# Mechanisms of Glucocorticoid Pro-Inflammatory Effects on CCL20: Crosstalk and Synergy

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# Plagiarism declaration

I, Tawanda Nhundu, know the meaning of plagiarism and declare that all of the work in the dissertation, save for that which is properly acknowledged, is my own.

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# Abstract

Glucocorticoids (GCs) are steroid hormones widely prescribed to treat inflammatory disorders and are regarded as anti-inflammatory molecules. GCs classically induce the expression of anti-inflammatory genes, while repressing pro-inflammatory genes via its endogenous cell receptor; the glucocorticoid receptor (GR). Emerging evidence, however, suggests that the mechanisms of GR action are more complex than previously assumed, with many reports of pro-inflammatory actions of GCs via the GR. While chronic exposure to GCs has been noted as anti-inflammatory, reports suggest that acute exposure can increase peripheral immune responses. Specifically, the GCs have been shown to positively regulate the innate immune response, which may be important in preventing the local, affected area from being immunocompromised. Furthermore, the GR can crosstalk with cell signalling pathways involved in pro-inflammatory responses, such as the TNFα pathway, to reciprocally modulate the expression of pro-inflammatory genes. The mechanisms behind the GR's pro-inflammatory actions and crosstalk with inflammatory inducers are not well understood.

GC's pro-inflammatory actions are attributed to GC insensitivity in asthma patients. The insensitivity is attributed to long-term GC usage, and the increase in $T_h17$ neutrophilic airway infiltration. A proposed hypothesis for the increase in neutrophils in the airways was that it was due to an increase in expression of chemokines by epithelial cells due to GC exposure. The pro-inflammatory, chemoattractant cytokine C-C motif chemokine ligand 20 (CCL20) has been previously shown to be induced by GCs and pro-inflammatory inducers in human bronchial cells, with positive modulation of their responses occurring with co-stimulation.

The present study investigated whether the GC dexamethasone (dex) and the pro-inflammatory inducer TNFα could induce CCL20 expression in a variety of human epithelial cell lines, and a simian fibroblast cell line. Using Real-Time Quantitative Reverse Transcription PCR (qRT-PCR), it was confirmed that dex can induce CCL20 mRNA expression, and modulate the TNFα-induced expression in some, but not all cell lines. Moreover, in the HeLa cell line, there was an apparent synergistic response between dex and TNFα, and modulation of the CCL20 response was observed between dex and the pro-inflammatory inducers phorbol 12-myristate

13-acetate (PMA), interferon γ (IFNγ) and lipopolysaccharide (LPS). The GR was shown to be required for the GC induction and modulation of CCL20 mRNA expression. Using promoter-reporter assays, the results showed that the NFκB binding site was necessary for the activation by the pro-inflammatory inducers, but not dex, while the STAT binding region was necessary for the IFNγ activation. Interestingly, lack of the STAT binding site on the promoter-reporter construct caused IFNγ to have repressive effects on CCL20 activation. Stimulation of cells by the pro-inflammatory inducers in the presence or absence of dex had no effect on the total levels of the p65 subunit of NFκB, while dex did appear to cause GR turnover as expected. The results show that dex, via the GR, is able to crosstalk with different pro-inflammatory inducers to induce and potentiate CCL20 mRNA expression and promoter activation. The mechanisms of CCL20 induction and crosstalk with the GR may be different for each pro-inflammatory inducer, however.

Regulation of CCL20 expression is complex, with many transcription factors converging on the promoter region to modulate its expression. This thesis shows that the NFκB binding site is important for the overall induction level of the promoter, however it is not necessary for the dex induced activation. The potentiation of the dex response by pro-inflammatory inducers may be due to the GR interacting with the AP-1 and C/EBP transcription factors, which have been shown to positively interact to increase gene expression. The potentiation of the dex response does not require the activation of NFκB, as IFNγ does not activate the transcription factor, yet can potentiate the dex response.

# List of Abbreviations

| Abbreviation | Full word |
|---|---|
| ANOVA | analysis of variance |
| AP-1 | activator protein 1 |
| C/EBP | CCAAT-enhancer-binding protein |
| cAMP | cyclic adenosine monophosphate |
| CBP | CREB-binding protein |
| CCL20 | C-C motif chemokine ligand 20 |
| ChIP | chromatin immunoprecipitation |
| COS-1 | African green monkey SV40 transformed fibroblast kidney cells |
| CREB | cAMP response element binding protein |
| dex | dexamethasone |
| DEPC | diethyl dicarbonate |
| DMEM | Dulbecco's Modified Eagle's Medium |
| EDTA | ethylenediaminetetra-acetic acid |
| End-1 | human HPV-16 E6/E7 transformed epithelial, endocervical cells |
| ERK | extracellular signal-regulated kinases |
| ERK-1/2 | extracellular-regulated kinase 1/2 |
| EtBr | ethidium bromide |
| EtOH | ethanol |
| FGF1 | heparin-binding growth factor 1 |
| GAS | gamma-interferon-activation sites |
| GC | glucocorticoid |
| GILZ | GC-induced leucine zipper |
| GPCR | G protein–coupled receptor |
| GR | glucocorticoid receptor |
| GREs | glucocorticoid response elements |
| HeLa | human cervix epithelioid carcinoma cells |
| hGR | human GR |
| HPA | hypothalamic–pituitary–adrenal |

| | |
|---|---|
| HSP90 | heat shock protein 90 |
| IFN | interferon |
| IL- | interleukin |
| IRAK | Interleukin-1 receptor-associated kinase |
| JNK | c-Jun N-terminal kinases |
| LPS | lipopolysaccharides |
| NFκB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| PKC | protein kinase c |
| PMA | phorbol 12-myristate 13-acetate |
| RTK | receptor tyrosine kinase |
| RU486 | mifepristone |
| SR | serum response element |
| TNF | tumour necrosis factor |
| U2OS | homo sapiens bone osteosarcoma epithelial cell |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| HDAC | histone deacetylase |
| HIV-1 | human immunodeficiency virus 1 |
| LBD | ligand-binding domain |
| MAPK | mitogen-activated protein kinase |
| MEK1/2 | MAPK/ERK kinase 1/2 |
| MOPS | morpholinopropanesulfonic acid |
| nGRE | negative glucocorticoid-response-element |
| NSC | non-silencing control |
| SRC | steroid receptor co-activator |
| STAT | signal transducer and activator of transcription |
| SGK | serum- and glucocorticoid-induced protein kinase |
| TFIID | transcription factor II D |
| TAT | tyrosine aminotransferase |
| TBP | TATA-box binding protein |
| TBS | Tris-buffered Saline |

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# Chapter 1: Literature Review

## 1.1 Inflammation

Inflammation is a biological response to harmful stimuli such as trauma, pathogen detection or irritants (Feghali *et al.*, 1997). Inflammation is a protective process, involving immune cells, blood vessels and inflammatory molecular mediators such as cytokines. Inflammation can be categorized into two phases, the acute and chronic phase (Feghali *et al.*, 1997). The acute phase is a rapid response, characterized by an increase in blood flow and membrane permeability, an increase in leukocyte trafficking and release of inflammatory mediators. The chronic phase is a long term inflammatory response, characterized by the development of a humoral response, mediated by B-cells, and involving the release of specific antibodies. The chronic phase also involves the activation of macrophages, natural killer cells and T-cells (Abbas *et al.*, 2009). In both acute and chronic inflammation, a host of inflammatory molecules are released such as cytokines, with some cytokines contributing to both phases of the immune response.

Cytokines which play a key role in mediating acute inflammation include IL-1β, TNFα, IL-6, IL-11, GCSF, GM-CSF and chemokines such as IL-8, CCL19 and CCL20. Cytokines important in mediating the chronic phase can be subdivided into two categories; those involved in cellular inflammation, and those in humoral inflammation. Examples of those in cellular inflammation are IL-2, IL-12, interferons (IFNs) such as IFNγ and TNF-α and –β. Those involved in humoral inflammation include IL-4, IL-10, IL-13, and transforming growth factor-β (TGF-β) (Feghali *et al.*, 1997; J.-M. Zhang *et al.*, 2007).

There is a functional difference in CD4+ T-cell responses involved in chronic inflammation based on their profile of cytokine secretion, namely Type 1 T helper (Th1) and Type 2 helper (Th2) cells. Th1 cells provide protection against intracellular bacteria, protozoa, fungi, and several viruses and produce IFNγ, TNFβ, IL-12 and 1L-2 which activate macrophages and cytotoxic T-cells. Th2 cells provide protection against multicellular parasites, extracellular bacteria, some viruses, soluble toxins, and allergens, and produce IL-4, IL-5, IL-10, and IL-13, which is responsible for strong antibody production by promoting B-cells and the actions of

the humoral immune response (Belardelli *et al.*, 2002; Romagnani, 1999). The actions of Th1 and Th2 cells inhibit each other, with IL-12, TNFβ and IFNγ inhibiting Th2 cells, and IL-4, IL-10 and IL-13 inhibiting Th1 cells (Mosmann *et al.*, 1996). Cytokines can also be classed as pro-inflammatory, such as IL-6 and TNFα, and anti-inflammatory, such as IL-10 and IL-4 (Elenkov *et al.*, 2002). The secretion of the two different classes of cytokines is important for fine-tuning and regulating the inflammatory response. The most potent antagonizers of both acute and chronic inflammation, however, are glucocorticoids (Barnes, 1998).

### 1.1.1 Inflammatory ligands

#### 1.1.1.1 LPS

The inflammatory response is critically important for protection against pathogens and their antigens. Cells at the site of an infection not only need to elicit an inflammatory response to protect against further cell and tissue damage, but also elicit the correct response for the particular pathogen (Medzhitov, 2008). For example, a response to a virus may require cell apoptosis, and the recruitment of NK cells, while a bacterial infection may require phagocytosis by macrophages. However, in both cases the acute and chronic immune response may be necessary. The ability of the local area to elicit the correct immune response relies on the detection of the cause of inflammation (Takeuchi *et al.*, 2010). Pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), play an important role in eliciting the appropriate immune response.

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria and is a potent activator of the immune response (Schletter *et al.*, 1995). LPS is recognized by Toll-like receptor 4 (TLR4), a membrane-spanning protein, and co-receptor with CD14, a glycosylphosphatidylinositol-linked cell surface glycoprotein necessary for sensitive responses to LPS (Ulevitch *et al.*, 1995). Activation of TLR4 leads to a signalling cascade which activates NFκB and causes the expression of mediators of inflammation such as IL-1β, IL-6, IL-8 and TNFα (Chow *et al.*, 1999).

### 1.1.1.2 IFNγ

IFNγ is the only member of the type II class of interferons and exists as a 17kDa dimer. IFNγ is important for maintaining chronic inflammation, and is produced by NK cells, $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T cells (Schoenborn *et al.*, 2007). IFNγ binds to its heterodimeric membrane cell receptor consisting of Interferon gamma receptor 1 (IFNGR1) and Interferon gamma receptor 2 (IFNGR2), which activates the JAK-STAT pathway (Aguet *et al.*, 1988; Schoenborn *et al.*, 2007). IFNγ is critical for immunity against viral and intracellular bacterial infections, and is involved in tumour control, while also being able to promote adhesion and binding required for leukocyte migration onto epithelial cells (Filipe-Santos *et al.*, 2006; Ikeda *et al.*, 2002; Romer *et al.*, 1995). IFNγ is the primary cytokine that defines Th1 cells, with IFNγ secretion causing the maturation of undifferentiated CD4+ cells (Th0 cells) to differentiate into Th1 cells, while also supressing Th2 cells (Mosmann *et al.*, 1996; J. Zhu *et al.*, 2008). It has been shown that IFNγ does not act via the NFκB transcription factor (Andreakos *et al.*, 2004; Cheshire *et al.*, 1997; Holden *et al.*, 2008). IFNγ has been shown to activate AP-1, however (Hu *et al.*, 2006).

### 1.1.1.3 TNFα

Tumour necrosis factor α (TNFα) is a cytokine involved in the acute immune response and is part of the tumour necrosis factor superfamily, whose other member is lymphotoxin-alpha, formerly known as tumour necrosis factor-beta (TNFβ). TNFα is mainly produced by macrophages but is also produced by many other cell types such as $CD4^+$ lymphocytes, NK cells and neutrophils (Beutler *et al.*, 1988). TNFα is a 17kDa protein and exists as a homotrimer (Smith *et al.*, 1987). The cell receptors for TNFα are the membrane-bound TNF receptor type 1 (TNFR1), which is expressed in most cells, and TNF receptor type 2 (TNFR2), which is typically expressed in immune cells (Ashkenazi *et al.*, 1998). TNFα can cause apoptotic cell death, induce fever, inflammation and inhibit tumorigenesis, while also inducing the production of IL-6 in most cell types (Warren, 1990). TNFα shares similar inflammatory effects with IL-1 and IL-6,

thereby creating a cascade of cytokines with overlapping properties, enhancing the acute inflammatory response (Feghali *et al.*, 1997). NFκB and AP-1 are both activated by TNFα (Karsan *et al.*, 2000).

#### 1.1.1.4 PMA

Phorbol 12-myristate 13-acetate (PMA) is a diester of phorbol, and a potent activator of the signal transduction enzyme protein kinase C (PKC) and its ability to activate PKC is due to its structural similarities to an endogenous PKC activator, diacylglycerol (Blumberg, 1988; Castagna *et al.*, 1982). As an activator of PKC, which is a mitogen-activated protein kinases (MAPK), PMA therefore acts as an inflammatory inducing signal (Robinson, 1992). PMA has been shown to copurify with PKC, and inhibition of PKC prevents the inflammatory actions of PMA (Niedel *et al.*, 1983; Tahara *et al.*, 2009). PKC plays an important role in many signalling pathways, including vital roles in innate and adaptive immune function (Seng-Lai *et al.*, 2003).

## 1.2 Glucocorticoids

Glucocorticoids (GCs), such as cortisol which is the endogenous GC in humans, are the most commonly used drugs in the treatment of inflammation (Barnes, 1998). Glucocorticoids are used to treat inflammation caused by the detection of antigens or pathogens, tissue injury due to various types of trauma, asthma, and various auto-immune diseases such as rheumatoid arthritis (Feghali *et al.*, 1997). Glucocorticoid-mediated effects are generally due to the suppression of inflammatory cytokines, such as IL-6 and TNFα, and the induction of anti-inflammatory cytokines, such as IL-10 (Ashwell *et al.*, 2000). Glucocorticoids have also been shown to have negative side-effects, especially in their long-term use, such as their induction of inflammatory chemokines, cytokines and innate immune-related genes. This has been hypothesised to be involved in the negative effects of glucocorticoids (Galon *et al.*, 2002; Moghadam-Kia *et al.*, 2010). Glucocorticoids are part of the hypothalamus-pituitary-adrenal

(HPA) axis with the GR playing a vital role in the immune system, homeostasis and metabolism (Del Rey *et al.*, 2008).

### 1.2.1 Receptor structure and domains

The glucocorticoid receptor (GR), also known as nuclear receptor subfamily 3, group C, member 1 (NR3C1) is the receptor to which GCs bind (Oakley *et al.*, 2013). The GR is a steroid receptor and therefore shares many structural similarities with other steroid receptors such as the progesterone receptor, the androgen receptor and mineralocorticoid receptor (Cato *et al.*, 2002). Through alternate splicing there exists 4 isoforms of the GR; GRα GRβ, GRγ, GR-A and GR-P, with GRα being the classical receptor subtype, and being the most extensively studied (Oakley *et al.*, 2011). For the purposes of this study, all subsequent mentions of GR will be referring to GRα. The glucocorticoid receptor consists of 777 amino acid residues with a weight of 95 kDa (Hollenberg *et al.*, 1985). The receptor can be divided into three functional domains namely the N-terminal domain (NTD), the DNA-binding domain (DBD) and the ligand-binding domain (LBD), with a hinge region between the DBD and LBD (Fig 1.1) (Griekspoor *et al.*, 2007). To date, no complete crystal structure of the GR has been solved, and this may be due to the unordered structure of the NTD of the receptor (Lu *et al.*, 2006). Other regions of the GR, however, have been resolved via X-ray crystallography, such as the LBD (Bledsoe *et al.*, 2002). The NTD has the highest level of variation between the different GR species and is directly involved in the mechanisms of GR transcription. The NTD is a site for protein-protein interactions with other transcription factors, such as TATA box-binding protein (TBP), cAMP response element binding protein (CREB)-binding protein (CBP) and steroid receptor co-activator 1 (SRC-1) (Kumar *et al.*, 2005). The region within the NTD that allows for these interactions is the transcriptional activation region 1 (AF1), which can constitutively act in the absence of the LBD and is also required for maximal transcriptional activity (Kumar *et al.*, 2001). This site is important for GR modulation and crosstalk with other pathways as it is a site for post-translational modifications, such as phosphorylation (Faus *et al.*, 2006). The DBD is the most conserved region between different GR isoforms and is responsible for direct DNA binding, receptor homodimerisation and certain protein-protein interactions, such as with c-

Jun (Yang-Yen *et al.*, 1990). Connected to the DBD via the flexible hinge region is the LBD, which is responsible for the binding of hormone ligands. The LBD consists of twelve amino acid residues, and four β-strands which form the ligand binding pocket of the receptor. The LBD has also been shown to be involved in nuclear translocation and homodimerization (Bledsoe *et al.*, 2002). The LBD also contains the ligand-dependent transactivation domain 2 (AF2), which is involved in co-factor recruitment and binding to heat-shock proteins (Bledsoe *et al.*, 2004).

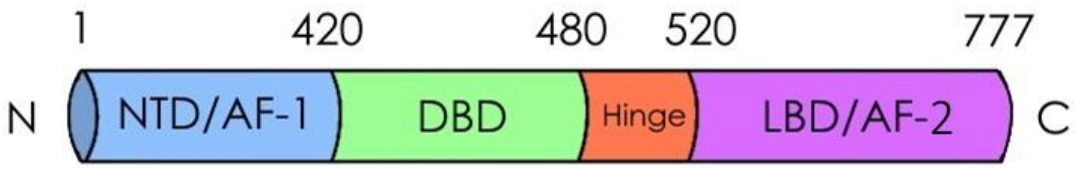



**Figure 1.1. Amino acid length and different mechanistic regions of the glucocorticoid α receptor.** NTD/AF-1, DBD, hinge region and LBD/AF-1 regions as described in (Griekspoor *et al.*, 2007).

## 1.2.2 Mechanisms of GR action

### 1.2.2.1 Ligand binding and nuclear translocation

In the absence of ligand, the majority of GR exists within the cytoplasm, although there is evidence to suggest unliganded GR shuttling in and out of the nucleus (Ritter *et al.*, 2014). While in the cytoplasm, the inactive GR monomer exists in a heteromeric multi-protein complex. It is bound with chaperone proteins such as heat-shock protein 90 (HSP90) and immunophilins such as FK506 binding protein 51 (FKBP51) (Dalman *et al.*, 1989; Murphy *et al.*, 2005; Zhou *et al.*, 2005). GCs are able to pass through the cell membrane by passive diffusion as they are lipophilic (Lu *et al.*, 2006). Once within the cell, GCs can bind the GR within the LBD, causing conformational changes of the receptor, and translocation to the nucleus (Flammer *et al.*, 2011). GR dimerization occurs within the cytoplasm before nuclear translocation (Savory *et al.*, 2001). Dimerization is classically needed for transactivation, but not necessarily for GR transrepression (Malkoski *et al.*, 1999). Two nuclear localization signals (NLs) are exposed during the conformational changes upon ligand binding. These are located in the DBD and the

hinge region of the GR and allow for the GR to enter the nucleus via the nuclear pore (Savory *et al.*, 1999). Fig 1.2 depicts the mechanisms of GR action. Mifepristone (RU486), is a potent antagonist of GR, and acts via binding to the LBD of the receptor (Bourgeois *et al.*, 1984).

