Nuclear Receptors in Immunity:
Molecular Mechanisms of GR, PPARγ and LXR in Inflammatory Gene Regulation

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Contents

Abstract

Nuclear receptors are known to be involved in many processes in the metabolism, reproduction, cell growth and immunity. Several nuclear receptors are indispensable factors in immune cells and modulate the inflammatory response by gene transcription. In this thesis we will focus on different mechanisms of inflammatory gene regulation of nuclear receptors. We will discuss the different mechanisms involved in gene regulation and describe the proposed models of gene (trans)repression in more detail. Furthermore, we will enlighten the molecular mechanisms of the glucocorticoid receptor, peroxisome-profilator-activated receptor γ and liver X receptor. These nuclear receptors are currently the most important regulators of the inflammatory response, which are most extensively studied. Reviewing, understanding and combining these important inflammatory gene regulating mechanisms of all three nuclear receptors might give insight into new treatment possibilities for certain diseases related to the immune system.

Chapter 1: The Nuclear Receptor Family

1.1 General Transcription

Transcription is the process of transcribing DNA nucleotide sequence information into RNA sequence information. Transcription is also referred to as RNA synthesis. The transcribed RNA can either be messenger -, transfer - or ribosomal RNA. All three types of RNA have an important function during the translation process, which is also referred to as protein synthesis. Messenger RNA (mRNA) carries information about protein sequence from DNA to ribosomes, which are the sites of translation. Ribosomes consist of ribosomal RNA (rRNA), acting as the catalytic component of a ribosome. Transfer RNA (tRNA) is a small RNA chain that transfers a specific amino acid to a growing polypeptide chain at the ribosomal site of protein synthesis, which is essential for proper translation of mRNA. Transcription of all RNA is dependent on RNA polymerase (RNApol), which is a very important enzyme. RNApol can be found in several types, each transcribing a different RNA. RNApol I synthesises pre-rRNA, eventually forming the major part of the ribosomes. In addition, RNApol II synthesises mainly mRNA, while RNApol III synthesises tRNA and little rRNA. As being the most studied type of RNApol and due to the high level of control required over transcription, combined with the fact that mRNA is the carrier of the information of the final translated protein, focus will be on both RNApol II and mRNA in the next explanation. RNApol II is essential for the start of the transcription progress of mRNA. The process of transcription involves three stages: initiation, elongation and termination. RNApol II is involved in all of these stages. RNApol II searches DNA for initiation sites (promoters) and unwinds double-helical DNA to produce single stranded DNA. Continuously, RNApol II detects termination signals to end transcription. Furthermore, interactions between RNApol II and activator - or repressor proteins modulate the rate of transcription initiation. These proteins are called transcription factors or trans-acting factors. Modulated transcription initiation results in different gene expression patterns. Gene expression is the combined process of the transcription of a gene into mRNA, the processing of that mRNA, and its translation into protein. Different gene expression patterns in cells lead to the formation of different cell function or modulated characteristics [1,2].

1.2 Control of Gene Expression

In the previous paragraph we have described that transcription can be controlled by transcription factors. This control of transcription can be achieved by promoting or blocking the recruitment of RNApol. An important factor in the control of transcription is the structure of DNA in a chromosome. If there is no transcription, DNA is wound around octamers. Octamers consist of two copies of each of the four core histone proteins, which form the spool around which DNA winds. The combination of DNA and an octamer is called a nucleosome. Changes in the complex structure of DNA and the other proteins that define a chromosome, which is called chromatin, play a major role in the regulation of gene expression. Initiation of transcription requires RNApol, which can only bind to the DNA if a specific DNA sequence is located. This DNA sequence is called a promoter, providing a binding site for RNApol and other transcription factors that can recruit RNApol. If RNApol is bound to DNA, the DNA unwinds and initiation of transcription becomes easier. Gene expression can also be regulated by enhancers, which are short DNA regions capable of binding transcription factors called activators. Enhancers have no promoter activity of their own and can increase activities of many promoters by changing the local chromatin structure. This allows interactions between RNApol, transcription factors and a gene or its regulatory sites, even when an enhancer is located at a distance of several thousand base pairs from the gene which is expressed. This

mechanism gives the enhancers the ability to act at a distance [2]. Transcription factors can also control transcription by formation of a large complex that interacts with the transcriptional machinery to activate or repress transcription. This mechanism is found in hormone signalling, where steroids bind to the nuclear hormone receptors which than act as transcription factors. These receptors are able to bind DNA in the presence or absence of bound ligands. Ligands are signalling molecules associated with a certain receptor. Binding of ligands changes the conformation of the nuclear receptor attracting the coactivators. The important function of coactivators is the addition of an acetyl group to lysine residues in the histone proteins (Figure 1). This reaction decreases the octamer-DNA binding, resulting in higher accessibility for gene transcription. Moreover, acetylation of histones attracts other components of the transcriptional machinery and initiates active remodelling of chromatin structure. These events open up sites on chromatin and can initiate transcription (Figure 1). Some nuclear receptors repress transcription in the absence of a ligand. This repression is mediated by the ligand-binding domain. If there is no ligand bound, the ligand-binding domain attracts corepressor proteins, repressing transcription. Only ligand binding can trigger the release of a corepressor. This allows new binding to a possible coactivator [2,121].

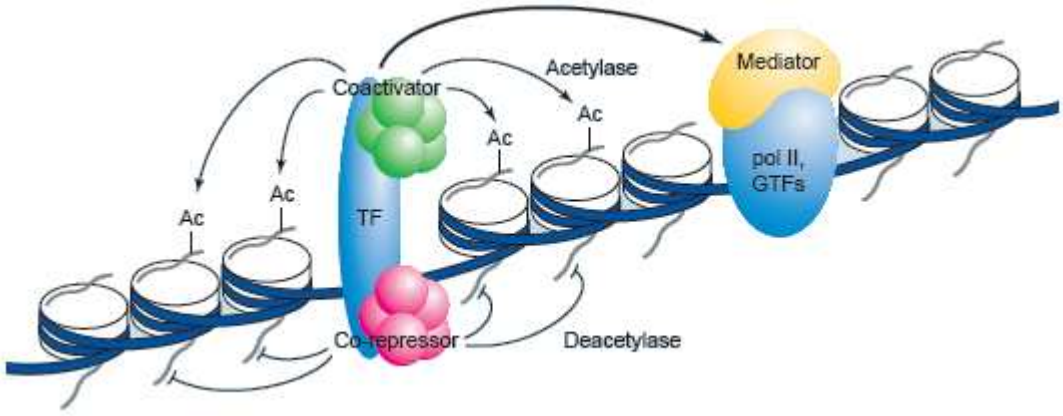

Figure 1. Basic transcription overview. Transcription is depicted schematically. A transcription factor (TF, blue) is shown with either an activator or repressor, bound to a regulatory element. Secondly, RNApol II (pol II, blue) and general transcription factors (GTFs, blue), bound to a promoter along the DNA (dark blue ribbon) is shown. Octamers with their acetylated (Ac) histone tails (grey) are shown as disks. An Activator stimulates transcription by interaction with a coactivator (green), which attracts acetylase, directly interacting with a mediator (yellow). A repressor interacts with a corepressor (pink), which recruits a deacetylase.
Adapted from: Kornberg RD 1999 [2].

1.3 Nuclear Receptor Family

The nuclear receptor family is a superfamily of ligand-dependent transcription factors regulating several developmental and homeostatic events. All nuclear receptors have own ligand- and DNA binding properties dividing them into three main classes [3,4]. The most extensively researched class of nuclear receptors are the steroid- and thyroid-hormone receptors [3]. In this thesis, the extensively studied glucocorticoid receptor (GR), which has several functions in immunity, will be described in detail [5,6]. The second class of nuclear receptors are the orphan receptors. They are structurally equal to the nuclear receptor superfamily. However, they have not been linked to ligands and can function in the absence of a ligand. Finally, the third class is an extension of the orphan receptors. These adopted orphan receptors were first described as orphan receptors. Studies revealed that adopted orphan receptors can be linked to naturally occurring ligands and therefore have their own physiological role. Several members of this subfamily have been linked to regulation of gene

expression in different immune cells and therefore are most interesting for this thesis. These include, peroxisome-profilator-activated receptor γ (PPARγ) and liver X receptors (LXRs) [7].

1.4 Nuclear Receptor Structure and Function

In this paragraph, we will describe the basic structure of nuclear receptors. Furthermore, we will discuss the similarities and differences in functioning and basic transcriptional activation between steroid-hormone receptors and the adopted orphan receptors PPARγ and LXR. The basic structure of all nuclear receptors is identical, consisting of several domains with accompanied functions (Figure 2). These domains include, activation function 1 (AF1), a DNA-binding domain (DBD), a ligand binding domain (LBD) and activation function 2 (AF2) [3]. The AF 1 is an activation domain found at the N-terminal, which is used during transactivation. Transactivation is one of several mechanisms which can start gene transcription. We will discuss transactivation in more detail in a later paragraph. Furthermore, the DBD in the nuclear receptor structure has the ability to recognise specific DNA sequences and therefore mediates binding of the nuclear receptor to response elements (REs) in enhancer or promoter regions of the target genes. Another function of the DBD includes contribution to the dimerisation of nuclear receptors, which leads to the forming of either monomers, homodimers or heterodimers. The LBD mediates the binding of a ligand to the receptor and can bind coactivators or corepressors. Similarly to DBD, the LBD also contributes to the dimerisation of the nuclear receptor. Finally, the AF-2 is also used during transactivation and can be found at the C-terminal [3].

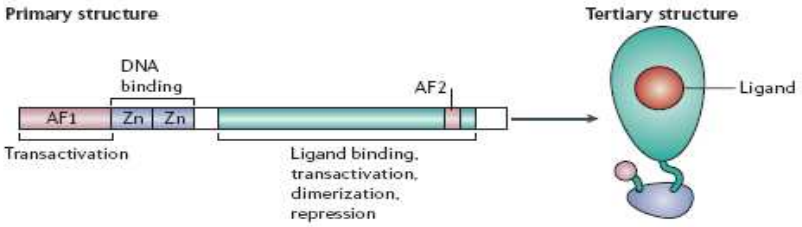

Figure 2. The primary and tertiary structure of a nuclear receptor. AF1 and AF2 (pink) are used during transactivation. DBD (blue) mediates binding to REs. LBD (green) mediates ligand binding, dimerisation and repression. *Adapted from: Glass CK et al. 2006,* [9].

