_2O before suspended into an equal amount of iFBS-EU and incubated 2 x 45 min at 56°C. The solution was pre-filtered using a 0.45 mm filter and filtered sterile through a 0.22 mm filter.

4.4 NF-κB activity assay

NF-κB activity assay was performed using THP1-Lucia (InVivogen) NF-κB reporter cells, with integrated NF-κB-inducible Luc reporter construct. The cells secrete luciferase into culture media.

Ligand modulation of TNFα response was performed with cells plated at 100 000 cells per well (n=5). ER and PGR ligands (1 nM-3µM) were added to the wells and incubated 48 hrs. TNFα (5 ng/ml) was added to the wells and incubated overnight.

Silencing of the *ESR1* and *PGR* was performed with HiPerfect tranfection reagent (Qiagen) and Silencer ® validated siRNAs (Ambion® Life Technologies) for *ESR1, PGR* and a scramble siRNA as a negative control. For the transfection mix, serum-free media was used. 112.5 ng/well (150nM) of siRNAs and 1.8 µl/well HiPerfect were incubated at RT for 5-10 min and siRNAs were transfected at 150 nM for 6 h, then diluted to 50 nM and incubated overnight. Transfected THP1-Lucia cells were plated at 60 000 cells per well, n=5. ER and PGR ligands (1µM) were added to the wells and left to incubate overnight. TNFα (5 ng/ml) was added to the wells and left again overnight.

For measuring accumulated NF-κB induced luciferase secretion, 10 µl of cell culture media was taken from the wells and transferred to an optical microplate. Luminescence was measured with a Victor x4 plate reader (1420, Perkin-Elmer) with a liquid dispenser (DISPENCER for 1420, 1420-2560, Perkin-Elmer). 50 µl Quanti-Luc solution was injected before a 4 s measurement. The luciferase reaction results were related to those of a fluorometric viability assay.

4.5 Viability assay

The luciferase reaction results were related to those of a fluorometric viability assay. alamarBlue™ Cell Viability Reagent (Invitrogen™, ThermoFisher) was used according to the instructions provided by the manufacturer and measured using excitation at 550 nm and emission at 580 nm wavelength in a Hidex Chameleon multiplate reader (Perkin-Elmer).

4.6 RT-qPCR

To verify the siRNA silencing effect by RT-qPCR 300 000 cells/sample were incubated with transfection mix as described in the NF-κB activity assay.

For cytokine expression analysis THP1-Lucia cells were plated at 100 000 cells/well (n=4) and incubated with ER and PGR ligands overnight. Cells were then stimulated with LPS (10 ng/ml) and incubated again, overnight. Expression was measured first in non-silenced cells, and later in ERα or PGR silenced human primary CD14+ monocytes. For these experiments, cells were plated at 300 000/well (n=1-4) and incubated overnight with ER and PGR ligands before stimulation with 10ng/ml LPS (8 h).

Cells were lysed using a guanidinium thiocyanate buffer with 1% β-mercaptoethanol, and RNA was extracted using NucleoSpin® RNA extraction kit (Macherey-Nagel) and reverse transcription was performed by High Capacity Reverse Transcription Kit (ThermoFisher). Cytokine expression analysis was performed by TaqMan® Gene Expression Assays (ThermoFisher) using *ACTB* as reference gene. All steps were performed according to the instructions of manufacturers.

4.7 Western blot

For Western blot analysis of the siRNA treatment, 10^6 cells/sample were incubated with transfection mix (serum-free medium, siRNA and HiPerfect transfrection reagent for 6 h in 150 nM and overnight in 50 nM siRNA (negative control, *ESR1* and *PGR*). Cells were lysed 24h after transfection in RIPA buffer and stored at -80°C. Proteins were quantified by Pierce BCA Protein Assay Kit (ThermoFisher), according to manufacturer's instructions and all samples were standardized to 50 µg total protein/lane. SDS-PAGE separated proteins were transferred (100 V, 1 h) by a wet-transfer system (Mini Trans-Blot® Cell, PowerPac™ and Basic Power Supply, Bio-Rad) onto PVDF membrane (Amersham™ Hybond® P Western blotting membranes, pore size 0.2 µm, Sigma) and blotted with primary antibodies Human/Mouse/Rat ER alpha /NR3A1 (R&D Systems) and Progesterone Receptor Antibody (MA1-410, Thermo) . Secondary Rabbit Anti-Mouse IgG H&L (HRP) (abcam) and detected using SuperSignal West Pico Chemiluminescent Substrate (TermoFisher) and LAS-4000 (Fujifilm Life Science) or Sapphire Biomolecular Imager (Azure Biosystems). The blot was analyzed using quantification of histogram area in ImageJ (Schneider et al., 2012).