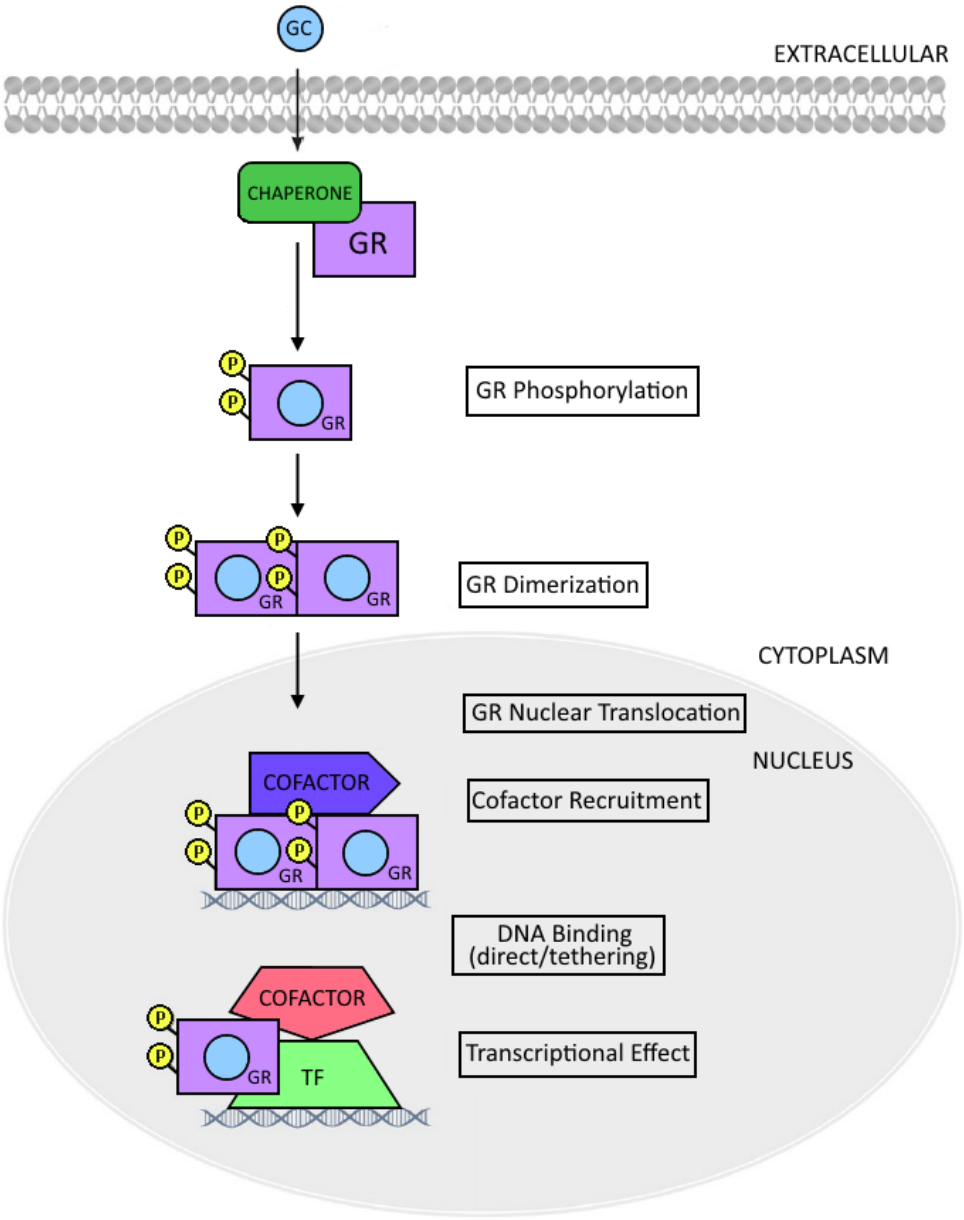



**Figure 1.2. Mechanisms of GR action.**
Simple, schematic diagram of the postulated GR mechanism of action. Shown is the GR either binding directly to the DNA, or via tethering to other transcription factors (TF) as described by (Savory *et al.*, 1999).

### 1.2.2.2 Transcriptional regulation

Once within the nucleus, the GR can cause its transcriptional effects by either binding directly to the DNA, or by tethering to other transcription factors and proteins in a tethering mechanism. Classically, direct binding to DNA results in transactivation and an increase in transcription, while tethering to other transcription factors leads to transrepression, and a decrease in transcription (Schäcke *et al.*, 2004).

#### 1.2.2.2.1 Direct DNA binding

The active GR once in the nucleus can bind directly to GREs, which are DNA target sequences, and cause transcription of GC-responsive genes. The consensus sequence of the GRE is the palindromic 15-bp sequence 5'-GGTACAnnnTGTTCT-3' (where n represents any nucleotide) (Barnes, 1998). Genes may contain one, or many simple-acting GRE sites within their promoter, such as the serine/threonine protein kinase (SGK1) promoter with one, while the mouse mammary tumour virus (MMTV) promoter contains many half-sites (Beato, 1993; Schoneveld *et al.*, 2004). Genes may also contain glucocorticoid response units (GRUs), where transcription requires not only GR binding to the GRE, but also the binding of other transcription factors, such as activator protein 1 (AP-1) proteins and TBP, to adjacent binding sites (Schoneveld *et al.*, 2004). The GR is also able to bind as a monomer to GRE half sites (Luisi *et al.*, 1991).
These effects classically lead to the transactivation of the particular gene, and therefore an increase in transcription. The GR does this by recruiting the basal transcription machinery, chromatin remodelling complexes, co-activators, as well as a variety of other transcription factors which facilitate GR-mediated transcription (Chinenov *et al.*, 2013).

The GR is also able to transrepress the expression of genes by binding directly to DNA via specific, negative glucocorticoid response elements (nGREs) (Malkoski *et al.*, 1999). The nGRE is similar to the GRE sequence, but contains more variability in its sequence, 5'-ATTACnnTnTGATCn-3'. The GR is able to transrepress genes by competing with positive acting transcription factors for promoter binding (Zhou *et al.*, 2005). The osteocalcin gene promoter

contains an nGRE which overlaps with the TATA box, thereby preventing the binding of TBP, and therefore repressing transcription (Strömstedt *et al.*, 1991).

#### 1.2.2.2.2 Tethering

In the classical model of transcriptional regulation, besides direct DNA binding, the GR can also bind to other transcription factors, via protein-protein interactions, to modulate genetic expression. This tethering to other transcription factors allows the GR to modulate the expression of genes that do not contain GREs or nGREs (Ratman *et al.*, 2013). Tethering is generally recognised as a mechanism for GR transrepression, as few examples of tethering causing transactivation to exist (Arambašić *et al.*, 2010; Johansson-Haque *et al.*, 2008). The prevailing mechanism for GR transrepression of pro-inflammatory genes is via GR tethering to the transcription factors activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (Adcock *et al.*, 2006). The precise mechanisms of how the GR interacts directly with AP-1 and NFκB is still unclear, although the DBD region of the GR has been shown to be vital for these interactions (Kassel *et al.*, 2007).

### 1.2.2.3 Post-translational modifications

The GR is a target for many post-translational modifications, which play an important role in modulating the activity of the receptor and also provides an important mechanism for crosstalk between cell signalling pathways (Faus *et al.*, 2006). Post-translational modifications affect the GR's transcriptional regulation, protein-protein interactions and degradation, via the phosphorylation, ubiquitination, sumoylation and acetylation of specific amino acid residues on the receptor (Duma *et al.*, 2006).

Phosphorylation of the GR plays an important role in GR activity, and also serves as an important marker for determining GR activity. Site-specific GR phosphorylation results in both

an increase (Kino *et al.*, 2007) and a decrease (Avenant, 2009) in transcriptional activity, suggesting regulation in a species- or cell- specific manner (Avenant, 2009; Kino *et al.*, 2007).

### 1.2.3 GR Crosstalk

Cell signalling crosstalk provides for fine-tuned and appropriate cell responses to stimuli. Cell signalling crosstalk can occur at many nodes (Campbell *et al.*, 2002). In fact, this is an important characteristic of cellular signalling, as it allows for many levels of interaction between signalling pathways (Taniguchi *et al.*, 2006). An example where cell signalling crosstalk is important is that of the immune response. Fig 1.3 depicts crosstalk between various cell signalling pathways with regards to inflammatory ligands, although this diagram is simplified from its true complexity. Cells must not only elicit an effective and efficient response to pathogens and trauma, but also prevent an overshooting of the response, which may lead to further tissue damage (Nathan *et al.*, 2010). Therefore, a high level of communication between signalling pathways involved in immune function is vital, with various levels of interaction that can either enhance, or repress specific the responses. When cells encounter pathogens, or a foreign antigen, an inflammatory response is elicited, resulting in the release or expression of cytokines, chemokines, adhesion molecules, receptors and enzymes critical to resolve the cause of inflammation (K. Newton *et al.*, 2012). Once the cause of inflammation has been appropriately resolved, the immune response needs to be repressed, to prevent detrimental effects and chronic inflammation. Glucocorticoids play an important role in the immune response, as they classically elicit an anti-inflammatory response, with both natural and synthetic glucocorticoids being the most prescribed as anti-inflammatory medication, due to their potent immunosuppressive properties. Glucocorticoids are able to induce the expression of anti-inflammatory genes such as GILZ and IL10, while also repressing the expression of pro-inflammatory genes such as IL6 (Waage *et al.*, 1990). Due to its important role in immune function, the GR is therefore able to affect, and be affected by other signalling pathways (Busillo *et al.*, 2013).

This can occur via transcriptional modulation of genes, but also through indirect methods, such as post-transcriptional and post-translational effects. This multi-levelled attribute of crosstalk allows for many nodes of input from multiple signalling pathways, and therefore a tightly regulated and sensitive network in response to many specific signals.

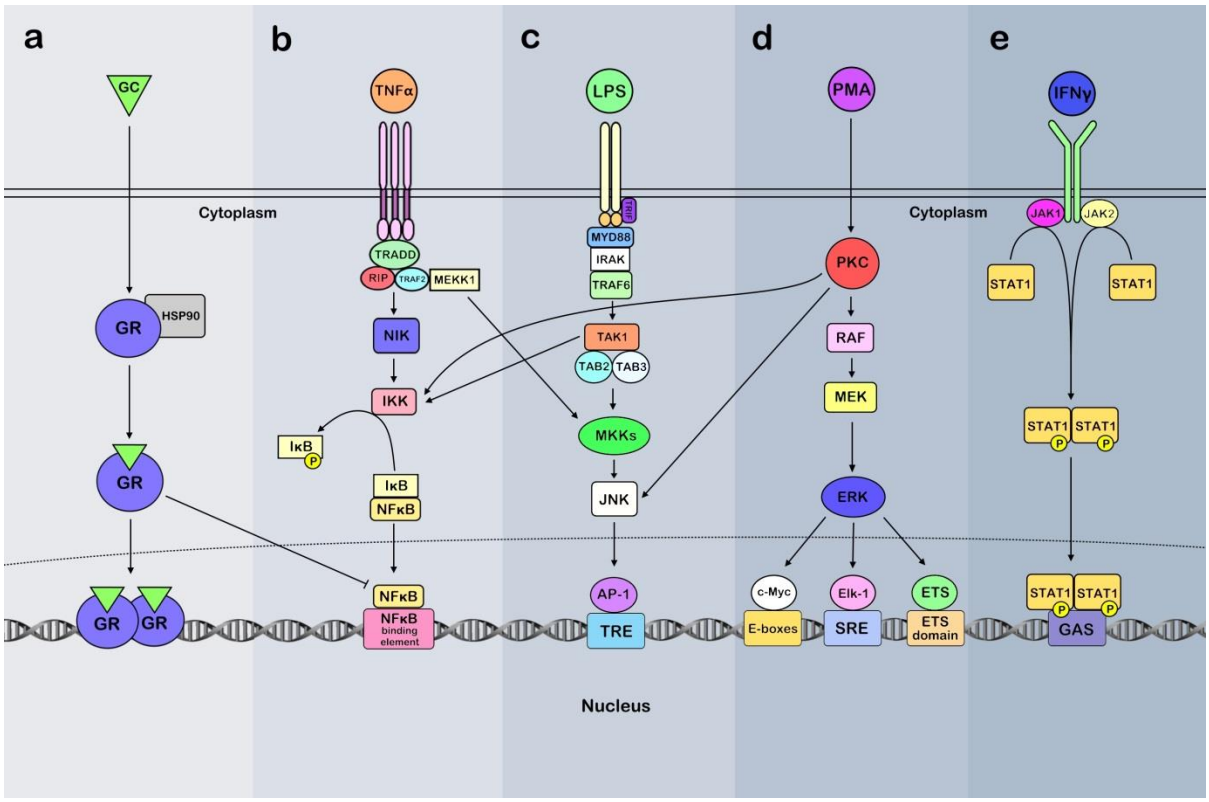



**Figure 1.3. Abridged, schematic model of cell signalling pathways for the GR and mediators of inflammation.**

GC (A), TNFα (B), LPS (C), PMA (D) and IFNγ (E) abridged cell signalling pathways and different promoter regions they interact with is shown (Chow *et al.*, 1999; Karsan *et al.*, 2000; Robinson, 1992; Savory *et al.*, 1999; Schoenborn *et al.*, 2007; Warren, 1990). The signalling pathways cause transcription factor binding to the CCL20 promoter either directly, or by the upregulation or modification of other proteins.

### 1.2.3.1 GR genomic actions

#### 1.2.3.1.1 Direct modulation

In response to stimuli, the GR is able to cause the transcription of genes important in immune regulation. As discussed in Chapter 1.2.2, the GR is classically activated by GC binding, causing its translocation to the nucleus, binding to GREs, and the increase of transcription of target genes such as GILZ and IL10 (Berrebi *et al.*, 2003). The activated GR is also classically able to repress the expression of genes, such as IL6 and IL1β, by tethering to other transcription factors (S. W. Lee *et al.*, 1988; Waage *et al.*, 1990). This direct modulation of genes is rapid, as the GR already resides in the cell in an inactive state and does not require *de novo* synthesis (Baschant *et al.*, 2010). GCs, instead of suppressing the inflammatory actions of TNFα, have been shown to cooperatively regulate TLR2 expression (Hermoso *et al.*, 2004). The NFκB site, STAT-binding element, and a 3' GRE on the TLR2 promoter have been shown to be important for this cooperative crosstalk.

#### 1.2.3.1.2 Indirect Modulation

The GR can also regulate the expression of genes, which in turn regulate the expression, or function, of other proteins. This indirect regulation allows for more nodes of crosstalk, and a larger network of regulated immune function genes. The GR is able to shuttle between the cytoplasm and nucleus and can affect the expression of genes even when it is unliganded (Ritter *et al.*, 2014).

An example of indirect modulation of the GR is via glucocorticoid-induced leucine zipper (GILZ), which is rapidly and ubiquitously induced by GCs. The GILZ protein is able to repress transcription of specific genes by interacting with TFs such as NFκB, AP-1 and C/EBPs (Ronchetti *et al.*, 2015). The implications of this are that GCs' upregulation of GILZ via the GR can repress

the expression of genes regulated by TFs such as NFκB, via the inhibitive interaction with GILZ. GCs via the GR are therefore able to modulate the expression of genes, and crosstalk with other signalling pathways, indirectly.

The GR is able to indirectly crosstalk with the TLR4 signalling pathway, by increasing the sensitivity of myeloid progenitors to LPS. Co-stimulation with GCs and LPS causes an overexpression of pro-inflammatory cytokines in these cells when differentiating into macrophages (T. Y. Zhang *et al.*, 2007). This result may be dependent on when cells are stimulated with GCs and LPS, as stimulation with GCs 1 h following LPS challenge is immunosuppressive, while administration of the GCs prior to LPS has been shown to increase the immune response and release of cytokines (Frank *et al.*, 2010).

GCs can indirectly crosstalk with the Janus kinase (JAK)/STAT signalling pathway via suppressors of cytokine signalling (SOCS). Interestingly, GCs have been shown to suppress the expression of SOCS-3 (Paul *et al.*, 2000). This in turn prevents the suppression of inflammation by SOCS-3 and has been thought to be a mechanism for GC resistance in asthma patients.

GCs are able to suppress the LPS-induced expression of cytokines TNFα and IL-6. GCs are able to indirectly modulate the activity of MAPKs, such as by increasing the transcription of dual-specificity phosphatases (DUSPs), which in turn dephosphorylate MAPKs (Tchen *et al.*, 2010).

The GR can also directly repress TLR4 signalling by interfering with the interferon regulatory transcription factor 3 (IRF3)/p65 interaction with Interferon-stimulated response element (ISRE) containing genes (S. Ogawa *et al.*, 2005).

### 1.2.3.2 GR Non-genomic actions

GR non-genomic actions are characterized whereby inhibitors of transcription and protein synthesis do not affect its actions (Falkenstein *et al.*, 2000). The non-genomic actions of the GR are rapid, occurring within minutes, and therefore make it difficult to be explained by genomic means (Croxtall *et al.*, 2000). The non-genomic activity of the GR can either occur via cytosolic GR, or membrane-bound GR (Bartholome *et al.*, 2004; Wehmeyer *et al.*, 2014).

The presence of GCs has been shown to modulate the activity of many signalling pathways in non-genomic manner (Song *et al.*, 2006). MAPKs are important in many pro-inflammatory

signalling pathways and are therefore an important target for GC repression of inflammation. The three main MAPK subfamilies in mammalian cells are the extracellular signal-regulated kinases (ERKs), the p38 MAPK, and the c-Jun $NH_2$-terminal protein kinase (JNK) (Cargnello *et al.*, 2011). GCs acting via the GR are also able to rapidly modulate MAPKs through non-genomic methods.

The MAPK p38 is rapidly phosphorylated via the actions of active DUPS1. This suggests a possible indirect mechanism for GR/TLR4 signalling pathway crosstalk (Bhattacharyya *et al.*, 2007). Activation of the GR has been shown to repress the Raf, MAPK/ERK kinase (Mek) signalling pathway in a non-genomic manner (Croxtall *et al.*, 2000). Stimulation of rat hippocampal cells with cortisol showed rapid activation of JNK and p38, although there are cases showing the opposite effects (Qi *et al.*, 2005). The non-genomic crosstalk between the GR and MAPKs is also reciprocal, with the MAPKs able to phosphorylate the GR (De Bosscher *et al.*, 2003). Activated GR has also been shown to interact with the phosphatidylinositiol 3-kinase (PI3K)/Protein kinase B (Akt) signalling pathway in a non-genomic, PKC dependant manner (Solito *et al.*, 2003). An example of a pathway that utilizes the PI3K/Akt signalling cascade is the epidermal growth factor receptor (EGFR). GCs have been shown to increase the activity of the EGFR, although it was not shown whether this occurred via non-genomic means (Sekiyama *et al.*, 2012). As stated previously, GCs can indirectly interact with the JAK/STAT pathway by inhibiting the expression SOCS-3. The GR is also able to directly interact with the SOCS-1 protein to crosstalk with the JAK/STAT pathway in a non-genomic manner. Activation of SOCS-1 by IFNγ was shown to inhibit GR activity via interaction of the LBD of the GR and the SH2 region of SOCS-1. SOCS-1 therefore plays an important role in the early phase of crosstalk between the GR and cytokine signalling (Haffner *et al.*, 2008).

Besides cytosolic located GR, non-genomic GR effects are also triggered by, or at least dependent on membrane bound GR (mGR). The mGR is able to interact with other signalling pathways such as G-protein coupled receptors (GPCRs) (De Bosscher *et al.*, 2000). An example of this is the interaction of mGR and the gonadotropin releasing hormone receptor (GnRHR) at lipid rafts (Wehmeyer *et al.*, 2014).

### 1.2.4 GR pro-inflammatory actions

Glucocorticoids have been shown to cause pro-inflammatory effects, which seems to contrast with their anti-inflammatory actions (Wiegers *et al.*, 1998). Interaction between the glucocorticoid receptor signalling pathway and inflammatory signalling pathways, such as the TNFR signalling pathway, has been shown to cause the co-regulation of many genes, with several of them being cooperatively expressed (Lannan *et al.*, 2012). Microarray data showed that glucocorticoids could induce the expression of chemokines, cytokines and other immune-related genes of immune cells (Galon *et al.*, 2002). Stimulation with GCs has also been shown to increase the expression of cytokine receptors, which correlates with an enhanced cytokine effect on target cells (Almawi *et al.*, 1996). Pre-treatment of macrophages with GCs was shown to induce cytokine overexpression and NFκB activation, further highlighting the paradoxical crosstalk between GCs and pro-inflammatory signalling pathways (Smyth *et al.*, 2004). Glucocorticoids may be positively regulating inflammation to prepare the immune system for a quick and effective response to pathogens. It is vital for the pathogen, or cause of inflammation, to be swiftly removed, so an immediate repression of the immune response by glucocorticoids would be unfavourable. Therefore, not only is it important for GCs to not repress the inflammatory response immediately, but specifically GCs should not repress the innate immune response before the cause of inflammation is resolved. GCs therefore have the ability to positively interact initially with the innate immune system and Th1 responses, while inhibiting the adaptive immunity, and Th2 responses, until homeostasis is restored (Galon *et al.*, 2002).

A previous study showed more than 800 genes were co-regulated significantly by dex and TNFα in microarray data, with over 300 of them being involved in inflammatory diseases (Lannan *et al.*, 2012). In the same study above, were also able to show that genes co-regulated by TNFα and dex could also be synergistically upregulated, such as serpinA3, SAA1 and SAA2. Synergism is defined as a response where the combined effect of two signals is greater than the sum of the individual responses, where each signal on its own is able to induce a response (Chou, 2006). If the overall level of the combined effect is not greater than the sum, then the signals may be combinatorial, or positively modulating their effects. When one signal, however, does

not induce a response on its own, but does increase the response of the second signal, this is referred to as priming or sensitizing.

CCL20 is a chemoattractant for neutrophils and Th17 cells and was shown to be expressed by the GC budesonide in human bronchial epithelial cells (Zijlstra *et al.*, 2014). Budesonide was also able to modulate the TNFα mediated expression of CCL20 when co-stimulated, highlighting a crosstalk between the signalling pathways. Wang *et al*. (1997) showed that dex can induce CCL20 expression in human bronchial epithelial cells, and that this most likely was a direct effect, as they co-stimulated the cells with cycloheximide, an inhibitor of *de novo* protein synthesis, to minimize potential secondary effects. Zijlstra *et al*. (2014) showed that GCs were able to modulate and enhance the TNFα induced expression of CCL20. In other words, co-stimulation with TNFα and GCs resulted in an overall level of CCL20 expression greater than either TNFα, or the GC alone.