Similar to the basic structure of nuclear receptors, basic functions are also the same. If a ligand is bound, the receptor forms a certain type of dimer and binds to REs on the DNA, eventually regulating gene expression. However, there are differences in the mechanisms between steroid-hormone receptors and adopted orphan receptors. A steroid receptor can only bind to DNA as a homodimer as its REs are usually arranged for homodimer binding, while PPARγ and LXR both bind as heterodimers in combination with Retinoid X receptor (RXR) [7,8]. Steroid receptors can only function in presence of a ligand as the LBD of a steroid receptor prevents DNA-binding in absence of a ligand. Interactions between the LBD and the heat shock protein (HSP) in the cytoplasm keep steroid receptors inactive. If a ligand binds to the steroid receptor, the interaction between HSP and steroid receptor is dissolved which leads to DNA-binding and transcriptional activation or repression (Figure 3A) [3,9]. In contradiction to steroid receptors, the LBDs of PPARγ and LXR do not mediate interactions between the HSP and receptor, but bind coactivators or corepressors in the nucleus. Two of those nuclear receptor binding corepressors, which will be of significant importance for this thesis are the nuclear receptor corepressor (NCoR) and the silencing mediator of retinoic-acid and thyroid-

hormone receptors (SMRT) [10,11]. The presence of a ligand is not always needed for either corepressor, coactivator or DNA-binding of LXR and PPARγ (Figure 3B).

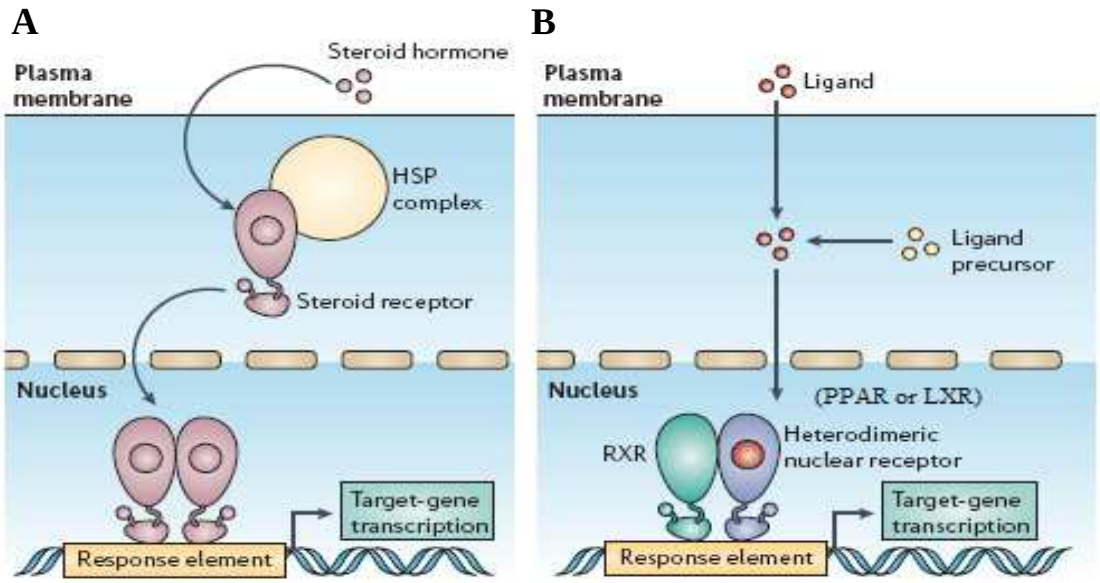

Figure 3. Schematic overview of DNA-binding and transcriptional activation by nuclear receptors. A) Hormone-binding dissociates HSP from the steroid receptor and enables DNA-binding and associated transcriptional activity. B) PPARγ or LXR form a heterodimer with RXR and bind DNA target genes in presence or absence of ligand. *Adapted from: Glass CK et al. 2006,* [9].

1.5 Nuclear Receptor Ligands

Ligands are important for nuclear receptor activity, as not all receptors function in the absence of a ligand. The steroid-hormone receptor only functions in the presence of hormones and regulates homeostasis and inflammatory gene expression. Many circulating steroid-hormones are produced under influence of the hypothalamic-pituitary adrenal axis (HPA axis). The HPA axis is a complex set of interactions between the hypothalamus, pituitary gland and adrenal glands. GR function is mediated by glucocorticoids (GCs), which is a steroid hormone class. In contradiction to the GR, both PPARγ and LXR are regulated by paracrine or autocrine produced ligands. Several fatty-acids can function as ligand for PPARγ, underlining the function of PPARγ in the fatty-acid metabolism [12]. Inflammatory responses can produce several fatty acids metabolites during inflammation which can activate the PPARγ receptor. 15-hydroxyeicosatetraenoic acid (HETE), 13 hydroxyoctadecadienoic acid (HODE) and 15-deoxy-delta-prostaglandin J2 (15dPGJ2) are all metabolites acting as ligand for PPARγ [12,13]. LXRs are involved in cellular cholesterol homeostasis and have several oxysterols as ligand, which are oxygenated derivatives of cholesterol. 24S-hydroxycholesterol and 22R-hydroxycholesterol act as ligands for LXR [14,15]. We will discuss the functions and ligands of GR, PPARγ and LXR in a later paragraph.

1.6 Nuclear Receptor Mechanisms

There are several distinct mechanisms involved in the regulation of transcriptional actions of nuclear receptors. Ligand-dependent transactivation is an important activity of steroid hormone - and most heterodimeric receptors, acting via direct binding of the receptor to REs in the promoter or enhancer regions of target genes (Figure 4A). As highlighted earlier, ligand-dependent transactivation attracts coactivator complexes, which modify the chromatin structure and the binding to the basal transcription machinery. Another mechanism which is involved in transcriptional regulation by nuclear receptors is ligand-independent repression.

Several nuclear receptors, including PPARγ and LXR can repress the transcription of direct target genes in absence of ligand (Figure 4B). A third mechanism of nuclear receptors involved in transcriptional regulation is ligand-dependent repression, where nuclear receptors are able to repress gene transcription. Binding of the receptor to negative regulatory elements (NREs) represses the transcriptional activity (Figure 4C) [16]. However, most nuclear receptors function via another mechanism, which is not fully understood. This activity is referred to as ligand-dependent transrepression or simply transrepression [17]. This negative regulation seems to result from inhibition of the activity of signal-dependent transcription factors (Figure 4D). In contrast to ligand-dependent transactivation, ligand-dependent repression and ligand-independent repression, transrepression does not function via interactions with (negative) response elements in promoter or enhancer regions. On the other hand, there is no full understanding if transrepression requires homo- or heterodimerisation [18-21]. We will discuss transrepression models in more detail in chapter 3.

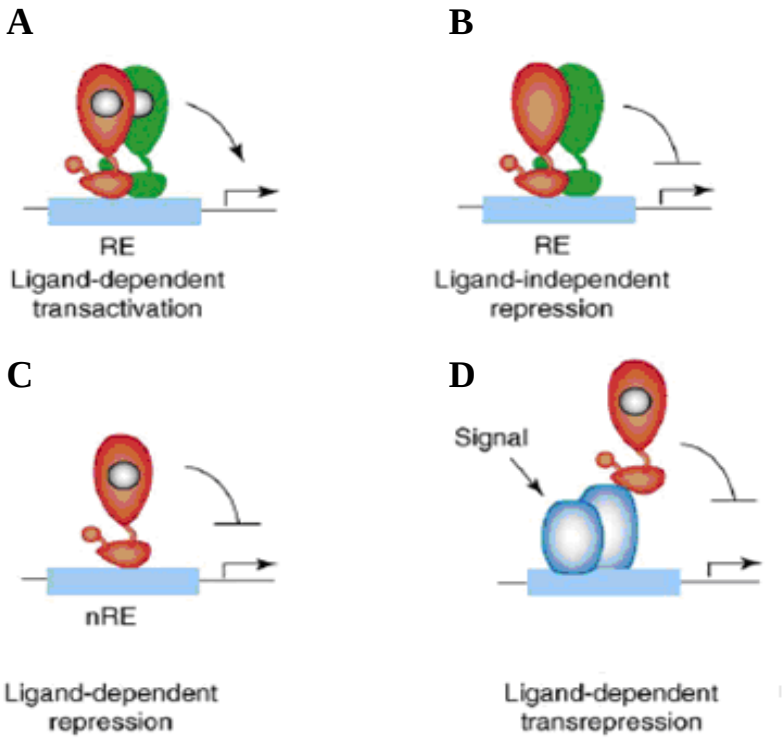

Figure 4. Nuclear receptor activity mechanisms. A) Heterodimeric ligand-dependent transactivation via RE binding. B) Heterodimeric ligand- independent repression via RE binding. C) Ligand dependent repression via NRE binding. D) Ligand-dependent transrepression via inhibition of signal-dependent transcription factors. *Adapted from: Pascual G et al. 2006,* [17].

1.7 GR Main Functions

Steroid hormones influence several metabolic, reproductive, immune and neuroendocrine responses and are the ligands of the steroid hormone receptors. Steroids are small lipophilic molecules, which are derived from cholesterol. Structure and biological activity determines the type of steroid. Those include: progestins, androgens, estrogens and corticoids. Corticoids can be subdivided in two other groups; mineralocorticoids (MCs) and GCs [22]. MCs are involved in the regulation of ion transport, while GCs have many other activities. Endogenous GCs are involved in resistance to stress, regulation of metabolism and in immunity and

inflammation [6,23]. Synthetic GCs are used for many clinical purposes, but usually as treatment for inflammatory diseases, including asthma and transplantation rejection. However, prolonged use of GCs can cause a wide variety of side effects, including hypertension or diabetes. Even resistance can occur if GCs are used long-term, making several inflammatory diseases untreatable with GCs. Therefore, interest in new synthetic GCs and the mechanisms involved is substantial. GCs are the ligands for the GR, functioning as a transcription factor in many processes (Figure 5). Similar to other nuclear receptor family members, GRs consist of an AF1, DBD, LBD and AF2 domain (Figure 2). Ligand binding converts the inactive receptor into an active transcription factor, which is found in the nucleus. Two groups of glucocorticoid receptor-regulated genes can be distinguished. The first group contains a glucocorticoid-response element (GRE) in their promoter - or enhancer sequence on the DNA. The GRE is a palindromic sequence binding the active GR homodimer. A few of these transcribed genes are glucose-6-phosphatase, tyrosine aminotransferase, glutamine synthetase and glucocorticoid induced leucine zipper (GILZ), which will be discussed later [24-27]. The second group of genes is regulated by a monomer of GR in absence of DNA-binding [28]. The GR inhibits the activity or activation steps of other transcription factors. In the previous paragraph, we stated that this was referred to as transrepression. Several transrepressed transcription factors include, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), activation protein 1 (AP-1), cAMP response element binding (CREB), CCAAT/enhancer binding protein beta (C/EBPβ) and members of the signal transducers and activators of transcription (STAT) family [6,18,29-31]. In the coming chapters we will go further into detail about these transcription factors.