4.8 Extraction and silencing of CD14+ human primary cells

For monocyte extraction, blood was drawn from one healthy human volunteer (adult male) to lithium-heparin tubes (Lithium Heparin Tube for Blood Collection, Henso Medicals) on three separate occasions. The peripheral blood mononuclear cells were separated by Fiqoll centrifugation technique and antibody labeled magnetic beads for CD14+ cells (MACS® Cell Separation, CD14 MicroBeads, human, Miltenyi Biotec). Cells were cultured at 37˚C and 5% CO_2 in αMEM without phenol red supplemented with 10% iFBS-USA, 0,05% glutamine and 50 µg/ml Pen-Strep. For ERα and PGR silencing, siRNA transfection mixes for negative control, *ESR1* and *PGR* (serum-free medium, siRNA and HiPerfect transfrection reagent x µl/well) was added after extraction (6 h at 150 nM and overnight in 50 nM siRNA concentrations). All CD14+ monocytes were activated with 50 ng/ml IFNγ overnight.

4.9 Statistical analysis

Level of significance was set to P≤0.05 and statistical significance is marked by asterisk (P≤0.05 *, P≤0.01 **, P≤0.001 ***). All statistical analysis was performed in Prism8 (GraphPad Software, Inc). Analysis of variance was tested by one- or two-way ANOVA for normally distributed data, while non-parametric Kruskal-Wallis test was used for analysis of non-normally distributed data. All analysis of variance was followed by a suitable post-hoc test. Dunnett's multiple comparisons test was used for analyzing difference to control, while Tukey´s or Sidak´s multiple comparison test was used for analyzing significant differences between all groups.

4.10 Ethical and confidentiality issues

Blood samples acquired for primary human CD14+ cell culture was taken by a licensed health care professional in accordance to the permission to collect and use human mononuclear cells from healthy volunteer donors for *in vitro* studies (Turku University Ethics Committee, statement 6/2017). No other personal information was stored, except for the sex and the age of the donor. The sample was not used for any other purposes, than this study project. SERM2 is a pharmaceutical compound under development by Forendo Pharma Ltd and thus the structure is strictly confidential.

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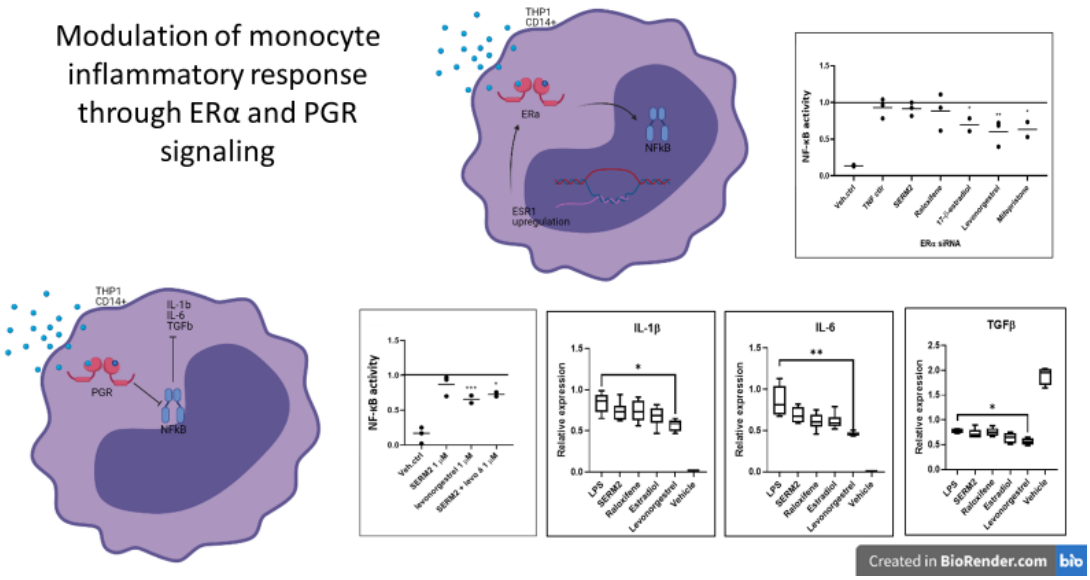
6. Appendices

6.1 Appendix 1: Graphical abstract



7. List of used abbreviations

AF	Activation function
APC	Antigen presenting cell
Arg	Arginase
CCR2	C-C chemokine receptor
CD	Cluster of differentiation
CD	Crohn´s Disease
CX3CR1	C-X3-C Motif Chemokine Receptor 1
DSS	Dextran sodium sulphate
E1	Estrone
E2	Estradiol
E3	Estriol
E4	Estrone
ER	Estrogen receptor (protein)
ERE	Estrogen response element
ESR	Estrogen receptor (gene)
FSH	Follicle stimulating hormone
GPER	G protein-coupled estrogen receptor
IBD	Inflammatory bowel disease
IL	Interleukine
IκB	Inhibitory kappa B

iNOS	inducible nitric oxide synthase
KO	Knockout
LH	Luteinizing hormone
LPS	Lipopolysaccharide
LVN	Levonorgestrel
Ly	Lymphocyte antigen
MPF	Mifepristone
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OVX	Ovariectomized
PGR	Progesterone receptor
PI3K	Phosphatidylinositol-3-Kinase
SERM	Selective estrogen receptor modulator
siRNA	small interfering RNA
Th	T helper (cell)
TIF2	Transcriptional Intermediary Factor 2
TLR	Toll-like receptor
UC	Ulcerative colitis

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