## 1.3 CCL20

### 1.3.1 CCL20 structure, receptor and promoter

C-C motif chemokine ligand 20 (CCL20), alternatively named macrophage inflammatory protein-3alpha, liver and activation-regulated chemokine (LARC), Exodus or SCYA20 is a chemoattractive cytokine that has been shown to be expressed by a variety of cell and tissue types such as PBMCs, epithelial cells, lung tissue and liver tissue (Rossi *et al.*, 1997). The mature CCL20 protein comprises of 70 amino acid residues, with a molecular weight of 8kDa, with cysteine residues at locations characteristic of CC chemokines (Hieshima *et al.*, 1997). CCL20 has been shown to have low levels of sequence similarity with other CC chemokines, while X-ray crystallography has shown that its structure is very similar to those of other CC chemokines (Hoover *et al.*, 2002; Yoshie *et al.*, 1997).

The full length, exon-encoded CCL20 mRNA is 821 nucleotides in length and contains polyadenylation signals in the 3'-non-coding region (Hromas *et al.*, 1997) (Fig 1.4).

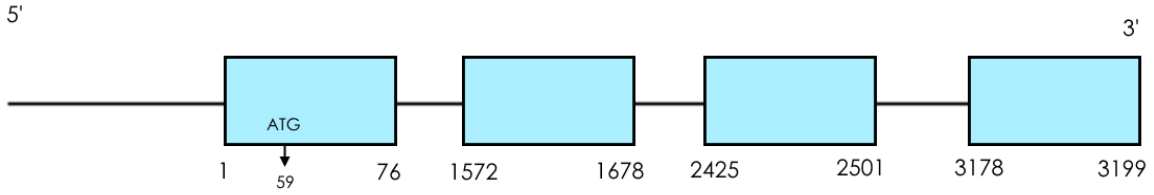



Figure 1.4. CCL20 mRNA exons and base pair length.
The CCL20 mRNA transcript is 3199 bp, with the translation start site (ATG) is marked at position 59 in the 5' to 3' direction as described by (Hromas *et al.*, 1997).

The receptor for CCL20 is the G-protein coupled receptor (GPCR) chemokine receptor 6 (CCR6) which is expressed on leukocytes such as T helper cells, monocytes, macrophages, and dendritic cells (DC) (Kucharzik *et al.*, 2002; Liao *et al.*, 1999). CCR6 plays a specific role in the induction of DC towards epithelial cells due to the invasion of pathogens and antigens (Dieu-Nosjean *et al.*, 1999). Recent studies have also shown CCR6 to be a specific marker for Th17 cells and regulatory T cells distinguishing them from other helper T cells (Hirota *et al.*, 2007). CCR6 has also been shown to be expressed on leukaemia and pancreatic cancer cells, however the exact role of the receptor on cancer cells is unknown (Imaizumi *et al.*, 2002; Kleeff *et al.*, 1999). CCL20 has the highest affinity for CCR6, with β-defensins-1 and -2 having much lower affinities (Williams, 2004; D. Yang *et al.*, 1999).

### 1.3.2 CCL20 Promoter and Binding Transcription Factors

Analysis of the CCL20 promoter region upstream of the transcription start site has shown putative binding sites for NFκB, C/EBP, AP-1, c-Ets, Sp1, STAT1 and GR within its first 700 base pairs (Table 1) (Hieshima *et al.*, 1997; Moore *et al.*, 2010; Sugita *et al.*, 2002). The CCL20 promoter contains a putative GR binding site (J. H. Kwon *et al.*, 2003). ChIP analysis determined GR binding on CCL20 (J.-C. Wang *et al.*, 2004).

Table 1. Transcription factor binding sites on the putative CCL20 promoter determined *in silico.*
Putative transcription factors from -647 to +58 base pairs of the putative CCL20 promoter (Hieshima *et al.*, 1997; Moore *et al.*, 2010; Sugita *et al.*, 2002).

| Name | Site | Binding Sequence |
|---|---|---|
| Translation Initiation Codon | +59 | ATG |
| TATA | -36 -41 | TATAAA |
| NFκB | -84 -93 | GGGAAAACCC |
| AP-1 | -114 -120<br>-267 -273 | TGACATG<br>TGGGTCA |
| STAT1 | -639 -647 | TTCTTAGAA |
| C/EBP | -95 -103<br>-416 -423 | TTGATCAAT<br>CCCTCCTC |
| GR | -501 -506<br>-637 -642 | TCTTCT<br>AGAAGA |
| Sp1 | -52 -58<br>-184 -193 | CGCCTTC<br>CCCACCCTGA |
| Ets | -144 -152<br>-157 -162<br>-168 -174 | CAGGAAGT<br>GAGGAA<br>CCTTCCC |

NFκB is a transcription factor protein complex, which plays a key role in the regulation of immune function genes (Gilmore, 2006). The NFκB complex contains the proteins p50 (NFkB1), p52 (NFkB2), p65 (RelA), c-Rel, and RelB (Baeuerle *et al.*, 1996). NFκB is a primary transcription factor, and therefore resides in the cytoplasm in an inactive state, sequestered by a family of proteins called inhibitors of κB (IκB) (S. Ghosh *et al.*, 1990). IκB is generally phosphorylated upon NFκB activation, leading to NFκB dissociation and nuclear translocation. Upon activation by pro-inflammatory ligands, the canonical NFκB p65/p50 subunit binds to NFκB promoter sites and causes transcriptional activation. NFκB also causes the transcriptional increase of its own inhibitor IκB. This creates a negative feedback loop, where the increased activity of NFκB causes its inhibition within the cytoplasm (Brown *et al.*, 1993). Phosphorylation of NFκB is also rapidly induced upon activation, with p65/RelA phosphorylation at S529 serving as a biomarker for NFκB activation (D. Wang *et al.*, 1998). Inflammatory ligands lead to the activation of

signalling cascades and cellular kinases such as protein kinase C (PKC). Mitogen-activated protein kinases (MAPKs) such as PKC has been shown to cause the dissociation of the NFκB/IκB complex (S. Ghosh *et al.*, 1990).

Specific binding of the p50/p65 heterodimer, and p65/p65 homodimer has been shown to bind to the CCL20 NFκB site in human cells (Fujiie *et al.*, 2001). Other possible TFs involved in CCL20 regulation are members of the forkhead box-containing protein O subfamily-1 (FOXO1). FOXO1 affects CCL20 gene expression in an NFκB dependent manner. TNFα-mediated CCL20 expression is enhanced by FOXO1 overexpression, and attenuated by FOXO1 silencing, although only when a functioning NFκB binding site is present. Furthermore, FOXO1 is not predicted to have a binding site on the CCL20 promoter (Miao *et al.*, 2012).

Activator protein 1 (AP-1) is a transcription factor protein dimer involved in the control of basal and inducible gene expression. The heterodimer consists of Jun and Fos family proteins and binds to TPA-responsive elements (TREs) on the DNA (Hess *et al.*, 2004). The Jun protein family, also known as c-Jun, consists of JunB and JunD, and the Fos protein family, also known as c-Fos, consists of FosB, Fra-1 and Fra-2 (Karin, 1995). Jun proteins can bind either as a homo- or heterodimer, while the Fos proteins can only form a heterodimer with Jun. Jun/Fos heterodimers, however, bind more strongly than Jun homodimers to DNA. Despite the high level of similarities between Jun and Fos proteins, differences in sequence specificity suggest differential dimeric complex regulation of distinct subsets of AP-1 regulated genes (Angel *et al.*, 1991). AP-1 is activated by a variety of cell signals in response to growth factors, inflammation and carcinogens. As expected for a transcription factor involved in many processes, AP-1 is tightly regulated via its expression, and post-translational modifications such as phosphorylation (Boyle *et al.*, 1991). MAPKs have been shown to be involved in AP-1 signalling, with $PKC^{-/-}$ in mice preventing AP-1 activity via the T-cell receptor (TCR) (Sun *et al.*, 2000).

(TCR)-induced activation of AP-1 has also been shown to have both positive and negative effects on the activity of other transcription factors, such as NFκB and the GR (Jonat *et al.*, 1990; Xiao *et al.*, 2004). While, classically, AP-1 and the GR negatively affect each other's activity, recent studies have shown the GR synergizing with AP-1 proteins in certain tissue or

cell types, with c-Jun homodimers in composite sites, or via tethering directly to AP-1 (Herrlich, 2001).

Signal transducers and activators of transcription (STAT) proteins are transcription factors that play an important role in the immune response and were discovered due to their key involvement in IFN signalling (Darnell Jr *et al.*, 1994). The members of the STAT family are STAT1, STAT2, STAT3, STAT4, STAT5 (STAT5A and STAT5B), and STAT6. STAT1 activation has been shown to be involved in both the synergistic activation, and suppression of gene expression, and therefore serves as an important member in crosstalk between signal transduction pathways (Ramana *et al.*, 2000). STAT1 is phosphorylated within the cytoplasm at S727 by receptor-associated Janus protein tyrosine kinases (Jaks), before nuclear translocation, and binds to the DNA as a homodimer (X. Zhu *et al.*, 1997).
A putative binding site for STAT1 is predicted within the CCL20 promoter, with STAT1 silencing causing a reduction in the expression of CCL20 mRNA (Moore *et al.*, 2010). Constitutively STAT1 expression in A549 cells was shown to cause an increase in CCL20 gene expression in rat pancreatic cells (Li *et al.*, 2007).

CCAAT/enhancer-binding proteins (C/EBPs) are a subfamily of the basic leucine zipper domain transcription factors. C/EBPs play an important role in the immune response, cellular differentiation and in metabolism (Ramji *et al.*, 2002). The members of the C/EBP family are C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ, C/EBPε, and C/EBPζ. C/EBPβ deficient mice have been shown to have a compromised immune system, highlighting the importance of the transcription factor in the immune response (Poli, 1998). Both C/EBPβ and C/EBPδ have been shown to be induced by glucocorticoid stimulation (Roesler, 2001). Glucocorticoid activation of C/EBPβ has been shown to involve the phosphorylation at T235, which increases the binding affinity of the transcription factor, and has been shown to be independent of C/EBPβ *de novo* protein expression (Berg *et al.*, 2005; Ramji *et al.*, 2002). Of particular interest, C/EBPβ has been shown to directly interact with other transcription factors, such as NFκB, AP-1 and the GR, on the promoter of genes (Hsu *et al.*, 1994; Johansson-Haque *et al.*, 2008; LeClair *et al.*, 1992). C/EBP has been shown to be a critical regulator of CCL20, being able to bind and induce expression of CCL20 mRNA (Sperling *et al.*, 2012). The GR and C/EBP proteins can activate transcription via composite response elements and protein-protein interactions, such as tethering (Fig 1.5A),

cause post-translationally modifications of C/EBP proteins (Fig 1.5B), or cause the upregulation of C/EBP proteins (Fig 1.5C) (Ramos *et al.*, 1996). C/EBPα and C/EBPβ are specifically implicated in the GC mediated induction of genes via C/EBP DNA binding regions (CCAAT box motifs) (Sai *et al.*, 2008; Z. Yang *et al.*, 2007)

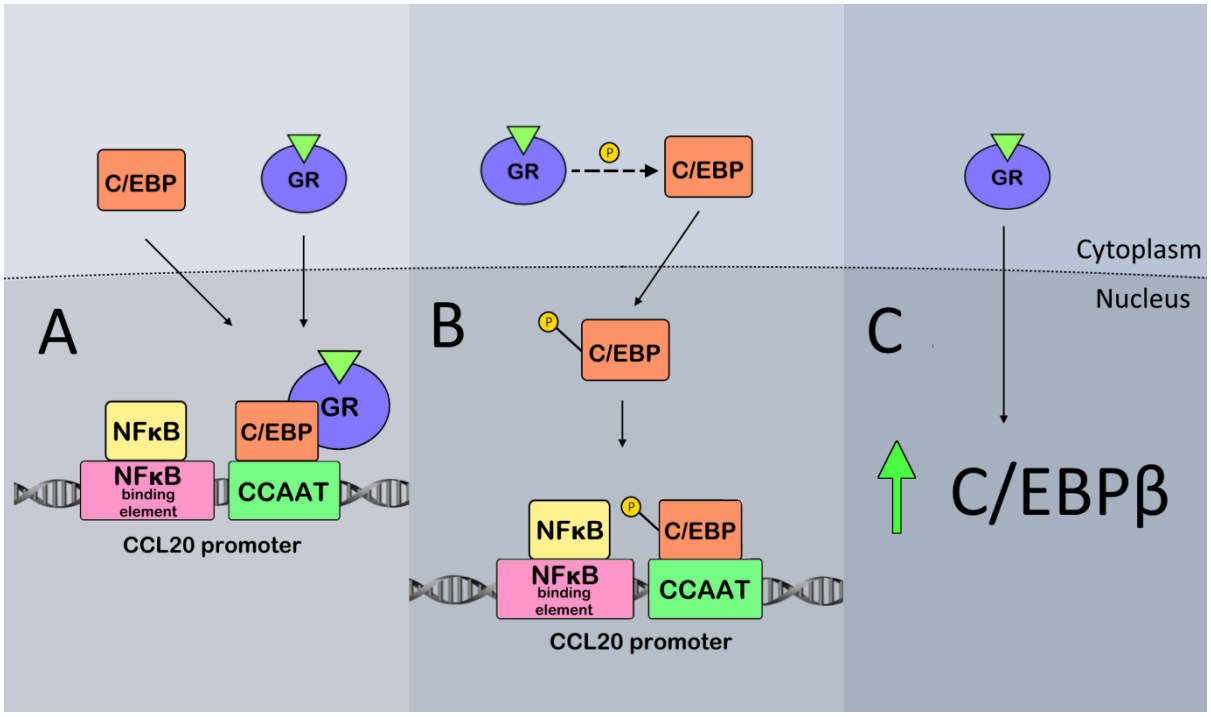



**Figure 1.5. Possible mechanisms which GR could positively interact with the C/EBP to cause the induction of CCL20.**
Transcription via composite response elements and protein-protein interactions (A), post-translationally modifications of C/EBPβ (B) and via the upregulation of C/EBPβ (C) (Ramos *et al.*, 1996).

It has also been reported that the specificity protein 1 (Sp1)-binding site within the CCL20 promoter is involved in regulating basal CCL20 transcription (J. H. Kwon *et al.*, 2003). Overexpression of Sp1, and site-directed mutagenesis of the Sp1 binding site caused respectively caused an increase and decrease of the activation of a CCL20 promoter-reporter system in Caco-2 cells (J. H. Kwon *et al.*, 2003). Kwon *et al*. also demonstrated that the E26 transformation-specific (Ets) transcription factor, epithelium-specific Ets 1 (ESE-1) is essential for CCL20 induction (J. H. Kwon *et al.*, 2003).

### 1.3.3 Biological Functions of CCL20

As a chemokine, CCL20 has been shown to be a strong chemoattractant for lymphocytes, dendritic cells, NK cells and neutrophils (Schutyser *et al.*, 2003). CCL20 can be expressed by a large variety of cells such as epithelial and endothelial cells; lung, liver, pancreatic and cells of the reproductive system (Giannini *et al.*, 2002; Hieshima *et al.*, 1997). This expression can be constitutive, while increased CCL20 expression can be elicited by microbial factors such as lipopolysaccharide (LPS) and by inflammatory factors such as tumour necrosis factor α (TNFα), causing migration of targeted leukocytes to the local inflammatory site (Schutyser *et al.*, 2000). Leukocyte responsiveness to CCL20 has been shown to depend on cell-type, sub-type, maturation and/or differentiation of the leukocyte.

Immature dendritic cells, including Langerhans cells (LC) have been shown to be responsive to CCL20, with a chemotaxis occurring (Charbonnier *et al.*, 1999; Dieu *et al.*, 1998). Functional CCR6 protein expression has been observed in CD34+ HPC-derived immature DC, as well as in immature monocyte-derived DC, and CCL20 acted as a chemoattractant for these cells (Power *et al.*, 1997; Vanbervliet *et al.*, 2002). Furthermore, CCL20 is a potent chemoattractant for freshly isolated, *ex vivo* LC, however, once these cells were matured with GM-CSF and TNFα, CCR6 expression, and CCL20 responsiveness decreased (Charbonnier *et al.*, 1999). Langerhans cells are present in the epidermis, and therefore target microbial antigens, becoming antigen-presenting cells (Katz *et al.*, 1979).

CCL20 is a strong chemoattractant for T-lymphocytes ($T_h17$), with $CD4^+$ cells expressing higher levels of CCR6 than $CD8^+$ T-cells (Liao *et al.*, 1999). These $CD4^+$ $T_h17$ cells and neutrophils are important for the innate immune system, and the immediate migration of these cells to the site of infection is vital as they are one of the first responders and are a hallmark for acute inflammation (Ouyang *et al.*, 2008). CCL20 is also a chemoattractant for both memory and naïve B-lymphocytes (Krzysiek *et al.*, 2000).

CCL20 is also implicated in the progression of pathological processes and negative side effects of drug use. Inflammation mediated by $T_h17$ cells has been implicated in GC insensitivity in asthma patients (Zijlstra *et al.*, 2014). Increased CCL20 expression due to GCs has been suggested as a reason for the increase in $T_h17$ cells in the lung. Colonic epithelial cells have

been shown to express CCL20, with higher amounts expressed in cells with colon and inflammatory bowel disease compared to normal cells, thereby increasing the leukocytes to their surface (J. Kwon *et al.*, 2002). An increase of leukocytes in the colon has been associated with colon cancer (Y.-J. Lee *et al.*, 2006). Increased CCL20 production is implicated in the progression of rheumatoid arthritis, and in immunopathogenesis of the necroinflammatory response in the liver (A. Lee *et al.*, 2014; Shimizu *et al.*, 2001). Furthermore, increased CCL20 expression at mucosal epithelial barriers is implicated in an increase in HIV-1 acquisition. This is due to an increased presence of Langerhans cells (LCs) in mucosal epithelia, such as of the cervix and oral cavity, which become targets for HIV-1 acquisition (Giannini *et al.*, 2002; Hosokawa *et al.*, 2005; Miller, 2007). Therefore, understanding the mechanisms in CCL20 regulation is important for knowledge of the pathological effects of the chemokine.

# Aims and Hypotheses

The aim of this study was to examine the mechanisms of crosstalk of the GR and inflammatory inducing signals on the pro-inflammatory gene CCL20. In particular, the study focuses on the expression of CCL20 mRNA in epithelial cell lines using tissue culture techniques. It has previously been shown that glucocorticoids, which are primarily noted for their anti-inflammatory actions, are able to induce the expression of pro-inflammatory chemokines and cytokines (Galon *et al.*, 2002). Zijlstra *et al.* (2014) showed that the expression of the pro-inflammatory chemokine CCL20 was induced by glucocorticoids in human bronchial epithelial cells. Furthermore, co-stimulation with glucocorticoids and the pro-inflammatory ligand TNFα caused an enhanced, modulatory effect on the expression of CCL20. The mechanisms of how glucocorticoids increase CCL20 expression and how they can crosstalk and modulate the induction of CCL20 by mediators of pro-inflammation have not been determined. Therefore, this study seeks to examine some of the potential mechanisms of this regulation. Due to CCL20's role in immune function, knowledge of the different inflammatory inducing signalling pathways and their crosstalk with glucocorticoids can provide more information on the mechanisms of CCL20 regulation. More specifically, this project investigated the following hypothesis; the pro-inflammatory chemokine CCL20 can be regulated in a variety of cell types by glucocorticoids and pro-inflammatory ligands, with crosstalk between them seen with co-treatment. The following aims/questions are addressed in this project;

i) Can the pro-inflammatory chemokine CCL20 be upregulated in a variety of cell types by inflammatory inducing signals, and by glucocorticoids?

ii) Does co-stimulation of cells with inflammatory inducing ligands and glucocorticoids have an enhanced, modulatory effect in CCL20 expression?

iii) Does the glucocorticoid-mediated increase in CCL20 expression occur in a GR-dependant manner?

iv) Does the observed increase of CCL20 expression occur at the level of transcription and, therefore, can glucocorticoids and inflammatory inducing ligands activate the putative CCL20 promoter on a reporter construct?

v) Is the NFκB binding site on the CCL20 putative promoter integral in CCL20 induction?

CCL20 is expressed in a wide variety of cells and tissue types, both constitutively and via induction by a broad spectrum of inducers. Besides its role in maintaining homeostasis of the immune system, CCL20 has been implicated in pathological processes, especially at epithelial surfaces (Schutyser *et al.*, 2000). Therefore, an understanding of CCL20 regulation, and its induction by GCs and inflammatory inducers is important in understanding its role in mediating pathology, and for the development of drugs without negative side effects due to CCL20 induction.

# Chapter 2: Materials and Methods

## 2.1 Cell Culture

Human epithelial cervical cancer cells (HeLa), African Green monkey kidney fibroblast cells (COS-1) and adenocarcinomic human alveolar basal epithelial cells (A549) were purchased from America Type Culture Collection (ATCC, USA). Human osteosarcoma cells (U2OS) were purchased from Sigma-Aldrich, South Africa. The cell lines mentioned above were cultured in 75 $cm^2$ flasks (Greiner Bio-one International, Austria) in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, South Africa) supplemented with 10% (v/v) fetal bovine serum (Highveld Biological, South Africa), 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco, Invitrogen, UK).

Human endocervical cells immortalized with human papillomavirus 16 E6/E7 (End1) cell line (Fichorova *et al.*, 1997) was obtained from Dr Fichorova, Brigham & Women's Hospital, Boston, USA. End1 cells were cultured in 75 $cm^2$ flasks (Greiner Bio-one International, Austria) in keratinocyte serum-free medium (KSFM) (Sigma-Aldrich, South Africa) supplemented with the provided keratinocyte growth supplement, 100 U/mL penicillin and 100 µg /mL streptomycin (Gibco, Invitrogen, UK).