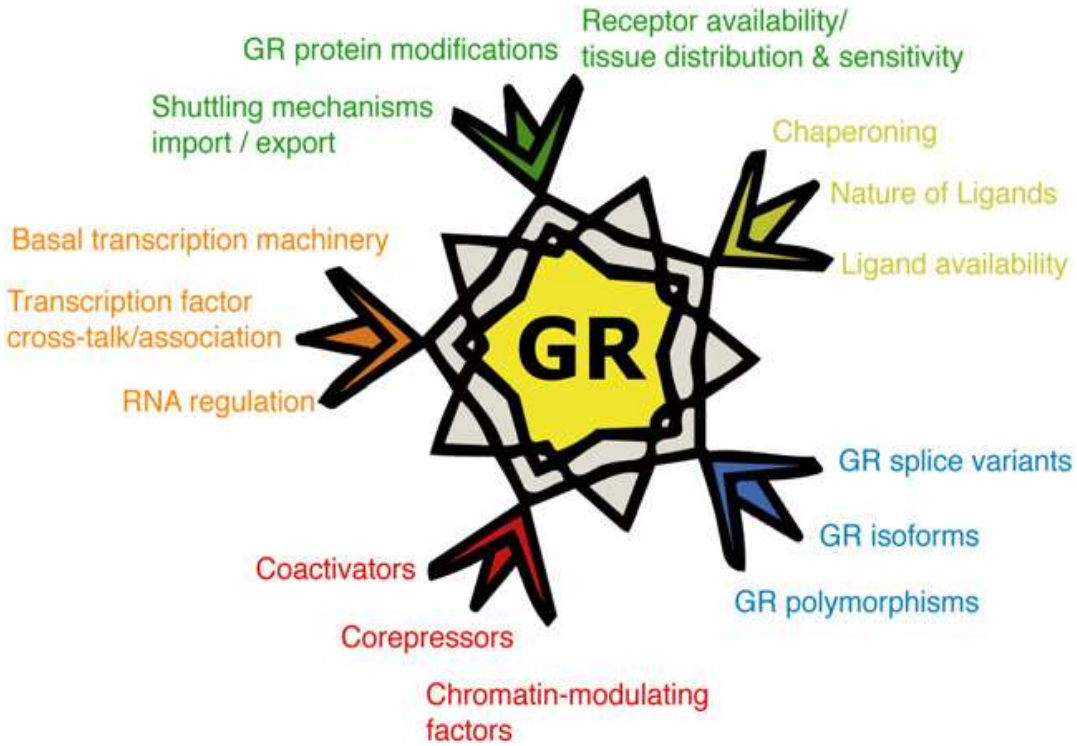

Figure 5. Overview of the multiple levels at which the functionality of the GR is modulated. The coloured triads represent: Control of GR signalling at systemic level (dark green triad), Control of GR signalling at cellular level (light green triad), Influences on the GR activity by different GR variants (blue triad), Influences on transcription outcome if GR acts as transcription factor (red triad) and the influences on GR activity by interactions with other transcription factors (orange triad).
Adapted from: de Bosscher K et al. 2007, [22].

1.8 PPARγ Main Functions

PPARγ is a member of the PPAR family, which includes PPARα, -γ and -β/δ. We will only focus on PPARγ in this thesis. This nuclear receptor is activated by several ligands and has a wide range of effects on metabolism, cellular proliferation, differentiation and immunity. PPARγ is predominantly known for its role in adipocyte differentiation and treatment of type II diabetes [32]. In addition, a role of PPARγ (and ligands) in immunity has also been suggested by PPARγ-dependent and independent pathways. In contrast to GRs, they form heterodimers with RXR (Figure 3B) and bind to PPAR response elements (PPRE) on the DNA to activate transcription [32]. Like all nuclear receptors, PPARγ consists of an AF1, DBD, LBD and AF2 domain (Figure 2). The ligand binding pocket of PPARγ is large compared to other nuclear receptors. Similarly to other nuclear receptors, ligand- or agonist-binding results in conformational changes in the structure of PPARγ, allowing binding of coactivators. Antagonist-binding results in a conformational change that favors binding of corepressors [33,34]. PPARγ-RXR heterodimers can bind to PPRE in the presence or absence of a ligand. Binding of a ligand results in ligand-dependent transactivation, while absence of a ligand on a PPARγ-RXR heterodimer results in ligand-independent repression by the binding of corepressors [35]. As described before, endogenous ligands of PPARγ include unsaturated fatty acids, HETE, HODE, 15dPGJ2 and oxidised low density lipoprotein (oxLDL). All endogenous ligands bind in different affinities, resulting in different transcription of genes. PPARγ is expressed in dendritic cells, macrophages, B- and T-lymphocytes and epithelial cells. This suggests several important roles in the immune system for PPARγ and its ligands.

1.9 LXR Main Functions

LXRs, including both LXRα and LXRβ are activated by several ligands and are central regulators of cholesterol metabolism [36]. Similarly to PPARγ, RXR forms a heterodimer with LXR (Figure 3B), which can bind to LXR response elements (LXRE). This event can activate transcription of target genes in their promoter regions. LXR also consists of an AF1, DBD, LBD and AF2 domain (Figure 2). The LXRE consists of two direct repeats of the hexamer sequence, AGGTCA. This sequence is separated by four base pairs. Like most other nuclear receptors forming heterodimers with RXR, ligand-bound-LXRs bind to the LXRE in complex with the corepressors SMRT or NCoR [11]. In the absence of a ligand, ligand-independent repression occurs via binding of corepressors. If a ligand is present, a release of corepressors can be triggered. As described before, endogenous ligands of LXRs include oxysterols and intermediates of the biosynthetic cholesterol pathway. 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 27-hydroxycholesterol, 24(S)epoxycholesterol, 25-epoxycholesterol and desmosterol are all recognised as natural ligands of LXRs [14,15]. LXRα and LXRβ are 77% structural identical. On the other hand, they have great differences in expression. LXRα is expressed in the liver, intestine, kidney, adrenal glands, adipose tissue and macrophages, whereas LXRβ is expressed throughout the entire body. The targets for both LXRα and LXRβ are highly overlapping, suggesting redundancy between both receptors [37]. LXRs regulate gene expression linked to the cholesterol metabolism in tissue-specific manner. LXR controls reabsorption of cholesterol in the intestine and regulates reverse cholesterol transport in macrophages. These events result in systemic activation of LXRs and therefore initiate transcriptional programs that regulate cholesterol content in the entire body [38]. In addition, LXRs are also important regulators of inflammatory gene expression and innate immunity. There are several indications that LXR inhibits inflammatory genes and chemokines [39,40]. Finally, a recent study revealed possible involvement of LXRs in the adaptive immune system [41].

Chapter 2: Immunity and Inflammation

2.1 The Immune System

Our body has the ability to protect itself against infectious disease. The immune system is the system that modulates the reaction against diseases. The immune system consists of two types of defences, a less specific component and a specific component. The less specific component is called innate immunity and provides the first line of defence against infection. Almost all components of innate immunity are present before infection and consist of non-specific disease-resistance mechanisms. Although not specific, these mechanisms function via recognition of specific molecules usually found on frequently encountered pathogens. Anatomic-, physiologic-, phagocytic/endocytic- and inflammatory barriers are all non-specific host defences. Few of these are phagocytic cells, like macrophages and barriers like the skin and they all play important roles in innate immunity [42,43]. The specific component of immunity is called adaptive immunity and only reacts if there is an antigenic challenge to the organism. The adaptive immunity has the ability to react in specific manner and can recall on memory. Normally, five days or more are needed to cause a reaction of the adaptive immune system against an antigen. A later exposure to the same antigen results in a faster memory response. This leads to a stronger and faster reaction against that antigen. The most important factors of adaptive immunity are lymphocytes and the antibodies [44,45]. Logically, innate immunity provides a first line of host defence and most pathogens are cleared by the innate immune system before they can activate the adaptive immune system [46]. We will discuss the properties of the adaptive immunity in more detail in the next paragraph.

2.2 Adaptive Immunity

The adaptive immunity is able to recognise and eliminate specific antigens. In contrast to innate immunity, adaptive immunity reacts to specific antigenic challenges. Four characteristics including, antigenic specificity, diversity, immunologic memory and self/non-self recognition define the adaptive immune system [47]. Antigenic specificity enables the adaptive immune system to define small differences between antigens. The antibodies produced during the immune response are able to recognise only single amino acid differences. The adaptive immune system can recognise many different molecules and therefore has a great diversity. If an antigen has been recognised once, the second encounter will induce a faster and heavier reaction due to immunologic memory of the adaptive immune system, providing long-term protection against a similar antigen. Finally, the immune system recognises only foreign antigens enabling self/non-self recognition. Outcomes are fatal if the adaptive immune system would react against own molecules. Adaptive immunity is dependent on innate immunity as non-specific phagocytic cells are essential for the start of the specific immune response [46]. For an effective immune response of the adaptive immune system, T lymphocytes and B lymphocytes are needed. Antigen presenting cells (APCs) are also needed to present foreign antigen. The most common APCs are the macrophages and the dendritic cells. T lymphocytes include T helper (Th) cells and T cytotoxic (Tc) cells [45]. B lymphocytes are needed for production of antibodies and for the immunologic memory. In the humoral response (antibody dependent) of the immune system, B cells interact with antigen and differentiate in antibody-producing plasma cells [44]. In the cellular response of immune system, T lymphocytes recognise antigens on APCs resulting in the production of cytokines by Th cells. Tc cells develop into cytotoxic T lymphocytes (CTLs), which mediate the killing of infected cells [45]. Both humoral and cellular responses cooperate together to remove foreign antigens in the adaptive immune response [47,122].

2.3 Inflammation

Inflammation is a complex response to a pathogen, cell damage or allergens. Inflammation is the protective reaction of an organism to remove injury and is also part of the healing process of the damaged tissue [48,49]. Infection is caused by an exogenous pathogen, which utilises host's resources to multiply. Inflammation is therefore often linked to the immune system if infection is the base of inflammation. The tight regulation of inflammation leads to successful wound healing and limited tissue loss. In some situations, uncontrolled inflammation can lead to certain type of diseases like hay fever. Inflammation can be divided in acute - or chronic inflammation, both showing several characteristics as discussed in the next section (Table A).