All cells were maintained at 37°C in a 5% $CO_2$ incubator. Cells were passaged with 0.25% trypsin/ 0.1% EDTA in PBS (Highveld Biological, South Africa). To stop the trypsinization, the process was neutralization with medium [DMEM (Sigma-Aldrich, South Africa), 10% (v/v) calf serum (Highveld Biological, South Africa), 100 U/mL penicillin and 100 µg /mL streptomycin (Gibco, Invitrogen, UK)]. The cell lines were regularly tested for mycoplasma infection by Hoechst staining (Freshney, 1987), and only mycoplasma-negative cells were used in experiments.

## 2.2 Compounds and Antibodies

Dexamethasone (dex) (D4902), tumour necrosis factor α (TNFα) (T7539), phorbol 12-myristate 13-acetate (PMA) (P1585), Mifepristone (M8046) (RU486), and lipopolysaccharide (LPS) (L2630) were from Sigma-Aldrich, South Africa. Interferon γ (IFNγ) (01-A0060-0100) was from ORF Genetics. The primary antibodies for GR (H-300; sc-8992), GAPDH (0411; sc-47724), c-Jun (H-79; sc-1694), c-Fos (K-25; sc-253) were from Santa Cruz Biotechnology, USA. The secondary antibodies anti-mouse HRP (sc-2005) and anti-rabbit HRP (sc-2313) were also from Santa Cruz Biotechnology (Santa Cruz, CA).

## 2.3 Plasmids

The pTAT-GRE-E1b-luc (TAT-GRE) plasmid containing the E1b promoter and two copies of rat GRE (Sui *et al.*, 1999), was a kind gift from Dr G. Jenster (Erasmus University of Rotterdam, Rotterdam, Netherlands). The pGL2-MIP-3α plasmid (the –871/+58 fragment of the human CCL20 promoter) and pGL2-MIP-3α/mκB plasmid (like the pGL2-MIP-3α plasmid, but with the NF-κB-binding site (nt –92 to –82) mutated through site-directed mutagenesis) (Imaizumi *et al.*, 2002) were a kind gift from Prof. N. Mori (University Graduate School of Medical Sciences, Nagasaki, Japan). The pcDNA3 (empty vector) plasmid was obtained from Invitrogen, UK. The steroid receptor plasmid pcDNA3-hGR (GR) (Verhoog N, 2011) was obtained from Prof. D.W. Ray (University of Manchester, UK).

## 2.4 Plasmid Transformation and Preparation

Plasmids were prepared by transforming them in competent *E. coli* DH5α cells, while the pGL2-MIP-3α and the pGL2-MIP-3α/mκB plasmids were transformed into *E. coli* JM109 cells (Promega). Transformation was done as according to the heat shock method of Sambrook *et al*. (Sambrook *et al.*, 1989). Briefly, 50 ng of plasmid DNA was added to 100 µl of competent cells and mixed, without vortexing. The cells were incubated for 20 mins on ice, heat shocked for 45 secs at 42°C, and incubated on ice for 5 mins. Thereafter, 1 ml SOC medium (2% (w/v)

tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 10 mM MgCl2 and 20 mM glucose) was added, and the mixture was incubated at 37°C for 60 mins, with shaking. Of the mixture, 200 µl was plated on an LB-AMP selection plate (1% (w/v) tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agarose containing 50 µg/mL of ampicillin) overnight at 37°C. A single colony was inoculated in 5 ml LB medium (1% (w/v) tryptone, 0.5% yeast extract and 1% NaCl) with ampicillin (100 ng/µl), and incubated at 37°C for 8 hours. To purify the culture, 1 ml was added to the Promega Pureyield Plasmid Midi-prep kit (Promega Corp., USA), according to the manufacturer's instructions. DNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies), while the integrity and purity of the plasmids was analysed by restriction enzyme digestion and agarose-gel electrophoresis (Sambrook *et al.*, 1989).

## 2.5 Transient Transfection of Cells and Luciferase Assay

For the promoter-reporter luciferase assay, HeLa cells were plated in 48-well plates (Sigma Aldrich, RSA) at a density of 5.0 x $10^4$ cells per well. The cells were transiently transfected the following day with either (250 ng DNA per well) pGL2-MIP3α or pGL2-MIP3α/κBm using XtremeGene9 (Roche Diagnostics, RSA) and incubated for 24 hours. Cells were washed once with PBS on day three, and treated with 100 nM dex, 20 ng/mL TNFα, 20 ng/mL PMA, 5 ng/mL LPS, or 20 ng/mL IFNγ, in serum free DMEM medium for 24 hours. The cells were harvested by washing twice with PBS and lysed in 50 µl 1X Reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity from the lysates was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and a Modulus microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA). Luciferase values were normalized to protein content per well using the standard Bradford assay (Bradford, 1976).

## 2.5 RNA Isolation and cDNA Synthesis

HeLa cells were plated at 5.0 x10 $^{4}$ cells per well, End1 cells at 2.0 x $10^{5}$ per well, and both COS1 and U2OS at 1.5 x $10^{5}$ cells per well in 12 well plates (Greiner Bio-one International, Austria). 24 hours later (and 48 hours later for HeLa), cells were treated with test compounds or vehicle for another 24 hours. Thereafter, cells were washed with PBS, and Total RNA was isolated from cells using TRI Reagent® (Sigma-Aldrich, South Africa) according to the manufacturer's instructions.

The isolated RNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies) and the integrity of the RNA was confirmed by the appearance of 18S and 28S ribosomal bands on denaturing agarose gel electrophoresis. Briefly, 15 µl sample loading buffer (12 % (v/v) DEPC water, 5 % (v/v) bromophenol blue solution, 7 % (v/v) glycerol, 10 % (v/v) 10X Morpholinopropanesulfonic acid (MOPS) buffer (0.2 M MOPS in DEPC water, 0.05 M sodium acetate and 0.01 M ethylenediaminetetra-acetic acid (EDTA)), 17 % (v/v) 12.3 M formaldehyde and 49 % (v/v) formamide) with 20 µg/mL ethidium bromide (EtBr) was added to 0.5 µg RNA, then electrophoresed on a 0.8 % agarose gel (70 % (v/v) DEPC water, 10 % (v/v) 10X MOPS buffer and 20% (v/v) formaldehyde) at 70 V for 45 mins.

Total RNA (500 ng) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions.

## 2.6 Real-Time quantitative Reverse Transcription PCR (qRT-PCR)

Equal volumes of synthesised cDNA were used for real-time qRT-PCR using the Sensi-Mix SYBR Green I system (Celtic Diagnostics, South Africa), or the FastStart Essential DNA Green Master system (Roche Applied Science, South Africa), and the Rotor-gene, RG-3000A (Corbett Research). Gene expression was measured using specific primer sets as described in Table 2 with GAPDH serving as the 'housekeeping' gene. The 20 µl PCR reaction mix contained 10 µl Sensi-Mix SYBR Green I, or the FastStart Essential DNA Green Master, 1 µl cDNA, 1 µl sense primer, 1 µl anti-sense primer and 7 µl PCR grade, RNA-free water. The PCR protocol was as follows: 95°C for 10 min followed by 40 cycles of 95°C for 10 secs, annealing for 10 sec and

72°C for 10 sec. To confirm the amplicon generated was the one desired, melting curve analysis and gel electrophoresis was performed on each sample. A standard curve was generated to determine the efficiency of each primer set, and this was used to determine relative transcript levels by the method described by Pfaffl et al. 2002 (Supp Fig 5). GAPDH was used to normalise transcript levels.

Table 2. Primer sequences and operating information.

| Gene | Primer Sequence (5' – 3') | Strand | Annealing Temp. (°C) | Final Conc. | Reference |
|---|---|---|---|---|---|
| CCL20 | CGAAGCAACTTTGACTGCTG | Forward | 58°C | 500 nM | (Miao *et al.*, 2012) |
| | CAAGTCCAGTGAGGCACAAA | Reverse | 58°C | 500 nM | |
| GAPDH | TGAACGGGAAGCTCACTGG | Forward | 58°C | 500 nM | (Ishibashi *et al.*, 2003) |
| | TGTCAGTTGATAAAACCGCTGCC | Reverse | 58°C | 500 nM | |
| IL6 | TCTCCACAAGCGCCTTCG | Forward | 60°C | 250 nM | (Wolf *et al.*, 2002) |
| | CTCAGGGCTGAGATGCCG | Reverse | 60°C | 250 nM | |

## 2.7 SDS-PAGE and Western Blotting

HeLa cells were plated at 5.0 x $10^4$ cells per well, End1 cells at 2.0 x $10^5$ per well, and both COS1 and U2OS at 1.5 x $10^5$ cells per well in 12 well plates (Greiner Bio-one International, Austria). 24 hours later (and 48 hours later for HeLa), cells were treated with test compounds or vehicle for another 24 hours. Thereafter, cells were washed once with PBS and lysed with 50 µl 2X SDS sample buffer (5 X SDS sample buffer: 100 mM Tris-Cl pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% ß-mercaptoethanol and 0.1% (w/v) bromophenol-blue) and boiled for 10 min at 100 °C. Equal amounts of the harvested samples were loaded on an 8% SDS polyacrylamide gel (PAGE) and separated at 120 V using a BioRad Mini Protean® II electrophoresis cell and a running buffer solution (25 nM Tris-HCL pH 6.8, 250 mM glycine and, 0.1% (w/v) SDS). The separated

proteins were subsequently electroblotted for 60 min, with a constant current set at 180 mA, onto a Hybond™ ECL™ nitrocellulose membrane (GE healthcare, Germany), contained within a BioRad Mini Trans-blot® cell, according to manufacturer's instructions, and submerged in ice-cold transfer buffer (25 mM Tris, 200 mM glycine, 10% (v/v) methanol). The membranes were blocked in 4% enhanced chemiluminescence (ECL) blocking solution (Amersham) and reconstituted in a Tris-buffered Saline and Tween-20 (TBS-T) solution (50 mM Tris, 150 mM NaCl and 0.1% (v/v) Tween) at pH 75 (Sambrook *et al.*, 1989). Monoclonal primary antibodies were diluted in 4% (ECL) blocking solution. Primary antibody solutions were added to the membrane and incubated overnight at 4°C on an orbital shaker at 65 rpm. After incubation, excess primary antibody was removed by washing the membrane for 15 min in 10 mL TBS-T at room temperature, followed by 2 similar TBS-T washes for 5 min each. Secondary HRP conjugated antibody, diluted in 5% (w/v) fat-free milk powder reconstituted in (TBS-T) solution was added to the membrane and incubated for 60 min at 65 rpm at room temperature. The membrane was washed again, as previously described, to remove excess secondary antibodies. Proteins were visualised using Pierce® ECL western blotting substrate (Thermo Scientific, South Africa), according to the manufacturer's protocol, and Hyperfilm MP high performance autoradiography film (Amersham, South Africa). Bands were scanned and quantified using the ImageJ software (National Institutes of Health).

Table 3. Dilutions of antibodies used in western blot analysis.

| Antibody | Dilution | Secondary antibody |
|---|---|---|
| GAPDH | 1 : 20000 | Mouse (1 : 5000) |
| GR | 1 : 4000 | Rabbit (1 : 10000) |
| HSP90 | 1 : 1000 | Mouse (1 : 5000) |
| c-Jun | 1 : 1000 | Rabbit (1 : 10000) |

## 2.8 Statistical Analysis

Statistical analyses were performed with GraphPad Prism software (version 5 and version 7) with different letters representing statistical significance by one-way ANOVA, with Tukey's Multiple Comparison Test, or a two-way ANOVA with Tukey's Post-Test with significance either denoted with different letters, or by #, ##, ### or ### indicating $P< 0.05$, $P< 0.001$, $P<0.0005$ or $P< 0.0001$, respectively (Tukey, 1949). Statistical significance by a t-test is denoted by *, **, *** or **** to indicate $P< 0.05$, $P< 0.001$, $P<0.0005$ or $P< 0.0001$, respectively. The statistical tests performed for each experiment are indicated in the respective figure legends. Both a one-way ANOVA and t-test was performed on the results obtained in the results throughout. While a t-test is more likely to show a significant difference between different conditions, it is more likely to produce a Type I error. Therefore, a one-way, or two-way ANOVA was used to control for this error as it is more stringent. However, although the t-test is more likely to produce Type I error, this does not necessarily mean that the statistical significance produced by this test is incorrect, therefore both tests are shown, and discussed.

# Chapter 3: Results

## 3.1 Dex and TNFα can induce CCL20 expression, and modulate each other's induction in a cell-specific manner

It has previously been shown in the lung epithelial 16HBE cell line that a glucocorticoid could induce the expression of CCL20 mRNA, and that this expression could be potentiated when co-stimulated with the mediator of inflammation TNFα (Zijlstra *et al.*, 2014). To investigate whether CCL20 mRNA induction and potentiation can occur in different cell lines, HeLa, End1, COS1, U2OS and A549 cells were stimulated with 100 nM dex, 20 ng/mL TNFα, or a combination of both for 24 hours in serum-free media, and CCL20 mRNA was measured using qRT-PCR, with fold induction measured relative to vehicle. The primer efficiencies for CCL20 and GAPDH primers were determined by generating standard curves (Supp Fig 5). In the HeLa cell line dex and TNFα were able to induce a 258- and 293-fold increase in CCL20 mRNA expression, respectively, compared to the vehicle (Fig 3.1A). Co-stimulation with dex and TNFα resulted in a significant 5177-fold increase in CCL20 mRNA expression, which was significantly different to that of TNFα alone indicating apparent synergism in the HeLa cell line (Fig 3.1A bar 4).

The previous experiment was repeated in the End1, A549, U2OS and COS1 cell lines. In the End1 cell line dex and TNFα were each able to induce a statistically significant 1.66- and 48.9-fold increase in CCL20 mRNA expression; with co-stimulation not causing an increase in induction compared to the TNFα response alone, which resulted in a 49-fold increase in CCL20 mRNA (Fig 3.1B). In the A549 cell line, dex and TNFα were able to induce statistically significant 4.22- and 1029-fold increases respectively in CCL20 mRNA expression; while co-stimulation with dex and TNFα did not cause an increase in induction compared to the TNFα response alone, with a 763-fold induction of CCL20 mRNA compared to the vehicle (Fig 3.1C). However, there was a high degree of error in the A549 result. In the U2OS cell line, dex and TNFα were able to induce 4.16- and 19.7-fold increase, respectively compared to vehicle although this was not statistically significant most likely due to experimental error (Fig 3.1D). Co-stimulation with dex and TNFα caused an increase in induction compared to the TNFα response alone, with a

statistically significant 37.7-fold increase in CCL20 mRNA compared to the vehicle (Fig 3.1D bar 4). The COS1 result was similar to the U2OS, with no significance seen most likely due to experimental error. Dex and TNFα caused 1.77- and 22.6-fold increases, respectively, in CCL20 mRNA, with co-stimulation resulting in a 33-fold increase relative to vehicle (Fig 3.1E).

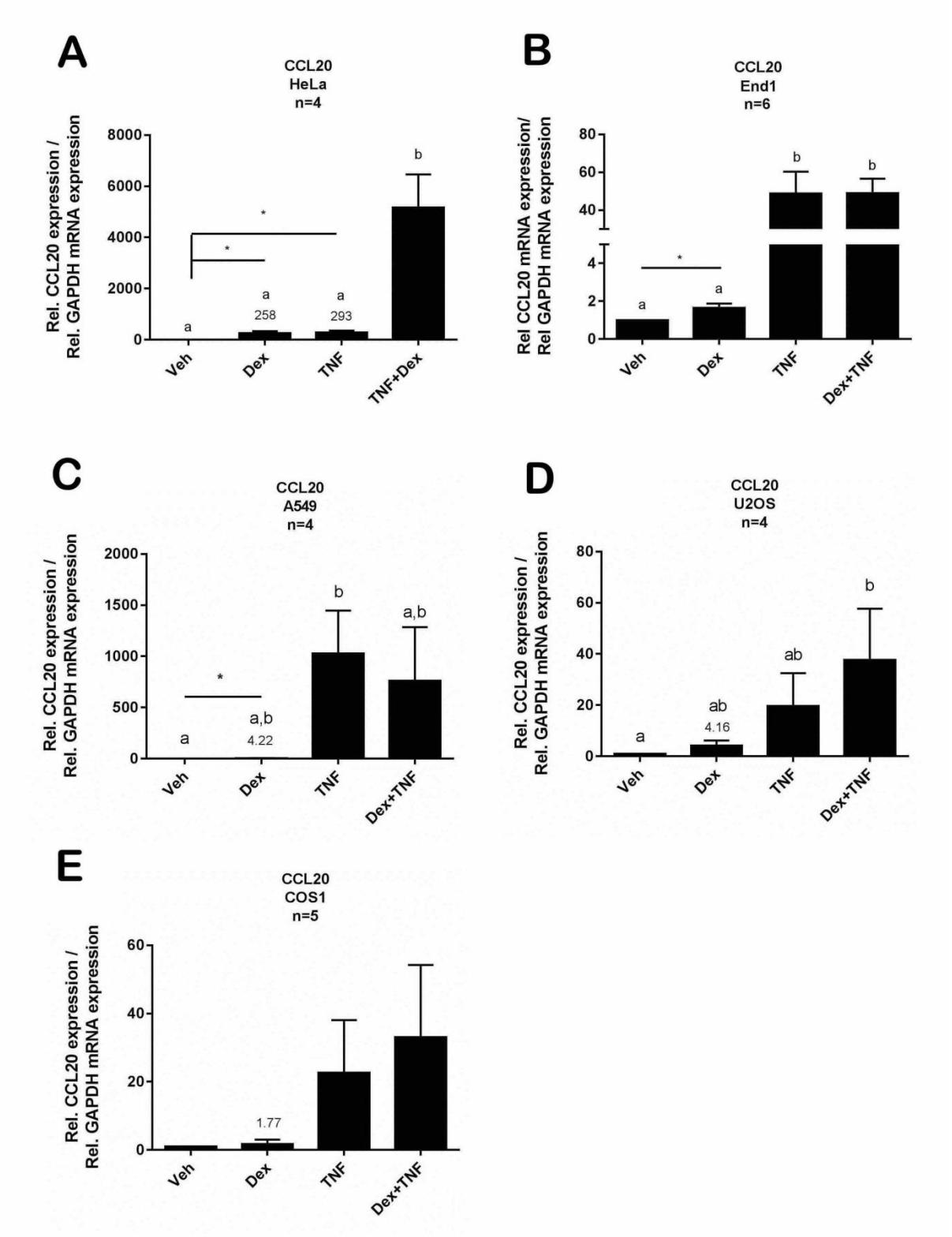



**Figure 3.1. Dex and TNFα highly induce the expression of CCL20 mRNA expression and co-stimulation has an apparent synergistic response.**

End1 (A), A549 (B), HeLa (C), U2OS (D) and COS1 (E) cells were seeded into 12-well plates at a density of 5 x $10^5$, 5 x $10^4$, 5 x $10^5$, 2.5 x $10^5$, 2.5 x $10^5$, cells/well, respectively and incubated for 48 hours. Cells were treated with 100 nM dex, 20 ng/mL TNFα, or a combination of both, as stated, in serum-free DMEM. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. CCL20 mRNA expression was measured by qRT-PCR and

normalised to GAPDH mRNA expression. Relative gene expression was normalized to basal activity (vehicle) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as a mean. Statistical analysis was carried out using GraphPad Prism™ software, with same letters representing no statistical significance and different letters representing statistical significance by one-way ANOVA, with Tukey's Multiple Comparison Test. Statistical significance by a t-test is denoted by *, **, *** or **** to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively.

Different levels of transcription factors between the various cell lines may contribute to the different responses seen. To examine whether the total levels of GR and p65 differed between the cell lines, HeLa (Fig 3.2A lane 1 and 2), End1 (Fig 3.2A lane 3 and 4), A549 (Fig 3.2A lane 5 and 6), U2OS (Fig 3.2A lane 7 and 8) and COS1 cell lines were probed for GR, p65 and GAPDH under basal conditions with two independent experiments per cell line. Results were scanned and quantified with GR (Fig 3.2B) and p65 (Fig 3.2C) relative to GAPDH protein levels as determined via western blotting and band density.

The HeLa and End1 cell lines have the highest levels of GR protein. U2OS and COS1 cell lines have very low levels of GR protein as expected. Statistical significance is only seen between the HeLa and End1 cell lines compared to the U2OS and COS1 cell lines (Fig 3.2B compare lanes 1 and 2 to lanes 4 and 5). The levels of total p65 were very similar in each cell line, with no statistical significance between the cells (Fig 3.2C).

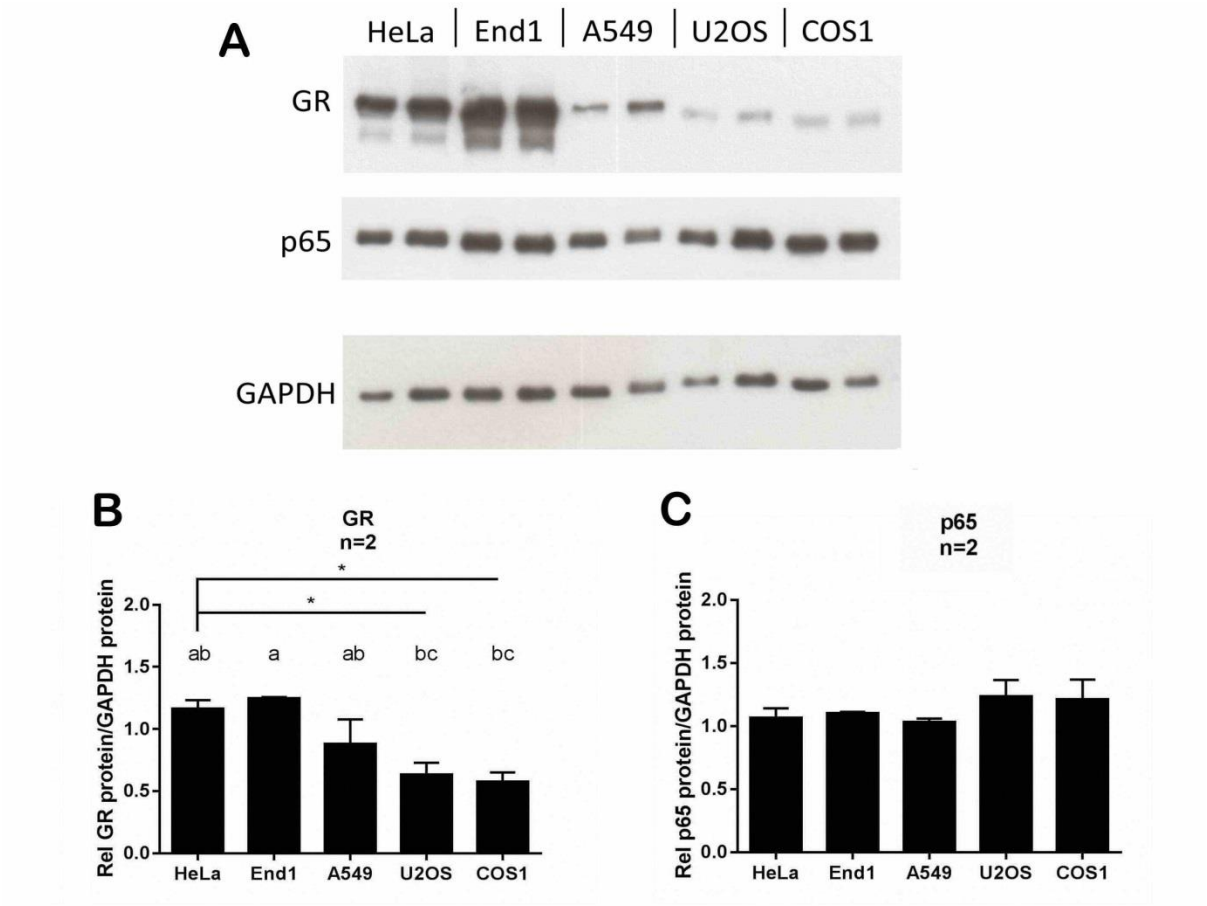



**Figure 3.2. Western blot analyses of GR and p65 under basal (vehicle) conditions, scanned and quantified.** HeLa (lane 1 and 2), Hend1 (lanes 2 and 3), A549 (lanes 5 and 6), U2OS (lanes 7 and 8) and COS1 (lanes 9 and 10) cells were probed to examine basal GR and p65 protein levels with each replicate an independent experiment (n=2). Equal amounts of cell lysates were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with antibodies to GR, p65 and GAPDH (loading control). Band density was quantified using ImageJ™ Software with GR (B) and p65 (C) relative to GAPDH. Statistical analysis was carried out using GraphPad Prism™ software and ImageJ software. Statistical significance by a t-test is denoted by *, **, *** or **** to indicate $P< 0.05$, $P< 0.001$, $P<0.0005$ or $P< 0.0001$, respectively.

TNFα and dex are able to induce CCL20 with statistical significance in the HeLa, End1 and A549 cell lines, with co-stimulation enhancing the effect with statistical significance in the HeLa cell lines (Fig 3.1). The differences in CCL20 expression between the cell lines when stimulated with dex may be due to the differences in total GR. The total p65 levels did not vary between the cell lines.

## 3.2 Other pro-inflammatory ligands besides TNFα can induce the expression of CCL20 mRNA expression and can modulate the dex response

To further investigate the crosstalk between dex and ligands that induce inflammation and therefore different signalling pathways, the PKC activator PMA, the $T_h1$ cytokine IFNγ, and the bacterial cell wall component LPS were used instead of TNFα. PMA was used as it acts directly on PKC, which is involved in inflammatory signal transduction pathways, while IFNγ was used as it is specifically part of the $T_h1$ immune response, and has been shown not to activate NFκB. LPS was used as it is exogenous, unlike the other ligands, and is of bacterial origin. HeLa cells were stimulated with 100 nM dex, a signal of inflammation (5 ng/mL PMA, 20 ng/mL IFNγ, 5 ng/mL LPS) or a combination of both for 24 hours in serum-free media (Fig 3.3A-C), and CCL20 mRNA was measured using qRT-PCR, with fold induction measured relative to vehicle. Stimulation with dex increased CCL20 mRNA by 253-, 290- or 227.6-fold (Fig 3.3A-C bar 2), while stimulation with PMA, IFNγ and LPS increased CCL20 mRNA by 85.9-, 2.61- or 4.62-fold, respectively (Fig 3.3A-C bar 3). Co-stimulation with dex and PMA, IFNγ or LPS increased CCL20 mRNA by 2039-, 581- or 647-fold, respectively (Fig 3.3A-C bar 4)

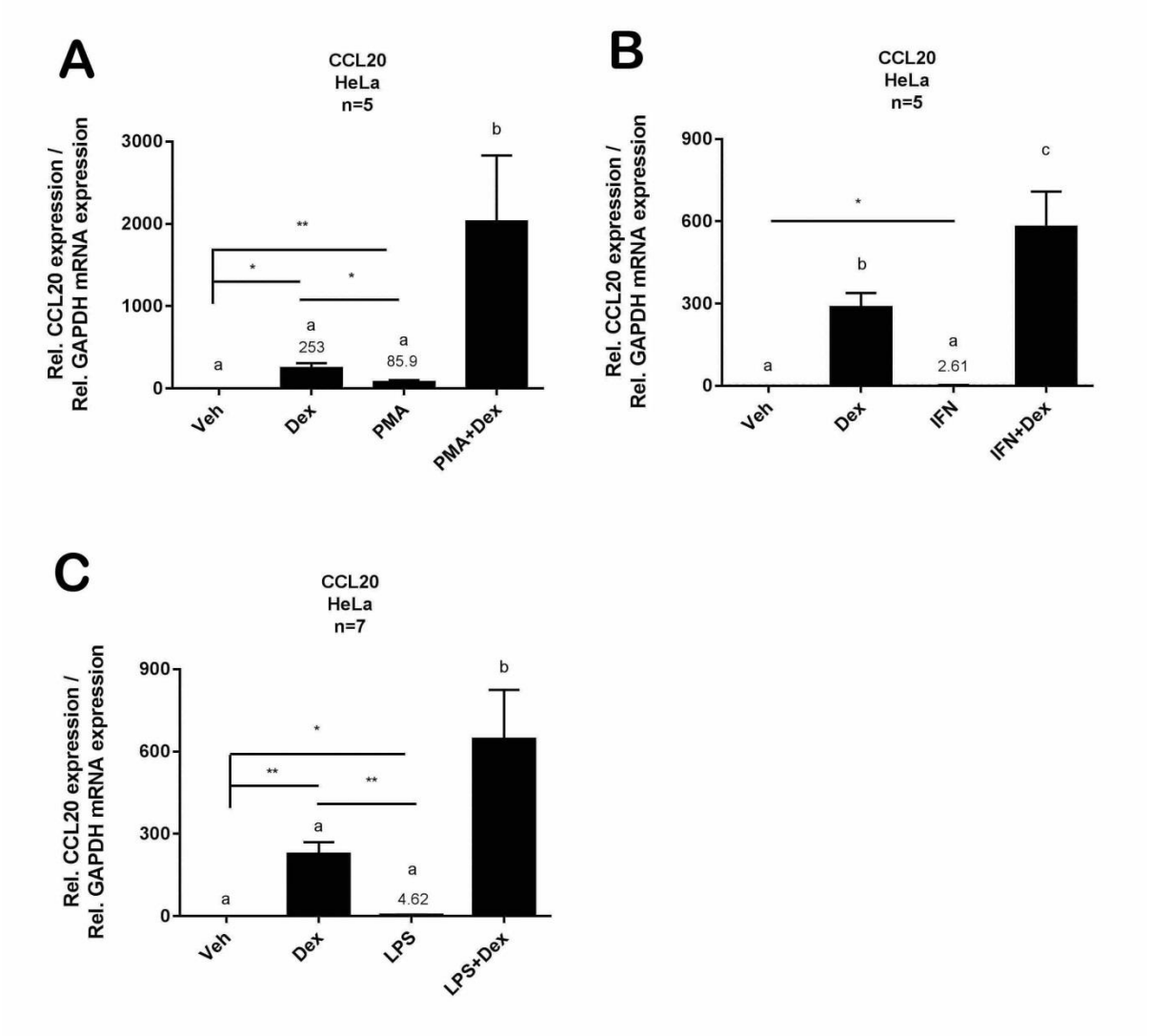



**Figure 3.3. The pro-inflammatory ligands PMA, IFN and LPS, and dex can induce the expression of CCL20 mRNA expression and can potentiate the dex response in the HeLa cell line.**

HeLa cells were seeded into 12-well plates at a density of 5 x $10^5$ cells/well and incubated for 48 hours. Cells were treated with 100 nM dex, and/or 5 ng/mL PMA (A), 20 ng/mL IFNγ (B), 5 ng/mL LPS (C), or a combination of both, as stated, in serum-free DMEM. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. CCL20 mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Relative gene expression was normalized to basal activity (vehicle) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean +/- SEM. Statistical analysis was carried out using GraphPad Prism™ software, with different letters representing statistical significance by one-way ANOVA, with Tukey's Multiple Comparison Test. Statistical significance by a t-test is denoted by *, **, *** or **** to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively.

The different ligands were also used on A549 cells as above (Supp Fig 2).

The different ligands used to stimulate the cells may affect the levels of protein involved in the signalling pathways that are a part of CCL20 regulation. To examine whether stimulation with the various ligands affected the total levels of GR and p65, HeLa cells were stimulated in the

absence or presence of 100 nM dex, and either 20 ng/mL TNFα, 5 ng/mL PMA, 20 ng/mL IFNγ or 5 ng/mL LPS for 24 hours in serum-free media. Fig 3.4A and B represent two independent experiments. Results were scanned and quantified for GR (Fig 3.4C) and p65 (Fig 3.4D) relative to GAPDH protein levels as determined via western blotting and band density. Stimulation with dex appeared to cause GR turnover, which was expected (Chapter 1.2), with a significant difference seen in in the TNFα compared to TNFα and dex result (Fig 3.4C compare lanes 9 and 10). The total levels of p65, however, did not appear to change under the various conditions (Fig 3.4D).

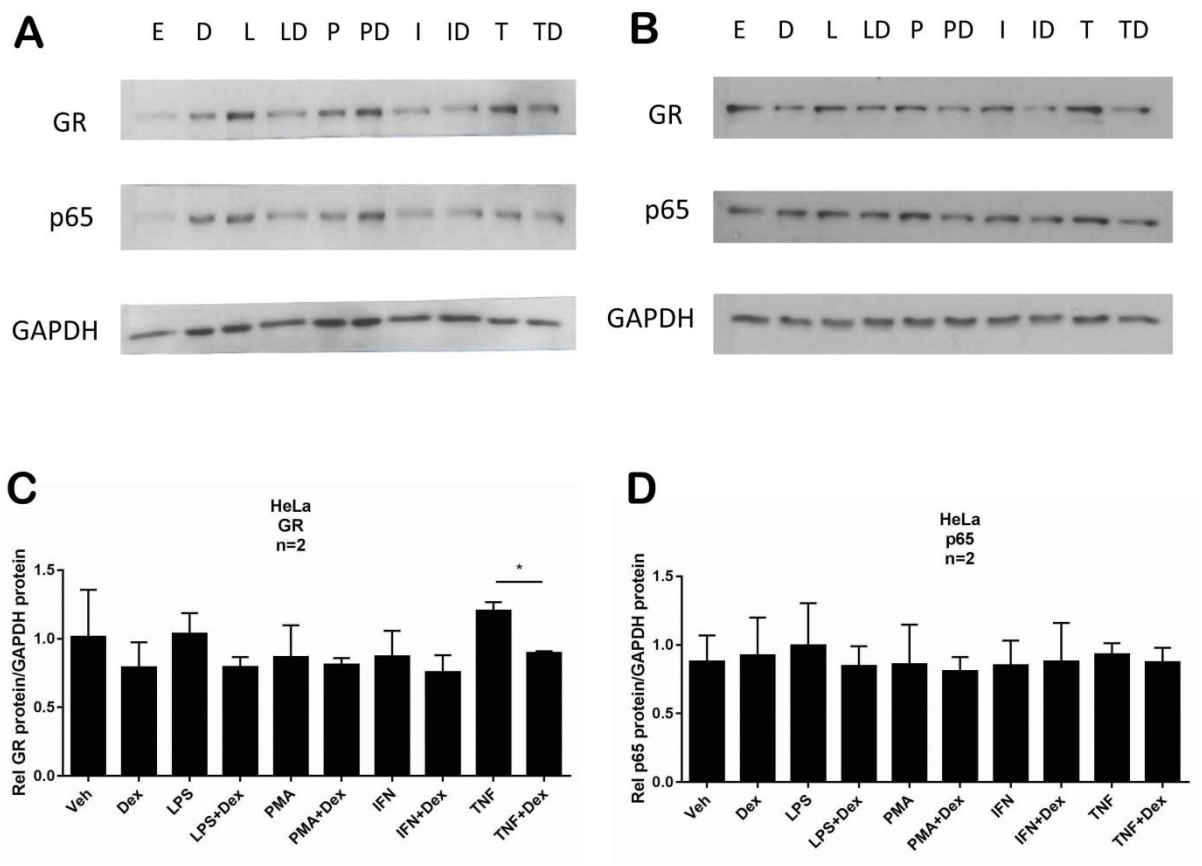



**Figure 3.4. Western blot analyses of GR and p65 under various cellular conditions, scanned and quantified.**

HeLa cells were seeded into 12-well plates at a density of 5 x $10^5$ cells/well and incubated for 48 hours. Cells were treated with either 20 ng/mL TNFα, 20 ng/mL PMA, 5 ng/mL LPS, or 20 ng/mL IFNγ, in the presence or absence of 100 nM dex, as stated, in serum-free DMEM. Equal amounts of cell lysates were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with antibodies to GR, p65 and GAPDH (loading control). A and B are 2 independent experiments. The quantified levels of GR (C) and p65 (D) protein levels are shown for A and B n=2. Statistical analysis was carried out using GraphPad Prism™ software and ImageJ software. Statistical significance by a t-test is denoted by *, **, *** or **** to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively.

Taken together, all four inflammatory inducers, TNFα, PMA, IFNγ and LPS, are able to induce CCL20 expression in the HeLa cell line (Fig 3.1 and 3.3). Furthermore, all inflammatory inducers are able to potentiate the dex response. Total GR levels did not change due to the inflammatory inducers used, but GR turnover was seen when cells were stimulated with dex as expected. Total p65 levels were not altered between different conditions. This indicates that there is crosstalk between the GR signalling pathway, and that of the inflammatory inducers used.

## 3.3 The GR is required for the dex response on CCL20 mRNA, with GR inhibition resulting in the loss of the modulatory effects of co-stimulation with pro-inflammatory ligands

To confirm whether the dex-induced increase of CCL20 mRNA is mediated via the GR, the GR antagonist RU486 was used to inhibit the receptor (Philibert *et al.*, 1990). Although RU486 also antagonizes the progesterone receptor (PR), it has been shown that HeLa cells do not express the PR (Bourgeois *et al.*, 1984). The potential of the unliganded GR to modulate the CCL20 mRNA induction of the pro-inflammatory ligands was also investigated.

HeLa cells were stimulated with 100 nM dex, 20 ng/mL TNFα, or a combination of both for 24 hours in serum-free media, in the presence or absence of 1nM RU486. CCL20 mRNA was measured using qRT-PCR, with fold induction measured relative to vehicle. On average, stimulation with RU486 increased basal CCL20 expression about 10-fold relative to vehicle (Fig 3.5A-D bar 2). The dex-induced expression of CCL20 mRNA was significantly reduced to basal levels in the presence of RU486 from 266.9- to 9.12-fold, 199.6- to 9.12-fold, 199.6- to 14.7-fold and 199.6- to 9.12-fold (Fig 3.5A-D, compare bars 3 and 4). RU486 significantly repressed the apparent synergy between dex and TNFα, PMA, IFNγ and LPS from 5637- to 671-fold, 1867- to 178-fold, 861- to 2.84-fold, and 697- to 13.2-fold, respectively (Fig 3.5A-D).

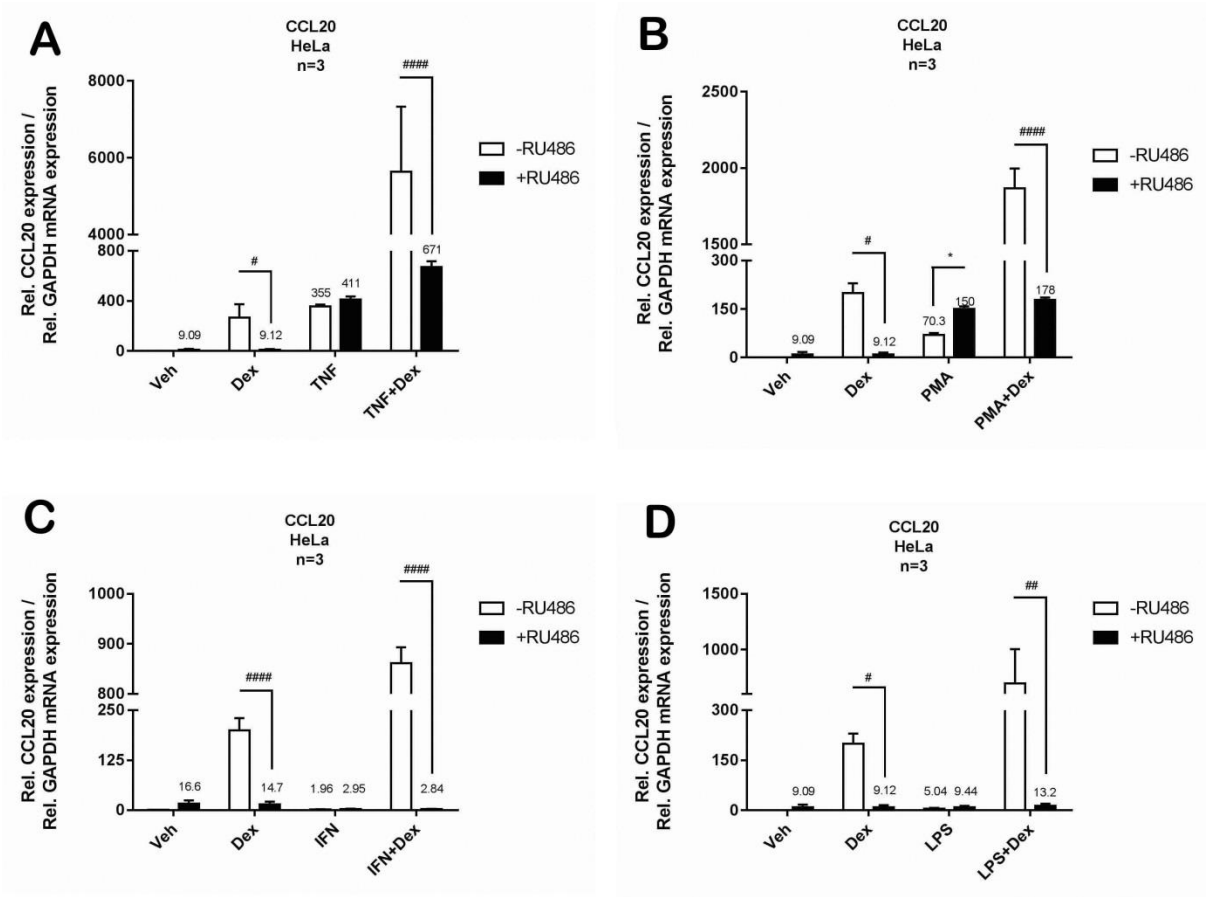



**Figure 3.5. The GR is required for the dex effect on CCL20 mRNA levels, as the GR inhibitor RU486 abolishes the response and the dex modulation of TNFα, PMA, IFNγ and LPS.**

HeLa cells were seeded into 12-well plates at a density of 5 x $10^5$ cells/well and incubated for 48 hours. Cells were treated with 100 nM dex 20 ng/mL and/or TNFα (A), and/or 5 ng/mL PMA (B), 20 ng/mL IFNγ (C), 5 ng/mL LPS (D), as stated in the presence or absence of 100 nM dex, or 100 nM dex alone. These conditions were further co-stimulated in the presence, or absence of 1 nM RU486, as stated, in serum-free DMEM for 24 hours. Statistical analysis was carried out using GraphPad Prism™ software, with Statistical significance by one-way ANOVA, with Tukey's Multiple Comparison Test denoted by #, ##, ###, or #### to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively. Statistical significance by a t-test is denoted by *, **, *** or **** to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively.