Table A

Characteristics of Acute Inflammation:	Characteristics of Chronic Inflammation:
Short-term reaction of the body	Long term reaction of the body
Increased movement of leukocytes to injury	Infiltration of mononuclear immune cells to injury
Characterisation by five cardinal signs (I-V)	No characterisation with five cardinal signs
Constant inflammatory stimuli needed	Oxidative stress and tissue damage
	Repair by local angiogenesis and connective tissue
	No repair leads to continuation of inflammation
	Periodic inflammatory reaction in some diseases

Acute inflammation is a short-term response of the body to injury and is achieved by increased movement of leukocytes to the injury site [50]. Complex biochemical reactions regulate the inflammatory response. The local vascular- and immune system are involved in these reactions. Acute inflammation is characterised by the five cardinal signs (Figure 6). The first two signs, heat (I) and redness (II) are caused by increased blood flow due to vasodilation and increased temperature at the injury site. Swelling (III) is caused by the accumulation of fluid due to increased permeability of the blood vessels at the area around the injury site. Pain (IV) is caused by the release of chemicals during inflammation, which stimulate the nerve endings. Finally, loss of function (V) can have multiple causes. Occasionally, several of these five signs will not appear in inflammation [51].

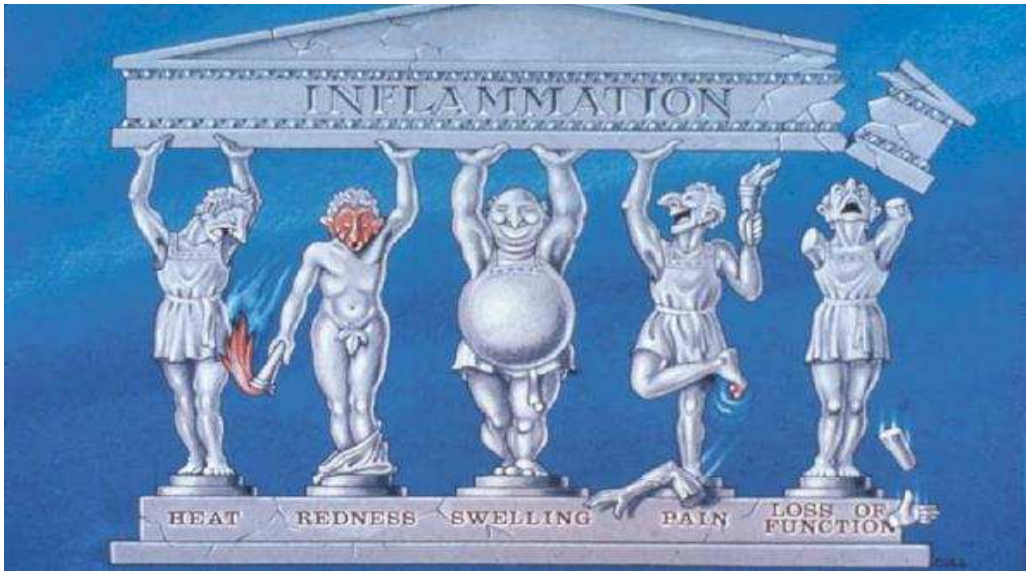

Figure 6. The five cardinal signs of inflammation. Heat and redness are caused by increased blood flow and increased temperature. Swelling is caused by accumulation of fluid. Pain is caused by the release of chemicals, while loss of function can have multiple causes. *Adapted from: Focosi D. 2005,* [123].

Acute inflammation is initiated by macrophages, dendritic cells, endothelial cells and mast cells. These cells are commonly present in all tissues. If these cells are activated, they release inflammatory mediators. Inflammatory mediators are often referred to as cytokines and chemokines and play a role in both acute - and chronic inflammation. In addition to mediators which originate from cells, acellular mediators also play a role in both inflammatory responses. These acellular mediators include the complement system and coagulation reactions. Acute inflammation needs constant stimulation by inflammatory mediators to maintain active. If the inflammatory stimuli disappear, inflammation will cease. In contradiction to acute inflammation, chronic inflammation is a long-term reaction of the body. The inflammatory stimulus is persistent in chronic inflammation, in contrast to acute inflammation. Therefore, the inflammation site is characterised by infiltration of different mononuclear immune cells. Mononuclear immune cells and other leukocytes cause oxidative stress and damage the body's own tissues. This leads to simultaneous cell-loss, an important characteristic of chronic inflammation. Another important characteristic of chronic inflammation is healing of the damaged tissue by local angiogenesis and fibrous connective tissue. During chronic inflammation, the body is not always able to repair tissue damage leading to continuation of the inflammatory cascade. In some diseases the chronic inflammation reaction can become only active periodically. Other than acute inflammation, chronic inflammation is not characterised by the five cardinal signs of inflammation described earlier [50,51,122].

2.4 Inflammatory Mediators

Inflammation is regulated by inflammatory mediators. In order to have an effective immune response it is critical that these mediators function properly in cell-cell communication. Inflammatory mediators bind to specific receptors on target cells and regulate gene expression via signal transduction [52]. Common receptors are the Toll-Like-Receptor (TLR), Tumor Necrosis Factor receptor (TNFr) and Interferon γ receptor (IFNγr). They bind inflammatory mediators and regulate further gene expression via different signalling pathways. The JAK-STAT signalling pathway is important for regulation of cellular responses to inflammatory mediators, as this pathway transduces a signal from membrane to nucleus. Binding of a inflammatory mediator to a receptor activates JAK proteins, which form tyrosine residues on the receptor. These tyrosine residues initially act as a binding site for STAT proteins. Phosphorylation by both JAK proteins and tyrosine residues leads to activation of STAT proteins. Finally, the activated STAT proteins form dimers and accumulate in the nucleus where they regulate gene expression [53]. As a consequence, most inflammatory mediators can therefore regulate cellular activity in a coordinated interactive manner. The most common inflammatory mediators are the cytokines. Cytokines can influence the activity of numerous cells involved in the entire immune response. Their action can either be autocrine, paracrine or endocrine. Cytokines can be divided into several classes (Table B).

Table B

Cytokine Class:
Interleukins (ILs)
Tumor Necrosis Factors (TNFs)
Colony Stimulating Factors (CSFs)
Interferons (IFNs)
Chemokines

Cytokines can also be classified by the difference of binding to their specific receptor. The cytokine receptors can be divided in type I and type II (Table C). The differences are based on structure and extracellular region motifs.

Table C

Cytokine Receptor Type I:	Cytokine Receptor Type II:
IL-4	IFN-γ
IL-6/12	IL-10
IL-2	IL-19
IL-7	IL-20
IL-9	IL-22
IL-15	IL-24
IL-21	IL-26
LIF	IL-28

All mentioned cytokines have specific functions and can be divided in either pro-inflammatory or anti-inflammatory cytokines (Table D). The regulation of the balance of both pro- and anti-inflammatory cytokines is essential for the inflammatory response [52].

Table D

Pro-inflammatory:	Anti-inflammatory:
IFN-γ	TGF-β
TNF	IL-4
IL-1	IL-5
IL-2	IL-10
IL-6	IL-13
IL-8	
IL-12	
IL-18	

Chapter 3: Nuclear Receptors in Immunity

3.1 Transrepression Models in Inflammation

In the first chapter we shortly described the transrepression mechanism of nuclear receptors and the accompanied ability to inhibit inflammatory gene expression. Seven models of inflammatory transrepression are currently studied and we will describe them in detail in this paragraph. The first model **(I)** involved in transrepression represents the induction of IκBα, which inhibits the entry of the NF-κB complex to the nucleus of a cell [54] (Figure 7A). In this model, the binding of a ligand forms a nuclear receptor dimer, which then binds to the IκBα promoter sequence on the DNA. Transcription and translation leads to formation of the IκBα protein. The IκBα protein binds to the NF-κB complex and inhibits the entry to the nucleus. The NF-κB complex consists of the protein subunits p65 and p50 and is a transcription factor involved in the transcription of pro-inflammatory mediators. Therefore, we can conclude that induction of IκBα inhibits the inflammatory reaction. We will discuss this again in a later paragraph. A second model **(II)** of transrepression suggests a coactivator competition model of transrepression, which represses many target genes (Figure 7B) [55,56]. In this model there is competition for a limiting pool of coactivators, including the CREB-binding protein (CBP). CBP is an transcription enhancing enzyme, which binds to CREB. Previously, we already mentioned that CREB binds to cAMP response elements on the DNA, thereby increasing or decreasing transcription. If there is heavy competition for such a low available coactivator it can be assumed that there is either slow or no transcription of many target genes. These observations might explain the antagonism between inflammatory transcription factors and nuclear receptor signalling, as the outcome of the competition model can either be transcription of inflammatory target genes or no transcription of those target genes. On the other hand, the exact physiological relevance of coactivator competition in the counter regulation of target genes by nuclear receptors is not clearly understood. A third model **(III)** of transrepression suggests that a nuclear receptor inhibits the binding of p65 to interferon regulatory factor 3 (IRF3), normally enhancing the transcription of the inflammatory mediators interferon-α and interferon-β (Figure 7C) [57,58]. P65 has a role as coactivator of IRF3 on genes containing interferon stimulated response elements (ISRE). In this model, interactions between p65 and a nuclear receptor inhibit IRF3 binding. These events prevent the activation of target genes of IRF3, which would have led to the transcription of inflammatory mediators. Therefore, this model can directly inhibit inflammatory gene transcription of IRF3 target gens. On the other hand, IRF3 does not always require p65 as coactivator. This might indicate that this transrepression model can be regulated in a signal-specific manner, still allowing inflammatory gene transcription by IRF3 via another coactivator [58]. Opposite to the previous model is the suggestion of the fourth model **(IV)**, where IRF3 acts as a coactivator in stead of P65. This model is based on the disruption of the coactivator complex. The disruption is initiated by the nuclear receptor being tethered to the P65 subunit of the NF-κB complex, inhibiting the recruitment of coactivator IRF3 to P65 (Figure 7D) [59]. If the interaction between P65 and coactivators like IRF3 is repressed, the transcription of inflammatory gene expression is inhibited. Similar to the third model we described, this model can also directly inhibit inflammatory gene transcription, although only of NF-κB target genes. Similar in function to the fourth model is the fifth model **(V)**, which is based on a block in RNApol II hyperphosphorylation. If a nuclear receptor binds to the P65 subunit of the NF-κB complex, recruitment of positive elongation factor b (P-TEFb) is inhibited (Figure 7E). P-TEFb is a very important transcription factor needed for phosphorylation of RNApol II and the accompanied activation of the elongation stage of transcription. If the recruitment of P-TEFb to RNApol II is disrupted, gene transcription is