The GR is required for the dex-induced increase of CCL20, with inhibition of the receptor with RU486 resulting in a loss of the dex response. Inhibition of the GR did not affect the inflammatory inducers, although it did prevent dex potentiating their response.

## 3.4 The pro-inflammatory ligands can potentiate the dex transactivation of the CCL20 promoter-reporter construct pGL2-MIP3α-LUC

Previous results have shown that both dex and the pro-inflammatory ligands; TNFα, PMA and LPS can induce endogenous CCL20 mRNA expression. Dex can also modulate the induction of CCL20 when co-stimulated with the pro-inflammatory ligands. To examine whether there is crosstalk between dex and the pro-inflammatory ligands on a minimal promoter, the pGL2-MIP3α-LUC plasmid reporter construct was used. To confirm the correct plasmid was used, a restriction enzyme digest was performed (Fig 3.6). The fragment sizes were confirmed to be similar to what was expected (Table 4). The reporter plasmid has the putative CCL20 promoter, -871 base pairs upstream, and +58 base pairs downstream of the transcription start site, and therefore only contains certain cis-regulatory elements (Sugita *et al.*, 2002). Knowledge of which transcription binding sites are present, and how dex and the different mediators of inflammation affect its transcription will help elucidate the different mechanisms of CCL20 gene regulation.

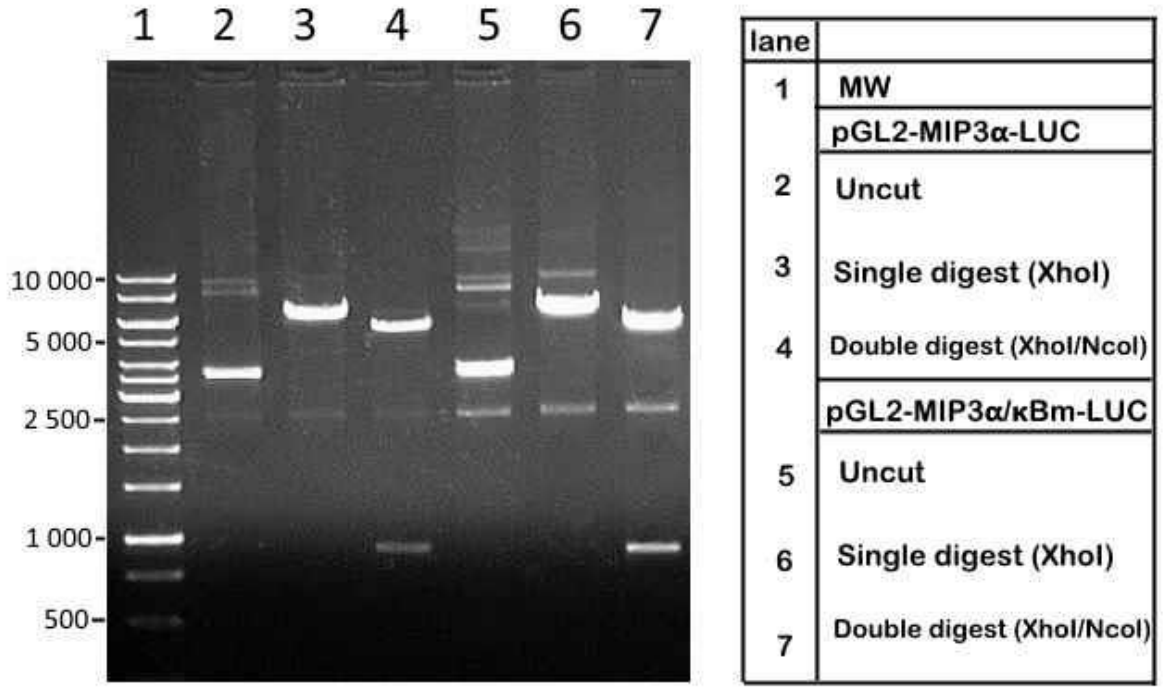



**Figure 3.6. Restriction enzyme digest of the pGL2-MIP3α-LUC and pGL2-MIP3α/κBm-LUC promoter-reporter constructs to confirm plasmid identity.**
Digested plasmid fragments of the pGL2-MIP3α-LUC and pGL2-MIP3α/κBm-LUC were separated via electrophoresis on a 0.8% agarose gel. 3 µl of a 1kb molecular weight marker (Fermentas Life Science O'GeneRuler™) was used in lane 1 to confirm plasmid fragment sizes.

**Table 4. Expected and obtained band sizes after restriction enzyme digest of pGL2-MIP3α-LUC and pGL2-MIP3α/κBm-LUC promoter-reporter constructs (Imaizumi *et al.*, 2002).**

| Lane | Expected fragment size(s) (bp) | Obtained fragment size(s) (bp) |
|---|---|---|
| 1 | MW ladder | |
| 2 | < 6629 | 9500, 8500, 3750 and 2500 |
| 3 | 6629 | 7000 and 2500 |
| 4 | 5693 and 936 | 5500, 2500 and 950 |
| 5 | < 6629 | 9500, 8500, 3750 and 2500 |
| 6 | 6629 | 9500, 7500 and 2500 |
| 7 | 5693 and 936 | 5500, 2500 and 950 |

HeLa cells were transfected with the pGL2-MIP3α-LUC plasmid reporter construct for 24 hours before stimulating with 100 nM dex, a signal of inflammation (20 ng/mL TNFα, 5 ng/mL PMA, 20 ng/mL IFNγ or 5 ng/mL LPS) or a combination of both for 24 hours in serum-free media (Fig

3.7A-D). As shown in Figure 3.7A, stimulation with dex and TNFα caused a statistically significant 2.6-fold and 9.7-fold increase of transcriptional activity, respectively, of the luciferase reporter gene (Fig 3.7A, bar 2 and 3). Co-stimulation with dex and TNFα significantly increased activation to 20.5-fold relative to vehicle (Fig 3.7A, bar 4). PMA caused a statistically significant 13-fold increase of transcriptional activity (Fig 3.7B, bar 3), while co-stimulation with dex and PMA increased activation, statistically significant, to 31.5-fold relative to vehicle (Fig 3.7B, bar 4). IFNγ repressed the basal activity on the promoter, with a statistically significant, 0.632-fold reduction relative to vehicle. (Fig 3.7C, bar 3). Dex relieved this repression, with a 1.28-fold induction of the promoter relative to vehicle when co-stimulated with IFNγ (Fig 3.7C, bar 4). LPS was unable to activate the pGL2-MIP3α-LUC reporter (Fig 3.7D, bar 3), although did modulate the dex mediated induction from 2.06- to 4.99-fold relative to vehicle (Fig 3.7C, bar 4).

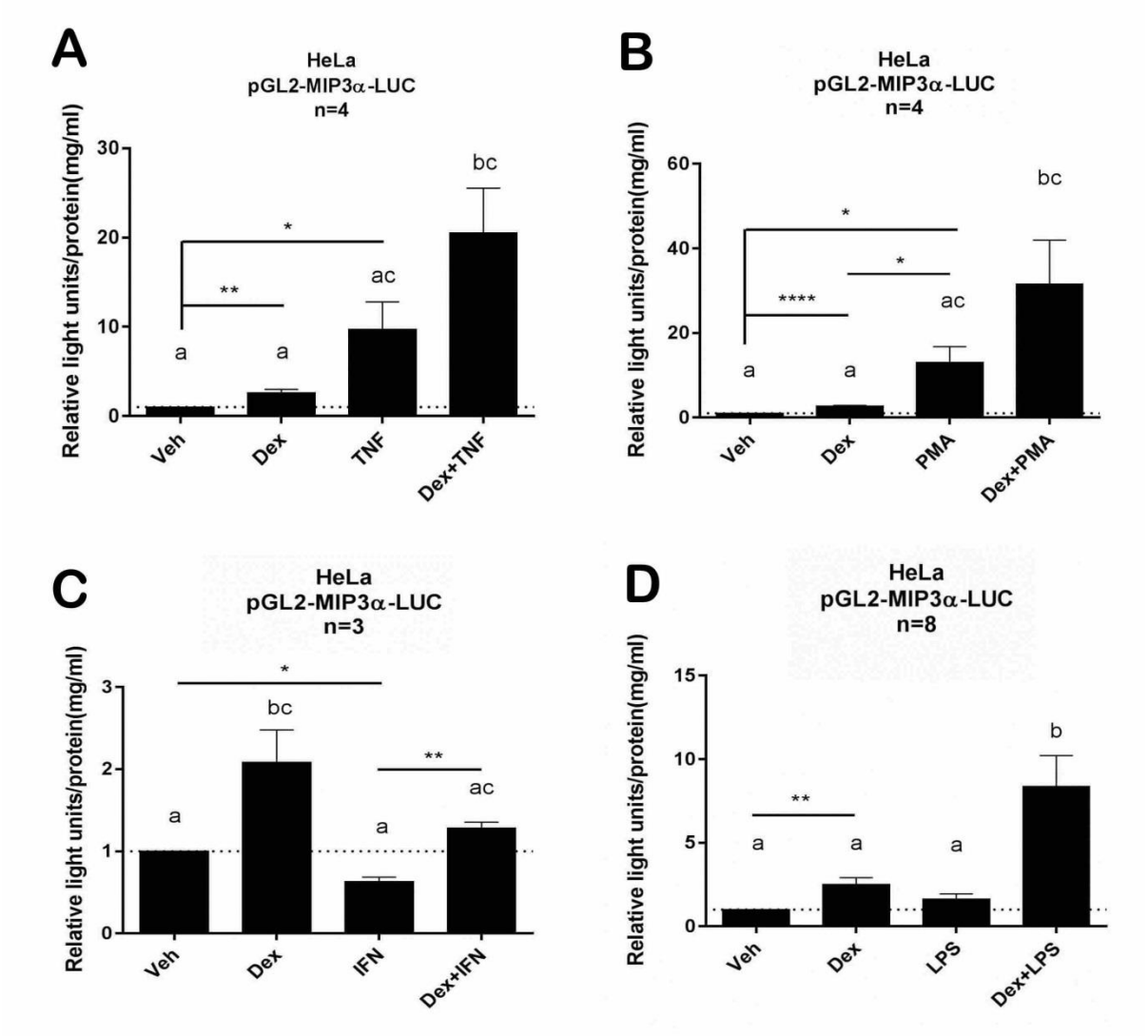



**Figure 3.7. The pro-inflammatory ligands can potentiate the dex transactivation of the CCL20 promoter-reporter construct pGL2-MIP3α-LUC.**

HeLa cells were seeded into 48-well plates at a density of 5 x $10^5$ cells/well. The following day, the cells were transiently transfected with 250 ng pGL2-MIP3α-LUC DNA. Cells were treated with 100 nM dex, 20 ng/mL TNFα, or a combination of both, as stated, in serum-free DMEM. Total cells were harvested, and RLUs measured via a luminometer and normalised to respective protein content, determined with a Bradford assay. Promoter activity was normalized to basal activity (vehicle) equal to 1, in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as a mean +/- SEM. Statistical analysis was carried out using GraphPad Prism™ software, with different letters representing statistical significance by one-way ANOVA, with Tukey's Multiple Comparison Test. Statistical significance by a t-test is denoted by *, **, *** or **** to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively.

Dex was shown to induce the pGL2-MIP3α-LUC promoter as it did to the endogenous gene. TNFα and PMA were able to induce promoter, with an apparent potentiation of the dex response. LPS however was not able to induce the promoter as it did the endogenous gene,

although it was shown to potentiate the dex response. Differing from the result seen on the endogenous promoter, IFNγ repressed the basal and dex mediated pGL2-MIP3α-LUC activity. This highlights the different transcription factor binding sites used between the ligands.

## 3.5 Mutation of the NFκB binding site on the CCL20 promoter-reporter construct affects the activation by dex and pro-inflammatory ligands compared to the wild-type promoter

The NFκB region has been shown to be important for TNFα induced activation of the CCL20 promoter (Sugita *et al.*, 2002). To determine whether the NFκB site of the CCL20 promoter is required for CCL20 regulation by dex and mediators of inflammation, the same reporter plasmid, with a mutated NFκB response element was used (Imaizumi *et al.*, 2002). HeLa cells were transfected with the pGL2-MIP3α-LUC reporter construct, and in parallel the NFκB mutated pGL2-MIP3α/κBm-LUC reporter construct for 24 hours.

To confirm the correct plasmid was used, a restriction enzyme digest was performed (Fig 3.6). The fragment sizes were confirmed to be similar to what was expected (Table 4). Cells were stimulated with 100 nM dex, and either 20 ng/mL TNFα (A), 5 ng/mL PMA (B), 20 ng/mL IFNγ (C) or 5 ng/mL LPS (D) in combination or alone (Fig 3.8A-D). The overall levels of luciferase transcribed were lower in the mutated plasmid compared to the wild type, with a significant decrease in basal activation relative to the pGL2-MIP3α-LUC (Fig 3.8B-D). This highlights the importance of NFκB for CCL20 activation. The dex activation of the promoter appeared to increase relative to vehicle, with a significant increase when the basal/vehicle level of transcription of the pGL2-MIP3α-LUC and the pGL2-MIP3α/κBm-LUC reporter is set to 1 (Supp Fig 4). A significant increase in the dex mediated induction of the mutated promoter construct compared to the wild-type is seen in Supplementary Figure 4. TNFα, LPS and IFNγ were unable to transactivate the mutated promoter (Supp Fig 4A-C). The mediators of inflammation were also unable to potentiate the dex mediated activation of the mutated CCL20 promoter. IFNγ was unable to transactivate either the wild-type or mutated CCL20 promoter and inhibited the dex response (Supp Fig 4C).

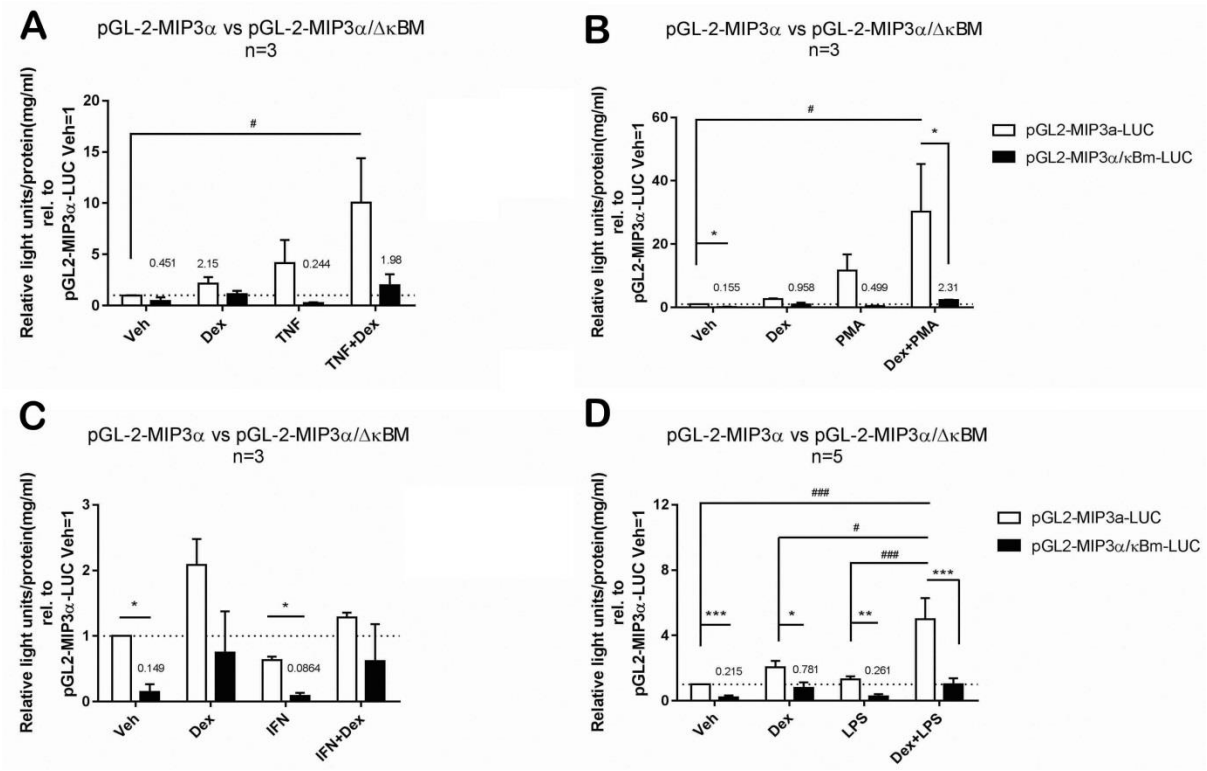



**Figure 3.8. Mutation of the NFκB binding site on the CCL20 promoter-reporter construct causes a reduction of activation by the pro-inflammatory ligands and dex compared to the wild-type promoter.**
HeLa cells were seeded into 48-well plates at a density of 5 x $10^5$ cells/well. The following day, the cells were transiently transfected with either 250 ng pGL2-MIP3α-LUC or pGL2-MIP3α/κBm-LUC reporter construct. Cells were treated with either 20 ng/mL TNFα (A), 20 ng/mL PMA (B) 5 ng/mL LPS (C), or 20 ng/mL IFNγ (D), in the presence or absence of 100 nM dex, as stated, in serum-free DMEM. Total cells were harvested, and RLUs measured via a luminometer and normalised to protein content, determined with a Bradford assay. Graphs represent pooled results of at least three independent experiments and are plotted as mean +/- SEM. Statistical analysis was carried out using GraphPad Prism™ software, with Statistical significance by one-way ANOVA, with Tukey's Multiple Comparison Test denoted by #, ##, ###, or #### to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively. Statistical significance by a t-test is denoted by *, **, *** or **** to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively.

Mutation of the NFκB binding site reduces the overall induction of the putative CCL20, and therefore highlights the importance of the transcription factor in the induction. TNFα and PMA are unable to induce the mutated NFκB promoter, and along with LPS are unable to potentiate the dex response. IFNγ still represses the mutated promoter. The dex-induced activation of the promoter is not via the NFκB binding site, as it is still able to activate the mutated promoter relative to vehicle.

# Chapter 4: Discussion & Conclusions

## 4.1 Discussion

### 4.1.1 GCs and TNFα Increase CCL20 expression in a cell-specific manner

Zijlstra *et al*. (2014) showed that GCs can induce CCL20 mRNA expression in primary bronchial cells and a human bronchial epithelial cell line. Therefore, the current study investigated whether the synthetic GC, dex, can induce CCL20 RNA expression in cells other than bronchial epithelial cells.

Research in the expression of CCL20 in other cell types besides those of the lung is important for the understanding of the effects of CCL20 in diseases and their progression throughout the body. It is known that epithelial cells of the female genital tract can express CCL20 (Cremel *et al*., 2006). This is significant as it has been suggested CCL20 may be involved in an increase of HIV-1 acquisition (Cameron *et al*., 2010). The End1 and HeLa cell lines were, therefore, used as in vitro models of the female genital tract, the main site of HIV-1 transmission. Furthermore, because HeLa cells are cancerous in origin they are routinely used as an in vitro model of human cervical cancer. CCL20 has also been implicated in the progression of rheumatoid arthritis, with bone tissue shown to express the chemokine (A. Lee *et al*., 2014). The U2OS cell line is a bone osteosarcoma epithelial cell line, and was used to investigate the mechanisms of CCL20 regulation in the bone. A549 are cancerous human alveolar epithelial cells and are a useful model in studying the regulation of gene expression in airways epithelial cells. A previous study reported that dex suppresses TNF-induced CCL20 expression in A549 cells (Lannan *et al*., 2012). However, it remains unclear whether dex can regulate basal CCL20 expression in this cell line. Further research into the mechanisms of CCL20 regulation in these cell types, and whether GCs may exacerbate the pathology of these tissues via increasing CCL20 expression, is required, as well as whether this has implications for diseases and their progression. To further try and elucidate the mechanisms of CCL20 regulation by GCs and pro-inflammatory ligands, COS-1 were used. This is a fibroblast-like cell line derived from African Green monkey kidney tissue and is a non-epithelial cell type. It has not been shown in the literature whether

COS-1 cells express CCL20, however African Green monkeys have been shown to express CCL20 in lung samples (Smits *et al*., 2011).

In Figure 3.1, dex was shown to significantly induce CCL20 mRNA expression in the End1, A549 and HeLa cell lines, with a 1.66-fold, 4.22-fold and 258-fold increase, respectively, with respect to basal (vehicle) expression (Fig 3.1A, B and C). Dex, however, was not able to cause a significant increase in CCL20 in the U2OS and COS1 cells lines (Fig 3.1D and E).

GCs, such as the endogenously expressed cortisol, or synthetic GCs such as dex are used to suppress the inflammatory response and prevent the detrimental effects of chronic inflammation such as tissue damage (Lawrence *et al.*, 2007). While GCs are important in suppressing inflammation, they have also been implicated in pro-inflammatory effects. This may be to prepare the immune system for a quick and efficient response to pathogens with acute exposure and suppress the immune system with chronic exposure to GCs (Cruz-Topete *et al.*, 2015). It may be important for GCs to still chemoattract leukocytes to the site of inflammation by inducing CCL20 expression, while also suppressing the expression of pro-inflammatory genes. This may be to prevent the site of inflammation becoming immunodeficient, which could increase the chances of a secondary infection occurring (Cutolo *et al.*, 2008).