inhibited. Similar to the previous described model, this transrepression model can also directly inhibit gene transcription of NF-κB target genes, thereby repressing the inflammatory response [60,61]. In contradiction with the previous five transrepression models, the final two models are corepressor-dependent. The sixth **(VI)** model is based on histone modification by histone deacetylases (HDAC). In the first chapter we already described that corepressors are involved in deacetylation of histones. We also highlighted the two important corepressors NCoR and SMRT, which can form complexes that contain HDAC. In this suggested sixth model a nuclear receptor dimer is formed, which interacts with HDAC2. This interaction inhibits gene transcription of NF-κB target genes by deacetylation of histones and the formation of a repressor complex (Figure 7F) [62-64]. Similar to the other models, this model can repress gene transcription of NF-κB target genes. The final and seventh **(VII)** model suggested is based on the inhibition of corepressor clearance by SUMOylation, which is a form of post-translational modification of proteins. In this model, a nuclear receptor (PPARγ in this example) interacts with protein inhibitor of activated signal transducer and activation of transcription 1 (PIAS1). If PPARγ is activated by rosiglitazone (Ro) or another ligand, a conformational change enables SUMOylation at position K367 (K) by E2 conjugation enzyme (UBC9). Small ubiquitin-like modifier 1 (SUMO1) modification of PPARγ initiates interaction with the NCoR-HDAC-TBL1 corepressor complex. This interaction blocks the recruitment of the Ubc5/19S proteosome machinery required for corepressor clearance, eventually keeping the NF-κB target gene transcription in a repressed state (Figure 7G) [65]. In addition to this model, a possible function of the NCoR-HDAC3-TBL1 complex as a repression checkpoint might be proposed as transducin β-like protein (TBL1) is shown to be involved in recruitment of ubiquitin conjugating machinery, removing the nuclear receptor corepressor complex [66]. Moreover, even a role in ligand-dependent negative regulation by other nuclear receptors may be suggested. All these findings suggest that this final model can inhibit inflammatory responses by blocking the signal-dependent clearance of NCoR corepressors [65,66]. In this paragraph we described seven models involved in transrepression by nuclear receptors and the table below gives an overview of the differences between all suggested models (Table E).

Table E

Model number	Characteristics	Mechanism	Direct DNA-binding of NR	Corepressor - dependent
(I)	IκBα induction	Keeps NF-κB complex in cytoplasm	YES	NO
(II)	Coactivator competition	Heavy competition for coactivators	NO	NO
(III)	Inhibition of IRF3 binding	Interaction between NR and P65	NO	NO
(IV)	Disruption of coactivator complex	NR is tethered to P65 inhibiting IRF3 binding	NO	NO
(V)	Block in RNApol II hyperphosphorylation	Binding between NR and P65 inhibits P-TEFb recruitment	NO	NO
(VI)	Modification by histone deacetylases	Interaction between HDAC and NR	NO	YES
(VII)	Inhibition of corepressor clearance by SUMOylation	SUMO1 modification of NR inhibits corepressor clearance	NO	YES

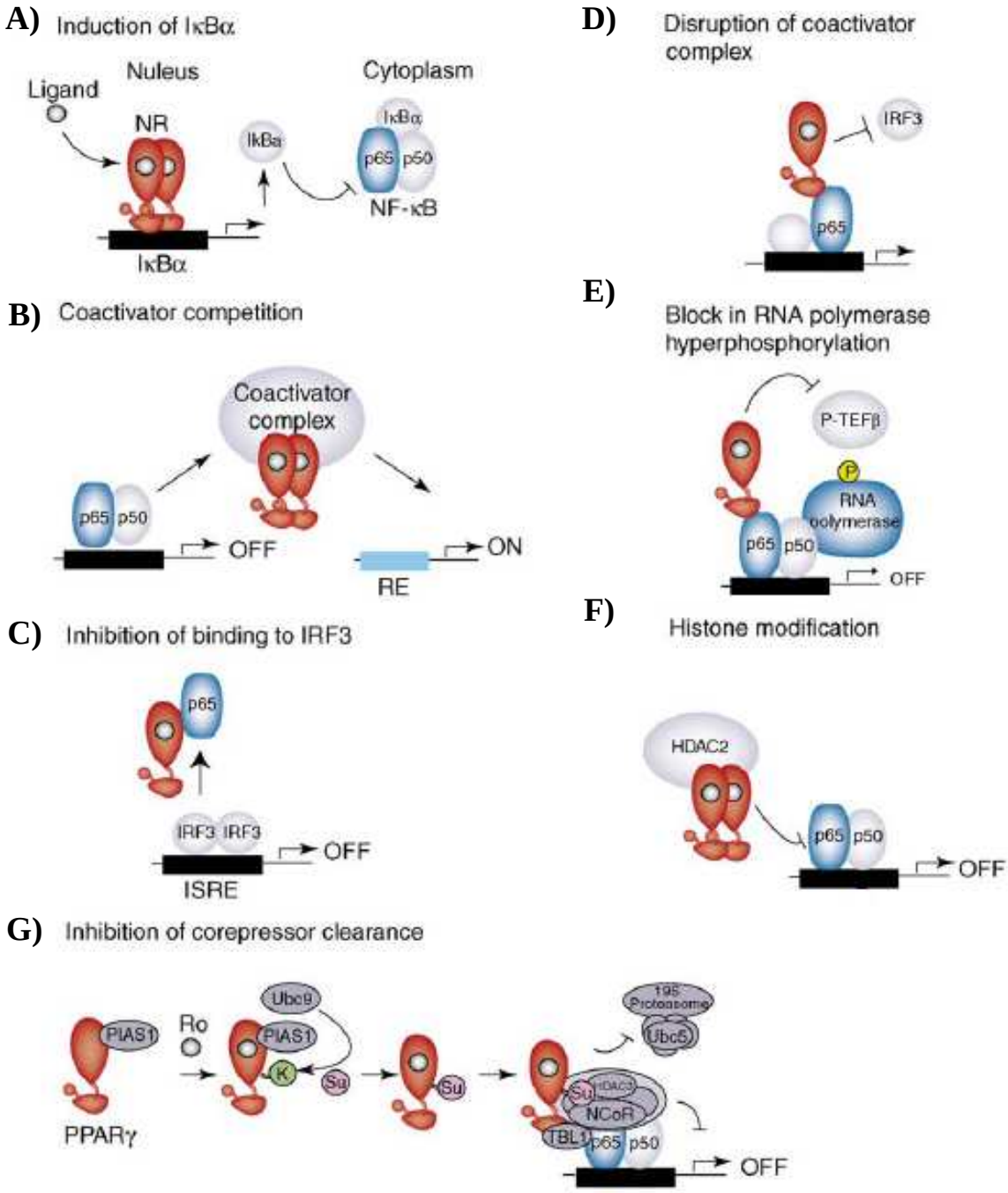

Figure 7. Models of transrepression by nuclear receptors. A) Induction of IκBα expression. B) Competition between coactivators. C) Inhibition of IRF3 binding by p65 interaction with a nuclear receptor. D) Inhibition of Coactivator recruitment to p65 and disruption of coactivator complex. E) Block in RNA polymerase hyperphosphorylation. F) Histone modification. G) Inhibition of corepressor clearance by SUMOylation. *Adapted from: Pascual G et al. 2006,* [17].

3.2 GR and the Regulation of Inflammatory Gene Expression

In chapter 1 we discussed main functions of GR and its ability to regulate gene expression. Furthermore, we described that GR can modulate inflammatory responses by the regulation of inflammatory gene expression in different cells. Finally, we have seen that GCs are the ligands for the GR. The presence of GCs can inhibit transcriptional actions of several pro-inflammatory mediators in many cells, including lymphocytes, macrophages, neutrophils, mast cells and eosinophils. There are indications that most anti-inflammatory actions of the GR are based on one or transrepression mechanisms (Figure 7) [67]. Many pro-inflammatory mediators induce transcription by the activation of signal-dependent transcription factors like NFκB, AP1 or STAT. These transcription factors are able to regulate the expression of a wide variety of cytokines, chemokines, growth factors and other inflammatory mediators in many inflammatory cells [68]. Repression of these inflammatory genes occurs via GR activity or via GR ligands [69,70]. In the next paragraph we will discuss the mechanisms involved in this repression.

3.3 Mechanisms Involved in Inflammatory Gene Repression by GR

The GR can affect the inflammatory gene expression of the signal-dependent transcription factors NFκB, AP1 or STAT in various ways (Figure 8). Either ligand-dependent transrepression or ligand-dependent transactivation has been described to be involved in inflammatory gene regulation (Figure 4A, D). The first studies described protein-protein interaction (transrepression) between AP-1 and GR [18,71,72]. Furthermore, there are two molecular mechanisms involved in NFκB inhibition by the GR. One mechanism involves physical protein-protein interaction between GR and NFκB [19,73-75]. The other mechanism involves upregulation of the IκB protein, which prevents NFκB transcriptional activation by retaining the NFκB protein in the cytoplasm. This upregulation of IκB occurs by ligand-dependent transactivation (Figure 7A) [54,76]. Interaction between GR and AP-1 or NFκB, or either upregulation of IκB results in inhibition of several cytokines like IL-1, IL-2, IL-5, IL-6, IL8, TNFα and IFNγ [61,77]. Interactions between GR and the STAT family members have also been described. These interactions either occur by modulation of several intracellular signalling pathways in which STAT family members are involved or by ligand-dependent transrepression (Figure 8) [78-80]. These interactions between GR and the STAT family result in inhibition of the transcriptional activity by STAT. The cytokines IL-4 and IL-12 are both regulated by the STAT family and can be inhibited. Subsequently, another mechanism possibly involving ligand-dependent transactivation by GR is the suppressors of cytokine signalling (SOCS) protein expression (Figure 8). SOCS contain SH2 domains which interact with JAKs, competitively blocking further interaction with STATs [81]. Expression of SOCS is increased if TLR ligands increase, leading to negative feedback [82]. In addition, SOCS are able to interfere with the TLR signalling pathway by degrading the Toll-Interleukin 1 Receptor Domain Containing Adaptor Protein (TIRAP) [83]. Moreover, ligand-dependent transactivation of the GILZ protein by the GR dimer also influences TLR mediated inflammation (Figure 8) [84,85]. GILZ is a small protein which has many anti-inflammatory actions. Studies showed that GILZ physically interacts with AP-1 and NFκB and inhibits DNA binding of both transcription factors [86,87]. Finally, GILZ inhibits several phosphorylation steps in TLR signalling, inhibiting activation of transcription factor AP-1 [88]. Because NFκB, AP-1 and STAT are major factors in inflammatory gene regulation, GR shows to be an important (trans)repressor of inflammation.

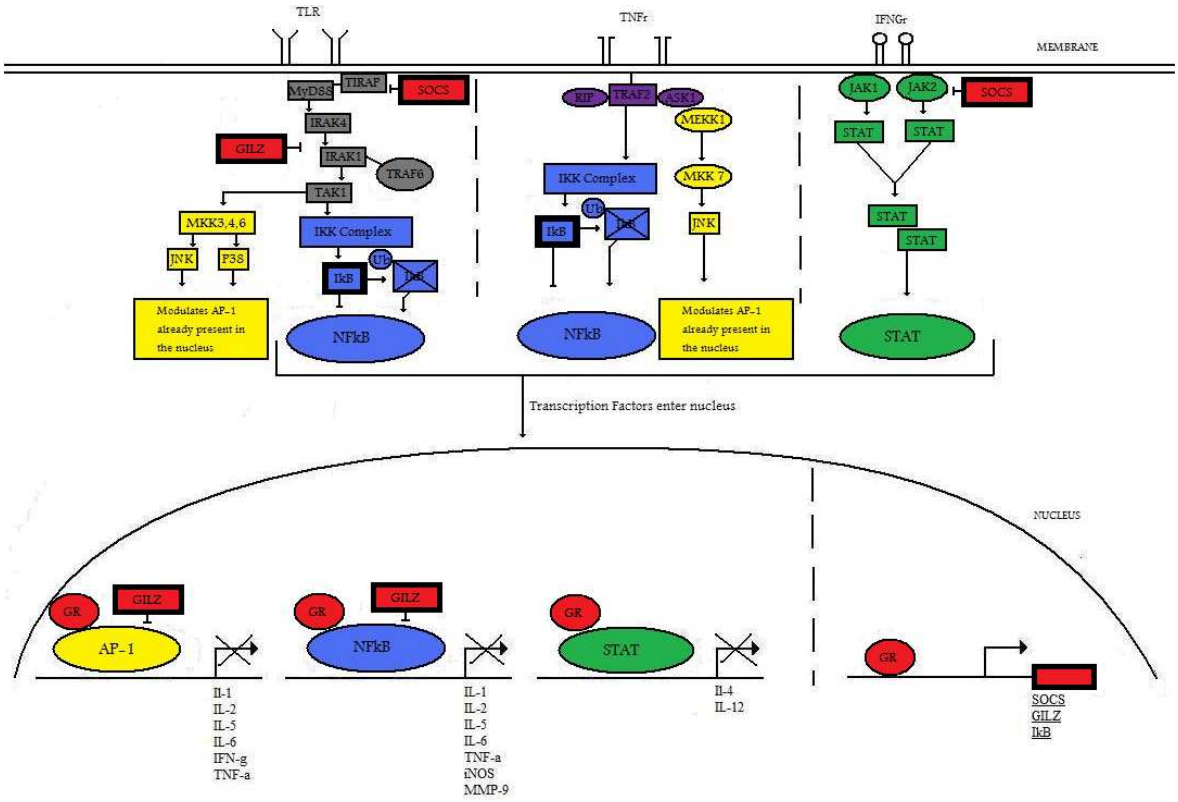

Figure 8. Overview of the mechanisms of inflammatory gene repression by GR . Signalling via TLR (grey), TNFr (purple) or IFNGr results in activation of AP1 (yellow), NFκB (blue) or STAT (green). Ligand-dependent transrepression of AP1, NFκB or STAT by GR (red) leads to inhibition of cytokine and other inflammatory gene transcription. Ligand-dependent activation of SOCS, GILZ or IκB results in inhibition in the signalling pathways. SOCS interacts with JAK proteins and degrades TIRAP, inhibiting appropriate signalling. GILZ inhibits DNA binding of AP-1 and NFκB and interferes with several phophorylation steps in TLR signalling. Upregulation of IκB keeps NFκB in the cytoplasm, inhibiting gene transcription.

3.4 PPARγ and the Regulation of Inflammatory Gene Expression

Similar to GR, PPARγ can play a role in the regulation of inflammatory gene expression, therefore influencing the inflammatory response [89,90]. In chapter 1 we have described that PPARγ and its ligands can inhibit the production of many inflammatory mediators and cytokines in various cells. These cells included monocytes, macrophages, dendritic cells, lymphocytes, smooth muscle cells, and endothelial cells. Moreover, studies showed that PPARγ has anti-inflammatory effects in several diseases like atherosclerosis, Parkinson, Alzheimer and diabetes [91,92]. There is evidence that PPARγ can inhibit inflammatory gene expression by several mechanisms, including competition of coactivators (Figure 7B) and direct protein-protein interaction with NFκB and AP-1 [93,94]. The next paragraph will describe the mechanisms in detail.

3.5 Mechanisms Involved in Inflammatory Gene Repression by PPARγ

In several studies PPARγ suppressed the upregulation of IL-1, IL-12, TNFα and inducible nitric oxide synthase (iNOS) [95,96]. PPARγ has also been shown to inhibit expression of IFNγ and IL-2 after T cell activation [97]. Even T lymphocyte proliferation and immune activation is regulated by PPARγ [89]. Most of the inflammatory gene repression by PPARγ is achieved via the transrepression mechanism. Direct interaction between PPARγ and AP-1, NFκB and the STAT family leads to downregulation of cytokines and other inflammatory mediators by inhibiting AP-1, NFκB and STAT DNA-binding (Figure 9) [98]. In a new study, the transrepression mechanism involves SUMOylation of the PPARγ ligand-binding domain by

the SUMO1 protein (Figure 7G & 9). This event targets PPARγ to corepressor complexes on inflammatory gene promotors. This leads to a repressed state of the promoter and inhibition of inflammatory gene expression [65]. This mechanism can explain the conversion of transcriptional activator to transcriptional repressor of NFκB and AP-1 target genes, which regulate inflammation. PPARγ ligands are capable of inducing anti-inflammatory responses through PPARγ independent mechanisms (Figure 9). 15dPGJ2 can directly inhibit NFκB signalling by the inhibition of IKK complex and the DNA-binding ability of NFκB (Figure 9) [99-101]. Furthermore, PPARγ ligands also inhibit JNK activity, necessary for AP-1 activation (Figure 9) [102].

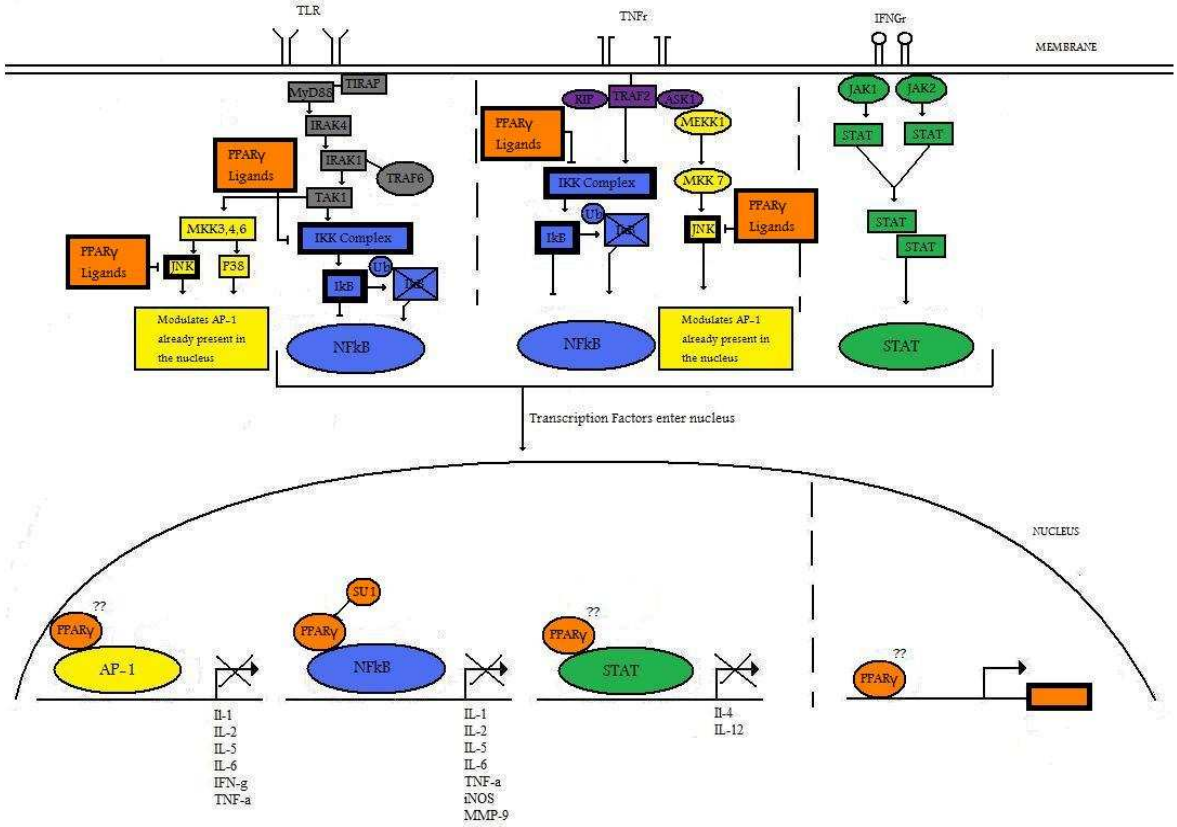

Figure 9. Overview of the mechanisms of inflammatory gene repression by PPARγ . Signalling via TLR (grey), TNFr (purple) or IFNGr results in activation of AP1 (yellow), NFκB (blue) or STAT (green). Ligand-dependent transrepression of AP1, NFκB or STAT by PPARγ or ligands (orange) leads to inhibition of cytokine and other inflammatory gene transcription. This transrepression is mediated by SUMOylation with the SUMO1 protein (SU1). PPARγ ligands interfere with JNK and IKK complex signalling, resulting in inhibition of AP-1 and NFκB respectively. There is no evidence of ligand-dependent activation of PPARγ, which would result in inhibition of inflammatory gene repression.