The differences in CCL20 induction in the End1, A549 and HeLa cell lines, which expressed CCL20 when stimulated with dex, could be due to a variety of reasons. Intrinsic differences between the cell lines may be a reason for why there are differences in the level of CCL20 expression. For instance, dex has been reported to upregulate basal as well as TNF/LPS-induced expression of CCL20 in human bronchial epithelial BEA-2B cell line, but not in A549 (Zijlstra *et al*., 2014; Lannan *et al*., 2014). The results presented herein show that the cell lines differ in level of expression of the GR. Hence, some cell lines may be more sensitive to treatment with dex than others. However, although HeLa and End 1 cells had similar levels of GR expression, the level of the response elicited by dex was much higher in HeLa than in End 1. This suggests that different mechanisms other than GC sensitivity may be vital for the upregulation of basal as well as TNF-induced expression of CCL20 in the cell lines. CCL20 has been shown to be

expressed in human kidney tissues, but microarray data has shown treatment with GCs reduces its expression (Cheng *et al.*, 2013). Therefore, it is likely that GCs also reduce the expression of CCL20 in the simian, COS-1 cell line. It is important to note that tissue culture work with cell lines will not always reflect what occurs *in vivo* as many other factors may not be present, and the cells may react differently to stimuli as they have been immortalised.

Cell-to-cell variations in levels of proteins such as enzymes, kinases and transcription factors can affect the expression levels of genes. The differences in CCL20 induction in the various cell lines due to stimulation with dex may, therefore, be due to differences in the relative GR protein levels. To examine whether the expression pattern of the GR varied between the different cell types, western blotting was performed. Cell lysates were probed for GR (Fig 3.2A). The U2OS and COS1 cell lines had visibly and statistically lower levels of GR than the others and may therefore be why dex is unable to induce CCL20 in these cell lines (Fig 3.1D and E). End1, A549, HeLa and U2OS cells are all cell lines of epithelial origin and COS1 cells are fibroblast, kidney cells. Epithelial cells, and specifically End1, A549 and HeLa cells, serve as cellular barriers to the outside environment and are, therefore, one of the first lines of defence against external pathogens, while the U2OS osteosarcoma cells may not respond due the gene being packaged into inactive chromatin. It may be essential for cells of epithelial tissue to excrete chemokines even when GCs are present to allow for neutrophils to be present in the area of high risk. Therefore, GCs are not necessarily immunosuppressive on all genes, at least with the respect to the innate immune system, and especially within epithelial tissues (Busillo *et al.*, 2013).

During inflammation TNFα is secreted by leukocytes and plays a major role in a systemic inflammatory response. Zijlstra *et al.* (2014) showed that TNFα caused an increase of expression of CCL20 in BEA-2B cells. This result was confirmed in Fig 3.1A, with TNFα causing about a 1000-fold increase in CCL20 mRNA expression, compared to vehicle. The TNFα mediated induction of CCL20 in the various cell lines, relative to vehicle, also showed variation as seen with dex stimulation. TNFα was able to cause a statistically significant increase of about 200-fold in the HeLa cell line and about 40-fold in the End1 cell line, while only causing less than a 50-fold increase in the U2OS and COS1 cell lines (Fig 3.1A-E).

A possible reason for the greater CCL20 response due to TNFα between the A549 cells and the HeLa and End1 cells may be due to intrinsic differences between the cell lines. As with the differences in GR protein levels seen between the cell lines being a possible contributing factor to the dex responses seen, differences in TNFα level of induction may be due to differences in amounts and activation of other transcription factors, and proteins involved. The TNFα pathway is involved in the activation of NFκB, therefore, the protein levels of the p65 subunit was probed. However, no differences, were seen between the different cell lines (Fig 3.2A). Although there were no differences seen in total p65 levels between the cell lines in Fig 3.2C, this does not exclude NFκB from playing a role in the differences in the TNFα mediated responses. As only total p65 levels were probed for, it is possible that there are changes in the activated/phosphorylated form of p65. Cells may also have different levels of IκB kinase (IKK), which, when activated, phosphorylated IκB, causing the release of NFκB to bind to the DNA (Karin, 1999). Therefore, a greater amount of IKK may allow for more active NFκB without changes in the absolute amount of NFκB. The cells may also difference in the level of expression of TNFR1/2.

### 4.1.2 The pro-inflammatory stimuli provided by PMA, IFNγ and LPS can induce CCL20 expression

To examine whether CCL20 gene expression can be regulated by other inflammatory ligands, and therefore different signalling pathways, other inflammatory molecules and mediators of inflammation were used to stimulate HeLa cells. TNFα, PMA, IFNγ and LPS can cause the activation of AP-1, while IFNγ has been shown to be unable to activate NFκB which the others can, and instead activate STAT1, which the others cannot (Chapter 1.3.2).

TNFα induces its induction of genes via the TNF receptor type 1 and 2 (TNFR1/2), activating mitogen-activated protein kinases (MAPKs) amongst other members in its signalling pathway. Activation of CCL20 by TNFα therefore may involve the activation of many members in its signalling pathway including MAPKs. To examine the effects of specifically activating the downstream, MAPK section of the TNFα signalling pathway, PMA was used to stimulate the

cells. PMA activates protein kinase C (PKC), which is a MAPK that plays an important role in several signal transduction cascades (Ali *et al.*, 2016).

Stimulation with IFNγ allowed for the activation of a different signalling pathway which does not activate NFκB as the other pro-inflammatory molecules do. This may, therefore, provide mechanistic insights into the regulation of CCL20 (Andreakos *et al.*, 2004). IFNγ is an endogenous molecule expressed in humans, and therefore would provide insights to CCL20 expression due to molecules expressed by Th1 leukocytes during an inflammatory response (Schoenborn *et al.*, 2007).

Inflammatory signals which induce the expression of chemokines can also originate from exogenous sources, such as from pathogens. Bacterial infections may require the recruitment of macrophages to phagocytose the pathogens. Cells can recognise components of bacterial cells walls using pattern recognition receptors (PRRs), such as toll-like receptor 4 (TLR4), which recognises lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria (Takeuchi *et al.*, 2010).

All pro-inflammatory inducers could induce a significant increase in CCL20 mRNA in the HeLa cell line (Figure 3.3), although the relative induction differed greatly between them. This is with accordance of CCL20 being a pro-inflammatory chemokine, therefore it has been shown to be induced by the pro-inflammatory stimuli used. Table 1 shows that the putative CCL20 promoter contains binding sites for transcription factors known to be activated by the inflammatory inducers used, namely NFκB, AP-1, and STAT1. The differences in the relative magnitude of induction compared to vehicle for each inflammatory signal may be due to other proteins and transcription factors activated by each signalling pathway. For example, LPS and TNFα phosphorylate IKK via transforming growth factor β-activated kinase 1 (TAK1) and NFκB-inducing kinase (NIK), respectively (C. Wang *et al.*, 2001; Zarnegar *et al.*, 2008). These different kinases may have different affinities for IKK, leading to different levels of activation of NFκB. It should be stressed however that the signalling pathways of the various ligands used are complex and can interact with various proteins within the cell. These cannot only affect the activation of various transcription factors, but also, for example, the stability of the mRNA expressed (Bellofatto *et al.*, 2011).

### 4.1.3 Co-stimulation with GCs and pro-inflammatory stimuli can enhance CCL20 expression

Having established that dex could induce CCL20 mRNA expression in the HeLa cell lines, it was determined what effects co-stimulation with TNFα would have. As seen in Figure 3.1A, dex and TNFα produced over a 4000-fold increase in CCL20 mRNA expression relative to basal in the HeLa cell line. This is much greater than the combination of the individual responses, showing apparent synergism on CCL20 expression. To confirm whether this is true synergy, a Chou-Talalay method can be used (Chou, 2010).

This apparent synergism, or modulation of the TNFα response of CCL20 expression, however, did not occur in the A549 cell line. Interesting to note is Lannan *et al.* (2012) showed co-stimulation of A549 cells with dex and TNFα caused a strong downregulation of CCL20. In the study of Lannan *et al*. (2012), however, study however used 1/10$^{th}$ the amount of dex (10 nM), 500X more TNFα (10 μg/mL) and 1/4 the stimulation time (6 h) than used in this study, and this may underlie the differences seen on CCL20 expression.
The apparent synergism or potentiation was not seen in the End1, COS1 and U2OS cell lines. This shows a cell-specific effect of modulation between dex and TNFα of CCL20 expression. These cell-specific differences may be due to different levels of receptors, members of the signalling pathway, or transcription factors (Gutierrez-Arcelus *et al.*, 2015).

Having demonstrated the cell-specific synergistic upregulation of CCL20 mRNA between TNFα and dex under these conditions, the effects of co-stimulation with dex and other inflammatory ligands were investigated in the HeLa cell line. When HeLa cells were co-stimulated with dex and PMA, the level of CCL20 expression was significantly higher than when stimulated with either ligand alone (Fig 3.3A). The potentiation was also seen in the co-stimulation with LPS and IFNγ in the presence of dex, although, because the expression caused by the inflammatory inducers alone was relatively low, it may be more accurate to say they are able to prime, or

sensitize, the dex response (Fig 3.3C and B). These results show clear crosstalk between the dex and the pro-inflammatory ligands TNFα, PMA, IFNγ and LPS.

A considerable amount previous research has focused on GC and inflammatory responses in the lungs, specifically looking at drug resistance (Giembycz *et al.*, 2015; R. Newton *et al.*, 2010). To examine whether other pro-inflammatory ligands could cause a synergistic upregulation of CCL20 in A549 cells when co-stimulated with dex, cells were stimulated with PMA, IFNγ and LPS as they were in the HeLa cell lines. No synergy was statistically seen with co-stimulation, although there did seem to be an apparent enhancement of the CCL20 induction with co-stimulation compared to each ligand on its own (Supp Fig 2). The differences in the responses to the pro-inflammatory ligands in the HeLa cells compared to the A549 cells, with the latter always having higher responses (compare Fig 3.1 with Supp Fig 2), highlights the cell- and ligand-specific effects on CCL20 induction.

The differences in the expression of CCL20 by the different inflammatory inducers may be due to the effects they have on the levels of certain transcription factors involved in their signalling pathways. The various inflammatory inducers did not cause any changes in GR levels in the HeLa cell line when compared to the vehicle, or each other (Fig 3.7A and B). There appeared to be a trend with dex reducing the GR levels in the presence or absence of inflammatory inducers, with only the TNFα condition with and without dex showing a significant drop in GR protein levels. This is an expected result as GC stimulation causes GR turnover (Chapter 1.2.2.3). There were no changes in the p65 levels under any of the conditions, but as explained in Chapter 4.1.1, this does not necessarily exclude the importance of p65 and NFκB in explaining the observed differences (Fig 3.7A and C).

The upregulation of pro-inflammatory genes by dex in the absence or presence of pro-inflammatory ligands suggests that GCs prepare the immune system for a quick and efficient response to acute exposure to pathogens (Cruz-Topete & Cidlowski, 2015). Previous studies have shown that treating HeLa and A549 cells with dex induces the expression of TLR2 thereby priming the cells towards TLR2-specific immune responses (Sakai *et al*., 2004; Imasato *et al*., 2004). Compared to controls, cells primed with dex have been shown to express more pro-inflammatory immune mediators in response to TLR2 ligands (Homma *et al*., 2004; Busillo *et*

*al*., 2011). Therefore, the pro-inflammatory effects of GCs may exacerbate immune responses or may prime and prepare the immune system respond promptly and clear invading pathogens through the expression of chemokines such as CCL20.

### 4.1.4 The GR is required for GC mediated induction of CCL20 and crosstalk with pro-inflammatory stimuli

Having established that dex can induce CCL20 mRNA expression, and that there is crosstalk between dex and pro-inflammatory inducers, it was next determined whether this occurred in a GR-dependent manner by using the GR inhibitor RU486. Although RU486 is also a potent antagonist of the progesterone receptor (PR), HeLa cells do not express endogenous PR. Therefore, it can be assumed any inhibitory effects of RU486 are via the GR (Giangrande *et al.*, 1997). Stimulation of cells with RU486 completely abolished the dex induced expression of CCL20, while having no effect on the expression due to the inflammatory inducers TNFα, PMA, IFNγ and LPS (Fig 3.5A-D and Supp Fig 3A-D). This would be expected as RU486 is specific for only the GR in these cells. This shows that the GR is required for the dex mediated induction of CCL20 mRNA, and dex does not induce CCL20 via other methods. Furthermore, without the ability to activate the GR, dex was unable to potentiate or crosstalk with the inflammatory inducers (Fig 3.5 and Supp Fig 3). This furthermore shows that the mechanism for dex to crosstalk with inflammatory inducers is via the GR. The loss of statistical significance in CCL20 expression, relative to vehicle, in TNFα, PMA, IFNγ and LPS stimulated cells, in the presence of RU486, may due to error introduced into the experiments (Supp Fig 3).

Interesting to note is that stimulation with RU486 caused an increase in basal/vehicle expression of CCL20, although this difference was not statistically significant. This may indicate that the unliganded, inactivated GR may a have slight repressive effect on basal CCL20 expression. These results indicate that the active GR is not only required for the dex response, but also for the crosstalk between dex and the inducers of inflammation. The GR is therefore directly involved in the crosstalk observed.

### 4.1.5 The putative CCL20 promoter is activated by GCs and some pro-inflammatory ligands on a reporter construct

Using a CCL20 promoter-reporter construct can reveal which regions on the promoter of CCL20 are critical for its induction, eliminate any effects of chromatin remodelling necessary for endogenous CCL20 induction, and remove any crosstalk between GCs and the mediators of inflammation that occur post-transcriptionally. The pGL2-MIP-3α construct used has the promoter region, from -871 to +58 base pairs upstream of the CCL20 promoter, attached to the luciferase gene as the reporter (Imaizumi *et al.*, 2002). Because the reporter construct only contains a section of the putative CCL20 promoter, regulatory insights can be determined from the identification of transcription factor binding sites. Fig 4.2 shows putative transcription factor binding sites determined *in silico,* showing the differences between the endogenous promoter from -1381 base pairs, and the pGL2-MIP-3α construct. Overall, the fold induction compared to vehicle in all stimulated conditions was lower than that observed on the endogenous gene (Fig 3.2 compared to Fig 3.4). This suggest that other cis elements not included in the reporter plasmid construct may be involved in CCL20 mRNA expression in the presence of the ligands. In addition, the cells may be limiting the synthesis of luciferase protein to prevent cell toxicity, which may restrict the ability of the luciferase assay to measure the activity of the promoter in the presence of the ligands. The differences in the response elicited by dex in the absence or presence of pro-inflammatory ligands on the endogenous gene and reporter plasmid may also be due to that some transcription factors play a role in recruiting other transcription factors to the promoters of target genes. For example, C/EBP and AP-1 have been shown to make the chromatin more accessibility to the GR, even where there are no GREs (Grøntved *et al.*, 2013; Biddie *et al.*, 2011). As shown in Fig 4.2, the endogenous CCL20 promoter has an extra C/EBP site compared to the reporter plasmid construct. This suggests that the GR, activated by dex, might interact with this and other such sites which are lacking in the reporter construct, to transactivate the endogenous CCL20 promoter in the absence or presence of pro-inflammatory ligands.

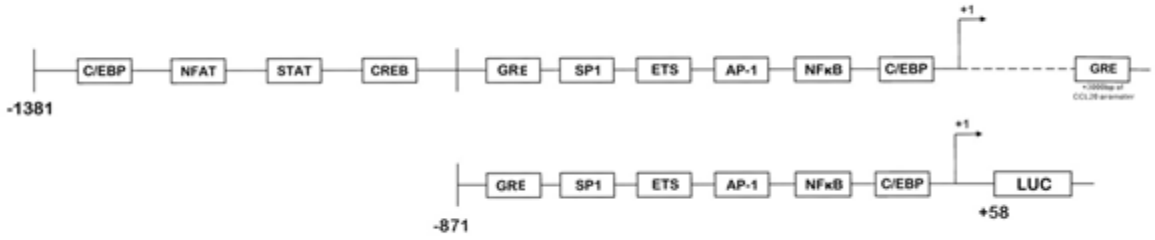



**Figure 4.1. Putative regulatory transcription factor binding sites for the pGL2-MIP-3α construct and the endogenous CCL20 promoter region.**
The putative regulatory transcription factor binding sites for the pGL2-MIP-3α construct are compared to the endogenous CCL20 promoter from -1381 base pairs upstream to the start of transcription site. The binding sites were determined in silico (Heinemeyer *et al.*, 1998).

Dex was shown to significantly induce the putative promoter of the pGL2-MIP-3α luciferase vector (Fig 3.4A-D). This therefore supports the possibility of a GRE within the putative CCL20 promoter. It is also possible for the GR to tether onto other transcription factors, such C/EBP to induce the promoter (Louw-du Toit *et al.*, 2014; Sai *et al.*, 2008). The pGL2-MIP-3α luciferase vector with the putative promoter was significantly induced in the HeLa cell line by dex and TNFα. Co-stimulation with dex and TNFα further increased the induction of the promoter, although this increase was only statistically significant compared to dex alone and not TNFα (Fig 3.4A). However, the result does closely mirror the result seen in on endogenous CCL20, and lack of significance may be due to experimental error. PMA, like TNFα, was shown to induce the reporter construct (Fig 3.4B). LPS, however, was not able to statistically significantly induce the construct, with a 1.63-fold increase (Fig 3.4D). This, however, was approaching significance with a p value of 0.0674. This suggests that the transcription factor binding sites necessary for the TNFα, PMA and LPS mediated activation of CCL20, and its crosstalk with the GR are present within the pGL2-MIP-3α promoter region, and are most likely NFκB, C/EBP and AP-1, and not CREB, STAT.

Surprisingly, IFNγ appeared to repress the activity of the pGL2-MIP-3α promoter, statistically significantly reducing the basal induction to 0.633-fold relative to vehicle (Fig 3.4C). The STAT binding site is not present in the pGL2-MIP-3α construct, while it is predicted in the endogenous putative promoter (Fig 4.2). The STAT binding site, therefore, may be required for IFNγ mediated induction, and potentiation of the dex response on the endogenous promoter, as the response is lost in the construct. Furthermore, the repressive effects of IFNγ on CCL20 are most likely not via the STAT binding site and may occur via another mechanism. Some studies have shown that both STAT and NFκB are involved in the IFNγ signalling pathway, with an NFκB loop positioned downstream of STAT (Ramana *et al*., 2002; van Boxel-Dezaire *et al*., 2007). Thus, it is possible that STAT is needed to induce the synthesis of a protein that activates NFκB or alternatively STAT and NFκB on the CCL20 promoter. Because STAT is absent from the pGL2-MIP-3α construct, such interactions between STAT and NFκB would no longer be possible and hence the activity of the pGL2-MIP-3α construct may then be repressed in the presence of IFNγ.

### 4.1.6 The NFκB binding site on the CCL20 promoter is important for CCL20 induction

NFκB plays a key role in the regulation of the immune response, and transcriptional activation of pro-inflammatory genes (Gilmore, 2006). The NFκB site has been shown to be necessary for the induction of pro-inflammatory genes when induced by signals of inflammation (Xie *et al.*, 1994). To further investigate the mechanisms of CCL20 induction by the GR and pro-inflammatory signalling pathways, the CCL20 reporter construct pGL2-MIP-3α/mκB was used. This construct is the same as the pGL2-MIP-3α construct, but with the NF-κB-binding site (–92 to –82) mutated through site-directed mutagenesis. NFκB would, therefore, be unable to bind and be involved in the activation of the CCL20 promoter in this plasmid. Compared to the relative light units measured in the basal level of expression in the presence of vehicle, the mutated NFκB binding site construct (pGL2-MIP-3α/mκB reporter construct) has a reduced activity compared to the wild type promoter (pGL2-MIP-3α reporter construct) (Fig 3.5A-D). This suggests that the NFκB binding site is important for the overall induction of CCL20.

Although mutation of the NFκB site causes a drop in the overall induction compared to wild type promoter, the promoter is still inducible, however, only weakly. When the graphs are re-plotted with both the vehicles of the wild type and NFκB mutated promoter set to 1, the dex-induced activation increases from 2-fold to 5-fold (Supp Fig 4). This increase in the dex response relative to their respective vehicles suggests that while dex can increase CCL20 induction via binding to sites such as the GRE and potentially C/EBP or AP-1, the dex response is repressed via NFκB. The repressive action of the GR on NFκB also occurs under basal conditions as, seen with an increase in the induction by the inflammatory ligands on endogenous promoter when cells were stimulated with the GR inhibitor RU486 (Fig 3.5B). Therefore, the dex response on CCL20 induction is most likely due to the net effect of;

i) activated GR binding directly to the GRE on the CCL20 putative promoter, and/or the positive interactions between the GR and other transcription factors such as C/EBPβ or AP-1, thereby increasing CCL20,
ii) the classical mechanisms of NFκB/GR mechanisms of co-repression and,
iii) NFκB activating the CCL20 promoter, allowing for relatively higher levels of induction.

Therefore, while the NFκB binding site is important for the overall magnitude of the reporter construct induction; it is not necessary for the dex mediated induction. Furthermore, GR does not interfere with the DNA binding properties of NFκB (Han *et al.*, 2001).
TNFα and PMA are apparently unable to induce the mutated reporter, therefore, it cannot be ascertained whether they can induce the mutated reporter, especially in the case of PMA (Supp. Fig 4B). It thus appears that TNFα and PMA require the NFκB binding site on the reporter construct for CCL20 activation. IFNγ still repressed the basal/vehicle induction of the CCL20 on the mutated promoter, it would appear that the repressive effects are, therefore, not via the NFκB binding site (Fig 3.5C and Supp).