3.6 LXR and the Regulation of Inflammatory Gene Expression

LXRs were characterised as nuclear receptors regulating cholesterol homeostasis [103]. If the ligands (oxysterols) bind to LXRs, they induce expression of a number of genes that protect cells from excess cholesterol [104]. LXRs are very important for cholesterol homeostasis in macrophages. This is seen in atherosclerotic development in mice, where treatment with LXR agonists inhibits the development of atherosclerotic plaques [105]. During atherosclerosis, the artery wall thickens and forms a plaque. This is the result of accumulation of fatty materials and cells including both cholesterol and macrophages. These findings suggest a possible role in regulation of inflammatory signalling in macrophages. As LXRs are expressed in many tissues throughout the body, it can be assumed that LXRs play a role in regulation of

inflammatory signalling in many other cells. LXR controls several genes, including IL-1, Il-6 and iNOS [106]. PPARγ and GR regulate similar genes and therefore LXR may have similar mechanisms regulating the inflammatory response. However, the mechanisms are not yet completely understood. The next paragraph will discuss the current knowledge of mechanisms linking LXR and the inflammatory response.

3.7 Mechanisms Involved in Inflammatory Gene Repression by LXRs

Ligands of LXR inhibit the inflammatory response by inhibition of several cytokines [39,40] (Figure 10). These cytokines IL-1, Il-6 and iNOS among others, are normally NFκB-dependent. This indicates that a similar mechanism like PPARγ and GR may inhibit NFκB via ligand-dependent transrepression. Moreover, evidence showed that parallel SUMOylation pathways mediate gene- and signal-specific transrepression pathways by either SUMO2 or SUMO3 (Figure 7G & 10). Similar to PPARγ, ligand-dependent conjugation of SUMO2,3 to LXR targets them to promoters of target genes. There, prevention of signal-dependent removal of NCoR corepressor occurs, which represses transcriptional activation [107]. Unlike PPARγ and GR, no transrepression mechanisms have been identified concentrating on AP-1 or the STAT family (Figure 10). On the other hand, LXR shows to have a role in the control of macrophage survival. LXRs seem to increase the expression of the anti-apoptotic AIM, while they reduce pro-apoptotic factors. This means that LXR enhances innate immunity for pathogens that evade the immune response by inducing apoptosis of macrophages [40,108].

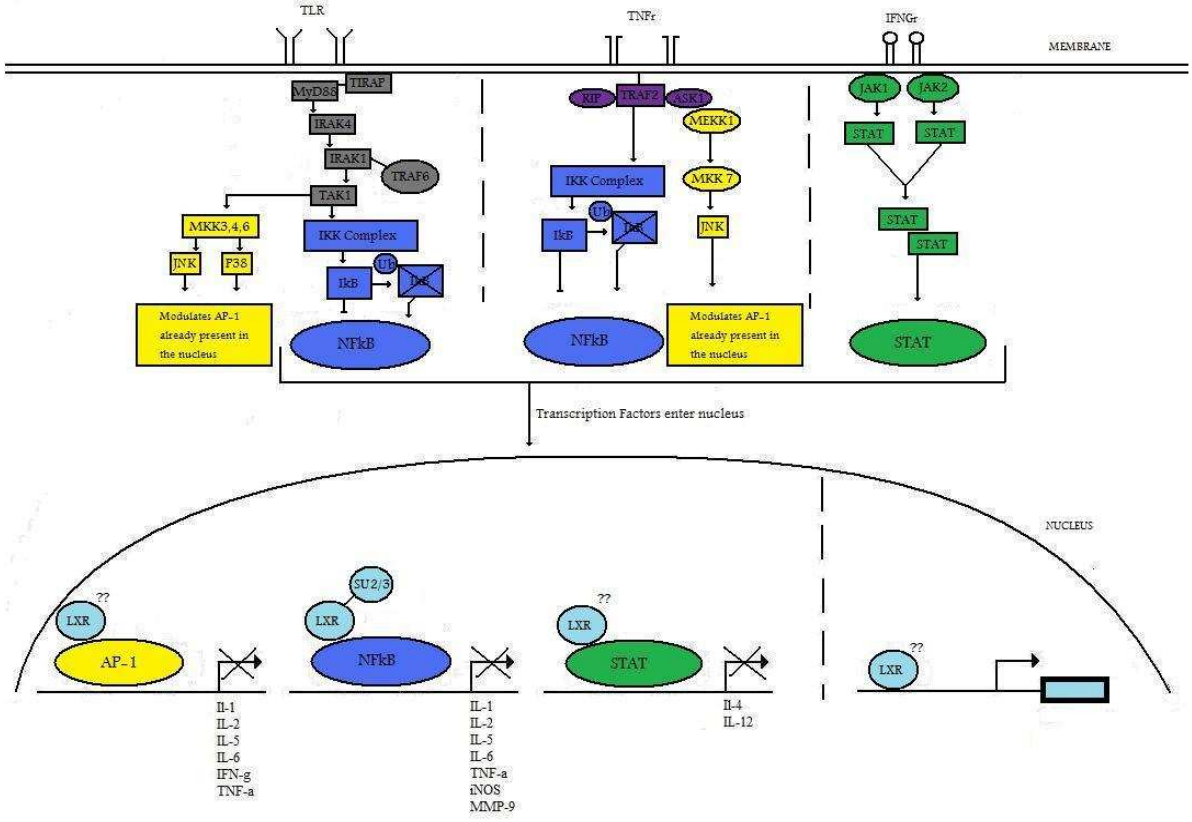

Figure 10. Overview of the mechanisms of inflammatory gene repression by LXR . Signalling via TLR (grey), TNFr (purple) or IFNGr results in activation of AP1 (yellow), NFκB (blue) or STAT (green). Ligand-dependent transrepression of NFκB by LXR (light blue) leads to inhibition of cytokine and other inflammatory gene transcription. This transrepression is mediated by SUMOylation with the SUMO2 and SUMO3 proteins (SU2/3). Ligand-dependent transrepression mechanisms of AP-1 and STAT by LXR has not been shown. There is also no evidence of ligand-dependent activation of LXR.

3.8 Differences Between Molecular Mechanisms of GR, PPARγ and LXR in Inflammatory Gene Regulation.

In the previous sections we described the mechanisms involved in inflammatory gene regulation for GR, PPARγ and LXR. Furthermore we described the mechanisms involved in inflammatory gene repression for all three nuclear receptors. Similar for all nuclear receptors is the ability to inhibit inflammatory signalling by the inhibition of NFκB. The inhibition of NFκB target genes can be regulated by one or more transrepression models described earlier in the first paragraph of this chapter. Similar for PPARγ and GR is the inhibition of cytokine transcription by transrepression of STAT family members and AP-1. It seems likely that LXR transrepresses these members via a similar mechanism, although that is poorly understood. Different from LXR and PPARγ is the ability of GR to directly bind to DNA and initiate direct transcriptional activation of SOCS, GILZ and IκB, which all inhibit either AP-1 or NFκB activation. Although the mechanisms of transrepression involved in AP-1 and NFκB are similar, mutations in the zinc finger of GR affect NFκB inhibition. AP-1 inhibition remains unaffected, showing non-identical requirements for transrepression between AP-1 and NFκB [109]. A unique feature for PPARγ ligands is the ability of modulating signal transduction, thereby inhibiting JNK activation and the IKK complex. Ligands of LXR and GR are not shown to inhibit inflammatory gene expression. The table below gives an overview of the differences between the mechanisms involved in inflammatory gene regulation (Table F).

Table F

Nuclear Receptor	Inhibition of:	Mechanism involved in inflammatory gene regulation:
GR	- NFκB - AP-1 - STAT	- Transrepression - Direct transcriptional activation of SOCS, GILZ and IκB
PPARγ	- NFκB - AP-1 - STAT	- Transrepression - Modulation of signal transduction by PPARγ ligands
LXR	- NFκB	- Transrepression

Previous findings suggest that there could be redundancy between the nuclear receptors GR, PPARγ and LXR in the inhibition of the target genes of NFκB, AP-1 and STAT. The transrepression mechanism seems to be important in the assumption of combinatorial control of inflammatory gene expression. Important evidence for this statement was published in a study by Ogawa *et al.* In this study, GR, PPARγ and LXR were compared by stimulation of the TLR pathway [58]. Stimulation of TLR pathways was achieved by the addition of lipopolysaccharide (LPS) and Poly I:C. In the previous paragraphs we have described that activation of a TLR pathway can lead to transcription of inflammatory genes if transrepression by a nuclear receptor is absent. Results of this study showed that GR repressed many functionally related inflammatory response genes by disrupting the binding of p65 and IRF as seen in the first paragraph (Figure 7C). On the other hand, PPARγ and LXR showed repression on overlapping but different p65/IRF-independent genes, indicating redundancy between nuclear receptors and their repressed target genes. Further research showed that 543 LPS responsive genes and 572 Poly I:C responsive genes were researched for sensitivity after simultaneous stimulation by combinations of GR, PPARγ and LXR. Stimulation by LPS showed that only 98 of the 543 genes are simultaneously responsive to GR, PPARγ and LXR, while stimulation by Poly I:C showed that only 44 of the 572 genes are simultaneously responsive to GR, PPARγ and LXR (Figure 11). Simultaneous activation of either GR and

PPARγ or GR and LXR still showed characteristic inhibition of TLR responses, indicating that there is combinatorial control of immunity and inflammation by two or more nuclear receptors. Other combinations between GR, PPARγ and LXR also showed redundancy between LPS- or Poly I:C-responsive genes. The exact number of responsive genes in combination with LPS- or Poly I:C stimulation are shown below (Figure 11) [58].

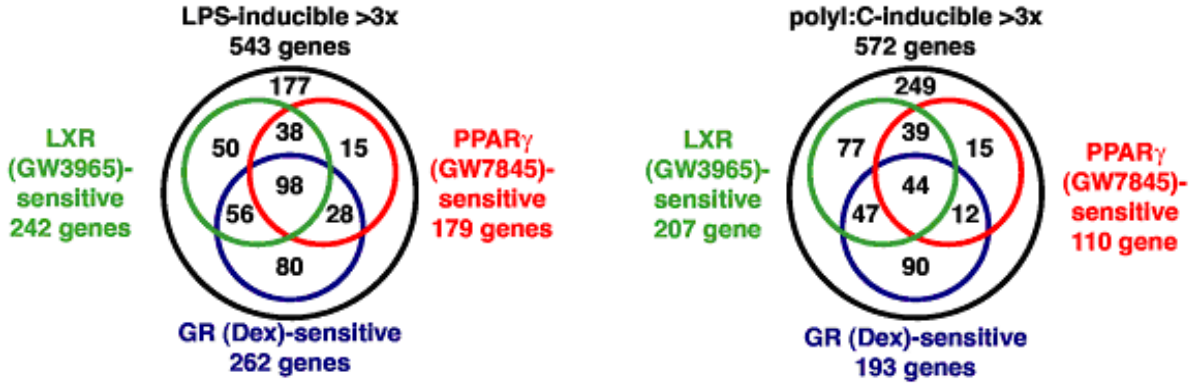

Figure 11. Venn diagrams of LPS and Poly I:C-responsive genes. The Venn diagrams indicate sensitivity of LPS- (right) and Poly I:C-responsive genes (right) to GR, PPARγ and LXR. Specific ligands were used for all three nuclear receptor (shown between brackets). Only 98 out of 543 genes are simultaneously responsive to GR, PPARγ and LXR if stimulated by LPS, while only 44 out of 572 genes are simultaneously responsive to GR, PPARγ and LXR if stimulated by Poly I:C. This still indicates combinatorial control of (inflammatory) genes by nuclear receptors. *Adapted from: Ogawa S et al. 2005,* [58].

The previous findings indicate that either GR, PPARγ or LXR only target a subset of the TLR-inducible NFκB target genes, which suggests that a higher number of activated nuclear receptors can more easily transrepress inflammatory gene transcription. This statement also clarifies the previous suggestion that there is redundancy between the nuclear receptors GR, PPARγ and LXR in the inhibition of the target genes of NFκB, AP-1 and STAT. Important future research should focus on similarities between transrepression mechanisms and targets of GR, PPARγ and LXR. Another possible research topic could include the sensitivity of STAT- or AP-1 pathway responsive genes to GR, PPARγ and LXR, similar to the study by Ogawa *et al.* Finally, a clinical trial should investigate if anti-inflammatory drugs like glucocorticoids could function at lower doses with fewer side effects by simultaneous administration with PPARγ or LXR ligands, since we have seen that more inflammatory genes can be transrepressed if a combination of GR, PPARγ and LXR is used.

Chapter 4: Discussion

In this thesis we described the mechanisms of GR, PPARγ and LXR involved in inflammatory gene regulation. GR is activated by endogenous GCs which are produced in the adrenal cortex, coordinating the regulation of gene expression in the whole body. In contrast to GR, PPARγ and LXR are activated by metabolites of fatty acids and cholesterol. Those are usually produced during local cytokine release and via other autocrine and paracrine mechanisms. We have seen that all three nuclear receptors have common mechanisms, including ligand-dependent transrepression to repress the inflammatory response. In addition, all receptors have receptor-specific mechanisms that can repress the inflammatory response. This indicates that each of the three discussed receptors has a certain effect and influence on the inflammatory response. Furthermore, we descibed that GR, PPARγ and LXR are expressed in macrophages, lymphocytes en dendritic cells, suggesting that there may be control of inflammation via inhibition of cytokine release by these nuclear receptors. Simultaneous stimulation of all three nuclear receptors also showed partly combinatorial control of inflammatory genes. This means that simultaneous activation of several nuclear receptors in a certain cell or cell-type leads to complex gene regulation in either local or systemic signalling pathways. Clearly, we described only few mechanisms controlling inflammatory gene expression in this thesis. Past studies revealed several other aspects of immune regulation which are dependent on nuclear receptors. LXR showed to directly target the arginase II (ArgG II) gene, is a enzyme that catalyses the conversion of L-arginine into L-ornithine and urea [110]. These conversions lead to the forming of polyamines. The Arg II gene can be anti-inflammatory by shifting the arginine metabolism towards polyamine synthesis, which reduces the nitric oxide (NO) production. In normal situation NO is needed for vasodilation in the inflammatory process. PPARα, which is another member of the PPAR family, induced expression of IκB in several cells. Mice experiments showed that PPARα possibly directly targets IκB, which then inhibits NFκB activation and DNA binding [111]. These findings indicate that there are probably more genes involved in inflammatory regulation, which are a direct target for a nuclear receptor. This makes the entire regulation system even more complex. An additional member of the nuclear receptor family, NR4A showed to have effect on NFκB responsive genes [112]. In contrast to the anti-inflammatory function of GR, PPARγ and LXR, NR4A showed to be pro-inflammatory in macrophages. Expression of NR4A in macrophages showed transcriptional activation of multiple genes involved in inflammation, apoptosis and cell cycle control. In contrast to GR and PPARγ, NR4A showed activation of the IKK-complex, which then activates NFκB. These results show a new role for NR4A in the regulation of inflammatory gene expression in macrophages. Moreover, these results imply that nuclear receptors can either be pro- or anti-inflammatory, indicating a more complex inflammatory regulation. In addition, PPARγ might have an influence on local immune response by affecting the cytokine release. By contrast, cytokines also regulate PPARγ expression in macrophages and other immune cells. It is shown that IL-4 upregulates expression of PPARγ in macrophages, while IFN-γ downregulates PPARγ expression [113]. These findings shown that PPARγ mediates anti-inflammatory activities of IL-4, while pro-inflammatory activity needs downregulation of PPARγ. Again, this study gives an example of complex regulation of pro- and anti-inflammatory activities of cytokines and nuclear receptors and additional gene regulation. All previous research can contribute to the development and application of nuclear receptor ligands in certain human diseases. Currently, treatments of several diseases are prone in becoming resistant to steroids and their accompanying receptors. Therefore, it would be beneficial to determine the sensitive or resistant inflammatory-response genes for a certain receptor. It is likely that a disease is GC resistant, if the pro-

inflammatory molecules are resistant to repression by synthetic GCs. This hypothesis indicates that it might be possible to match specific nuclear receptors with a specific disease or -state. This speculation is based on the expression patterns of the receptors and the targeted regulatory genes in certain cells. Currently, there are still no reliable research model systems available as our knowledge is not yet sufficient enough to create appropriate animal models. In contrast to this, research has been done by use of synthetic PPARγ ligands in animals. Improvement after treatment with PPARγ ligands has been found in the inflammatory state of researched animal models in either atherosclerosis, inflammatory bowel disease, arthritis and psoriasis [91,114-116]. In addition to these studies, a clinical trial has been performed where PPARγ ligands reduced the intensity of inflammation in patients suffering from ulcerative colitis [117]. These studies show the potential attractiveness of PPAR ligands as drug targets. On the other hand, creating a therapeutic effect with a natural or synthetic nuclear receptor ligand is very difficult. In many cases undesirable or unacceptable side effects occur, limiting treatment possibilities. Even the widely used GCs can occasionally cause or increase susceptibility for hypertension, type 2 diabetes mellitus or obesity. Improved knowledge of selective modulators of nuclear receptors, which alter the specificity of either coactivator or corepressor attraction would give new insight in development of possible treatments [118]. GR- , PPARγ- and LXR ligands are proven to repress partly overlapping inflammatory genes by different molecular mechanisms. This indicates a possibility that combinations of ligands for GR, PPARγ and LXR might have characteristic effects [58]. These features of nuclear receptor agonists might lead to a desirable treatment effect, while side effects can be decreased. This might be achieved by treating (chronic) inflammatory diseases with a combination of GCs, PPARγ- and LXR ligands at lower doses, minimising the side effects. If we want to expand the potential of therapeutic use of nuclear receptors, we need to fully understand the biological roles and underlying mechanisms of all nuclear receptors. Subsequently, development of new model systems are needed for appropriate research and further development of possible new synthetic ligands. A first step could be a study targeting the importance of the SUMOylation process in inflammatory gene regulating of the nuclear receptors. It was shown that SUMOylation processes prevented clearance of the NCoR complex from signal-responsive promoters by SUMO1, -2 or -3 (Figure 7G) [65,107]. Experiments targeting other nuclear receptors (e.g. GR) and associations with SUMOylation might give insight in the regulation of inflammatory gene expression. This suggestion is strengthened by possible cooperation of different nuclear receptors as both SUMO-LXR and SUMO-PPARγ seemed to interrupt distinct signalling inputs leading to NCoR clearance [58]. A possible experimental setup studying covalent modification between SUMO1, -2 or -3 and nuclear receptors in immune cells could include an in vitro assay of lymphocytes, macrophages and dendritic cells. In this experimental set-up, any nuclear receptor of choice can be labelled, followed by an assay including immunoprecipitation and immunoblotting [119]. For detection of a SUMO1, -2 or -3/Nuclear receptor complex, a protein complex immunoprecipitation (Co-IP) can be used. In addition to this experiment, an enzyme activity assay can be added. SUMOylation is a process which needs several enzymes, including the E1 enzyme and the conjugating enzyme Ubc9. An example studying enzymatic activity could include a suspension of any nuclear receptor of choice, and the SUMO protein. During spectrophotometric analysis, all substances which are needed for the SUMOylation process can be added. The assay eventually shows enzymatic activity and the course of the entire SUMOylation reaction [120]. Further research should focus on the identification of molecular targets of SUMO-LXR and SUMO-PPARγ. This research could lead to possible explanation of their role in the inhibition of NCoR clearance. Another important step would be the development of conditional knock-out animal models. Currently, there are no models available with null alleles for either GR, PPARγ or LXR as such a mutation is often lethal for

embryos. The best option could be a knock-in mouse model with one or more nuclear receptor genes mutated. If this can be achieved, these generated models can be used during experiments with cell-specific knock-outs for any of the 48 nuclear receptors currently known. A possible study could focus on the effect of the mutation of one or more nuclear receptors on the inflammatory gene regulation. An example could include a knock-in mouse model mutating either GR, PPARγ or LXR individually, or a combination of those nuclear receptors. The outcome of these experiments could reveal more about the exact (physiological) functions of one of these receptors. Another outcome could give more insight in the redundancy between nuclear receptors, showing how inflammatory gene expression is regulated if two or more nuclear receptor genes (e.g. PPARγ and LXR) are mutated. Moreover, other proteins involved in inflammatory gene regulation (e.g. SUMO) can be knocked-out in order to study certain aspects. If we are able to successfully perform these tasks, it should be possible to formulate new questions regarding the function of nuclear receptors in the immune system and to study these questions in effective and proper experimental setups.

Acknowledgements

So here we are then, at the end of my Master's thesis. It has been a nice period and I have learned and expanded my scientific writing and reading. It sometimes was a tough and stressful period as well, combining sports, hobbies, work, sleep, social life, other BMS courses and the writing of this thesis in just 168 hours a week!! But in the end it leads to an interesting thesis, al least from my point of view. First of all, I would like to express my gratitude to Eric Kalkhoven for being my supervisor during the writing of this thesis. Your advice, assistance and suggestions really were the biggest factors in completing this thesis.
I know that it can be time-consuming to supervise a student and I found it nice to be advised in a kind and honest way. Thank you again.

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