In summary (Fig 4.2), dex, via the GR, can induce both the endogenous, pGL2-MIP-3α and pGL2-MIP-3α/mκB CCL20 promoter. Kwon *et al*. (2003) determined *in silico* that the CCL20 promoter contained a GRE, the DNA target sequence for GR binding. It is therefore likely that the GR binds to this GRE, to elicit a response on CCL20. The presence of a potential GRE on a

pro-inflammatory cytokine, although counter-intuitive, can be supported by the pro-inflammatory effects of GC on the innate immune system, and especially within epithelial cells (Busillo *et al.*, 2013; Cruz-Topete *et al.*, 2015). Furthermore, So *et al*. (2007) described a GRE binding site within an intron 4409 base pairs downstream of the transcription start site via a ChIP assay, but the regulatory properties of this GRE have not been extensively studied. Other mechanism for the GR to induce CCL20 expression is via interaction with the C/EBP transcription factors, or via interaction with AP-1 proteins in certain circumstances (Chapter 4.1.5). Furthermore, the NFκB binding site is not necessary for the dex-induced activation of the CCL20 promoter, but rather confers a repressive effect. TNFα and PMA are able to induce both the endogenous and pGL2-MIP-3α CCL20 promoter, with LPS approaching statistical significance (Fig 3.7). TNFα, PMA and LPS cannot induce the NFκB mutated promoter (Fig 3.8). Therefore, the transcription factors used to induce the endogenous and reporter promoter are most likely the same; via NFκB. The mechanism for potentiating the dex response is also most likely similar; both the GR and NFκB/AP-1 are bound to the CCL20 promoter and produce an enhanced effect on CCL20 induction. IFNγ can induce expression and potentiate the dex response on the endogenous reporter, most likely via the STAT binding site, and potentiate the dex response when the GR is also bound to the promoter. On the pGL2-MIP-3α promoter, however, where the STAT binding site is not present, IFNγ represses the basal level of expression and dex activation of the promoter. This repression function of the CCL20 promoter does not require the STAT, or NFκB binding site as seen on the mutated pGL2-MIP-3α/mκB reporter construct.

Data in this thesis has shown that dex is able to activate the CCL20 via the GR, but not necessarily via the GR binding to a GRE on the putative promoter. While the NFκB binding site is important for the induction by pro-inflammatory ligands, dex is still able to induce the promoter. Therefore, targeting the dex mediated activation of CCL20 should not necessarily be done by modulating the GR/ NFκB interaction, but rather by GR interacting with other transcription factors, such as AP-1 and C/EBP. It has also been shown the CCL20 promoter is most likely induced, and suppressed by some ligands at the same time, with the overall resulting induction a combinatorial effect. This is seen with IFNγ repressing the induction when the STAT binding site is not present in the reporter plasmid (Fig 3.7C).

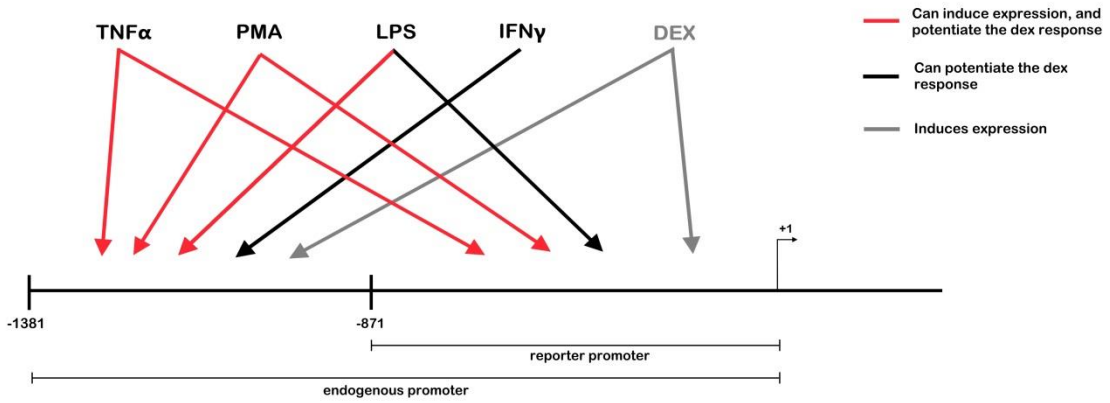



**Figure 4.2. Observed effects of endogenous CCL20 mRNA and pGL2-MIP-3α promoter activity.**
The effect of different ligands on the expression and activation of the putative endogenous CCL20 promoter and the pGL2-MIP-3α construct.

### 4.1.7 Physiological relevance of CCL20 regulation

CCL20 is implicated in the progression of many diseases, such as rheumatoid arthritis and inflammatory bowel disease, and also in GC insensitivity in asthma patients (J. Kwon *et al.*, 2002; A. Lee *et al.*, 2014; Zijlstra *et al.*, 2014). Understanding the mechanisms and signalling pathways behind the regulation of CCL20 by inflammatory inducers can provide better insights on how to prevent such pathological effects of CCL20. Furthermore, the fact the GCs, which are classically used to treat inflammation and cytokine production, enhance the expression of CCL20 and potentiate the effects of inflammatory inducers in a variety of cell types, warrants further investigation into the regulation of CCL20 expression.

An undesirable side-effect of GC use is GC insensitivity, which is thought to be due to an increase in $T_h17$ cells in the lungs of asthma patients. Inflammation mediated by $T_h17$ cells has been implicated in GC insensitivity in asthma patients (Chapter 1.3.3).

Since CCL20 is a chemoattractant for $T_h17$ cells, and GCs can regulate and potentiate the TNFα and LPS responses on CCL20 expression, an understanding of the mechanisms of GC regulation of CCL20 is important. The designing of GCs which have little or no activity on CCL20, or the use of 'add-on' therapies to potentiate the desired anti-inflammatory effects of GCs such as long-acting β2 adrenoreceptor agonists (LABAs) and phosphodiesterase 4 inhibitors can be more effective with the knowledge of the underlying mechanisms.

CCL20 may be implicated in an increase of HIV-1 acquisition at mucosal epithelial barriers such as the cervix, or oral epithelial cells (Chapter 1.3.3). This is further exacerbated at inflamed epithelial cells, such as during menstruation (Dieu-Nosjean *et al.*, 2000; Evans *et al.*, 2012). CCL20 has been shown to be a possible anti-HIV-1 molecule, via direct interaction with HIV-1 (M. Ghosh *et al.*, 2009). CCL20, however, still causes an increase in $T_h17$ cells and may therefore still increase the rate of HIV-1 infection, especially if the HIV-1 titre is high. There is a potential for contraceptives such as medroxyprogesterone acetate (MPA), which has been reported to have GC-like properties, to increase CCL20 expression. MPA can therefore potentiate the CCL20 response due to inflammatory inducers such TNFα, which expressed during menstruation (Govender *et al.*, 2014). Therefore, a possible mechanism for MPA or GCs to increase HIV-1 acquisition in the female genital tract is via an increase CCL20 production.
The female genital tract hosts a microbial flora and, even if a female is not menstruating, the presence of LPS may potentiate GC's response on CCL20 induction. Infection with pathological microbes has been shown to increase HIV-1 susceptibility (Y. Ogawa *et al.*, 2009). This can be exacerbated by the presence of GCs as, via CCL20, these may cause the migration of HIV-1 target cells to the female genital tract.

Greater insight into the mechanisms of CCL20 regulation may lead to the development of more effective treatment strategies for diseases and production of drugs lacking undesirable side effects. The observation that different signalling pathways can produce different effects, such as IFNγ compared to the other inflammatory inducers on the pGL2-MIP3α-LUC promoter (Fig 3.7C), highlights that an understanding of mechanisms in CCL20 promoter regulation can provide insights in designing more effective treatments.

## 4.2 Conclusions

GCs, acting via the GR, are involved in a multitude of cellular functions and responses, such as metabolism, cardiovascular function, homeostasis and immune function. Tightly controlled cell signalling crosstalk between multiple signals is required for appropriate cellular responses to maintain the health of the system. Although the GR is ubiquitously expressed in cells throughout the body, much regarding the receptor is still not understood due to the complexity of genetic regulation, and the fact that it can crosstalk and modulate the function of other signalling pathways. An example of this is the GR's role in the inflammatory response. GC's have classically been established as a mediator of anti-inflammatory cellular responses; however, recent research has highlighted many pro-inflammatory GC effects (Cruz-Topete *et al.*, 2015). The pro-inflammatory chemokine CCL20 has been demonstrated to be induced by GC's, and potentiates the response due to inflammatory inducers, such as TNFα, in the HeLa cell line. Furthermore, it was established that co-stimulation with GCs and a variety of pro-inflammatory inducers can cause a synergistic or at least enhanced expression of CCL20. This potentiated response resulted in a greater expression of CCL20 compared to either GCs, or pro-inflammatory inducer alone. It was established that the GR is required for this induction, as inhibition abolished this effect. To further examine the mechanisms behind this crosstalk between GCs and pro-inflammatory ligands, a reporter construct with the putative CCL20 promoter was expressed in the HeLa cell line.

The most significant findings in this study is that only certain binding elements and interactions between these elements were required were required for the crosstalk between dex and pro-inflammatory ligands to upregulate CCL20 expression. This was determined from the CCL20 promoter-reporter construct. The results shown herein demonstrate that TNFα, PMA and LPS require the NFκB binding site on the CCL20 promoter to induce its expression, while IFNγ requires the STAT binding site. The lack of a STAT binding site in the CCL20 minimal promoter may have resulted in IFNγ repressing the activity of the CCL20 reporter-promoter. This present study demonstrates that the NFκB binding region is important for the activation of the CCL20 promoter by some pro-inflammatory ligands, both in the presence or absence of dex. This

study also shows that the GR activates CCL20 expression, most likely via a binding to a GRE or via interacting with the AP-1 or C/EBP sites on the promoter.

GCs most likely induce CCL20 expression to maintain a protective state, especially in immunocompromised areas, where pro-inflammatory inducers will also be present. Acute exposure to GC has been shown to be involved in an enhancement of the immune response, especially in peripheral regions (Dhabhar, 2002). Therefore, knowledge of GC mediated and potentiation with inflammatory inducers can lead to the development of more effective treatment strategies which do not have the negative, pathological effects of increased CCL20 expression. Due to CCL20 being implicated in various diseases such as GC insensitivity in asthma, rheumatoid arthritis and inflammatory bowel disease, understanding the mechanisms of CCL20 regulation may provide practical medical insights for a variety of diseases. This is important especially for chronic diseases such as AIDS, which increased levels of CCL20 expression due to GCs and pro-inflammatory inducers, may increase acquisition of HIV-1, due to the migration of leukocytes to the epithelial barriers.

CCL20 is a chemoattract and its sole cognate receptor CCR6 is expressed predominantly on Th17 cells. There is evidence suggesting strong correlation between Th17 cells and cancer patient survival (Punt *et al*., 2015). That is, high numbers of Th17 cells in squamous cervical cancer improves patient survival, whereas similar numbers of Th17 in other cancers may result in poor outcome (Punt *et al*., 2015). In patients with cancer, synthetic GCs can be a part of the cancer treatment because of their pro-apoptotic and anti-inflammatory action (Cook *et al*., 2016). The results shown in this study suggest GCs can crosstalk with pro-inflammatory immune activators in the tumour microenvironment to upregulate CCL20 expression. The result shown herein suggest this may be specific for some and not all cancer cells. However, it remains unclear what are the direct effects of elevated CCL20 levels on tumour cells.

The results show that the GR and NFκB are required in the co-regulation of CCL20 expression by dex and select pro-inflammatory ligands. This suggests inhibiting the GR or NFκB may mitigate the effects on disease outcome mediated by high levels of CCL20. In addition, when designing drugs to prevent GC insensitivity on asthma and its effects in induce CCL20 implicated in the progression of rheumatoid arthritis, inflammatory bowel disease and an increase in HIV-1 acquisition, one should not necessarily target the NFκB pathway. Rather the

drugs should either potentiate the anti-inflammatory effects of GCs, such as by including LABAs, or reduce the pro-inflammatory effects of GCs. The latter could be achieved by preventing the GR from interacting with transcription factors such as AP-1 or C/EBPβ. However, if the overall goal is to supress the expression of CCL20 regardless if cells are stimulated with dex or pro-inflammatory ligands, the targeting of NFκB would result in a large decrease in the overall induction of CCL20 as seen in the mutated reporter result. Due to the NFκB pathway being involved in a variety of cellular functions, the molecules which target this pathway must be specific for the intended use with few side effects. A potential source for the discovery of molecules that target NFκB can be from pathogenic viruses and bacteria which have been shown to produce a large variety of molecules that target NFκB (Rahman *et al*., 2011).

## 4.3 Future Perspectives

An important question for elucidating the mechanism of GR regulation of CCL20, is finding which trascription factors are present on the promoter during its induction. A ChIP assay, probing for specific proteins such as the GR, NFκB, C/EBPβ, STAT1 and other co-factors involved in their binding, would provide mechanistic insights into CCL20 induction by GCs and pro-inflammatory inducers. ChIP assays were attempted but they proved unsuccessful, most likely due to issues with the reagents in the assay used. Therefore, it could not be determined which proteins were bound to the CCL20 promoter under specific conditions. Due to the possibility of the GR binding on other transcription factors to possibly cause the potentiated response seen, a ChIP done, first targeting the GR, then other transcription factors such as NFκB, AP-1 C/EBPβ and STAT1 can be performed for the CCL20 promoter.

Changes in the protein levels of transcription factors involved should also be examined. Although there were no significant changes in GR or p65 due to stimulation of cells with the different ligands, there may be changes in other transcription factors such as AP-1 or MAPKs involved in the respective signalling pathways. Post-transcriptional changes such as phosphorylation of the GR, or IKK should be investigated, as these affect the activity of these proteins, and, therefore, the expression of genes in their signalling pathways. GR post-

translational modifications have been shown to be biomarkers for GR activation (Chapter 1.1.2.3). Therefore, Western blot analysis of GR phosphorylation, specifically at S211 and S226 under different conditions would provide insight as to changes in GR conformation, and activity.

The involvement of members of various signalling pathways could also be investigated such as via the use of inhibitors to kinases. Activated, phospho-p65 levels should also be examined, and the activation of IKK and other kinases within the signalling pathways.

The present study showed apparent synergism in CCL20 induction between dex and the pro-inflammatory inducers, TNFα, LPS and PMA. This was not shown to be true synergism as a Chou-Talalay analysis was not performed (Chou, 2010). Therefore, to show this is indeed true synergism, the experimental set-up could be structured to statistically and quantifiably show synergism between these factors. An understanding of which treatments result in true synergy may give insight into which pathways and mechanisms are involved in causing an increase in CCL20 expression, which could possibly be involved in disease progression. These pathways could then be drug targets to reduce the level of CCL20 induction, and the possible adverse side-effects associated with it.

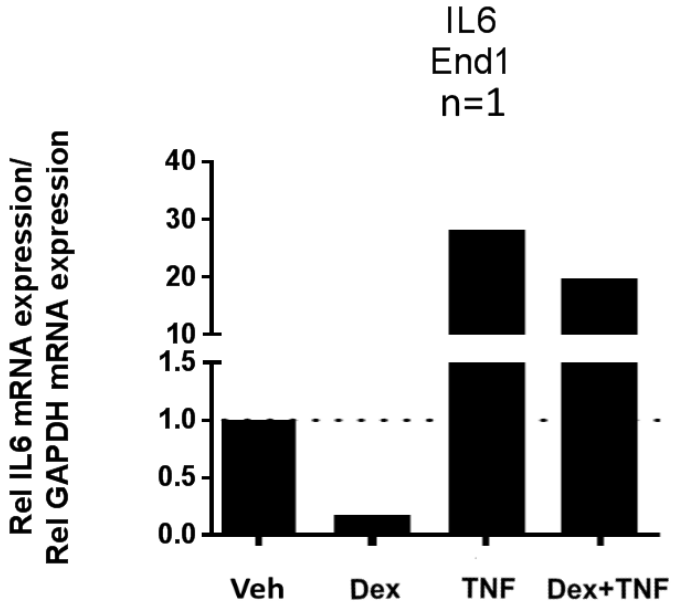
Once it has been determined which transcription factors are directly involved in CCL20 induction by the GR, other pro-inflammatory genes that the GR induces can be examined for similarities or differences in transcription factor recruitment. Furthermore, any differences or similarities in co-factor recruitment on the promoter of pro-inflammatory genes on with which synergy occurs between GCs and pro-inflammatory inducers should be examined.

The differences in cell-specific responses should also be compared. The experiments previously mentioned could, therefore, be explored in the different cell lines.

It would also be important to determine whether the changes in CCL20 mRNA expression result in changes in CCL20 protein, and whether this protein is indeed released by the cells, for it is still possible to have post translational CCL20 regulation. This could be done in tissue culture via enzyme-linked immunosorbent assays (ELISAs), to determine whether the induced mRNA is expressed into protein. To further examine whether these effects occur in the body, more

physiological models could be used, such as the use of explant tissue. Furthermore, samples from blood or biopsies could be tested for CCL20 protein after exposure to GCs and/or pro-inflammatory ligands. A migration assay could also be performed to examine whether the CCL20 produced by the cells could cause the migration of leukocytes.

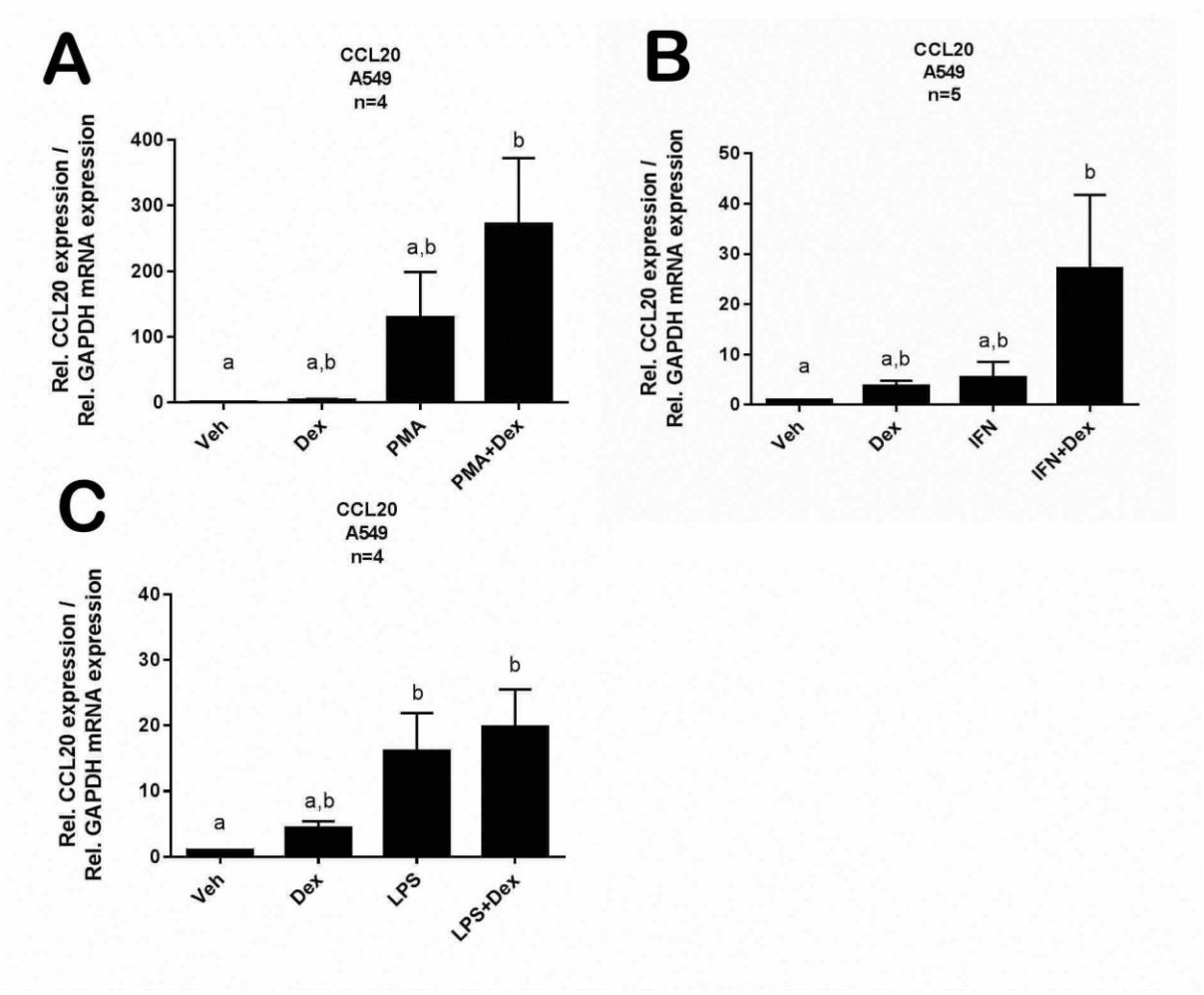
## Supplementary Figures




**Supplementary Figure 1. Changes in expression of IL6 when stimulated with dex, TNFα, or a combination of both.**

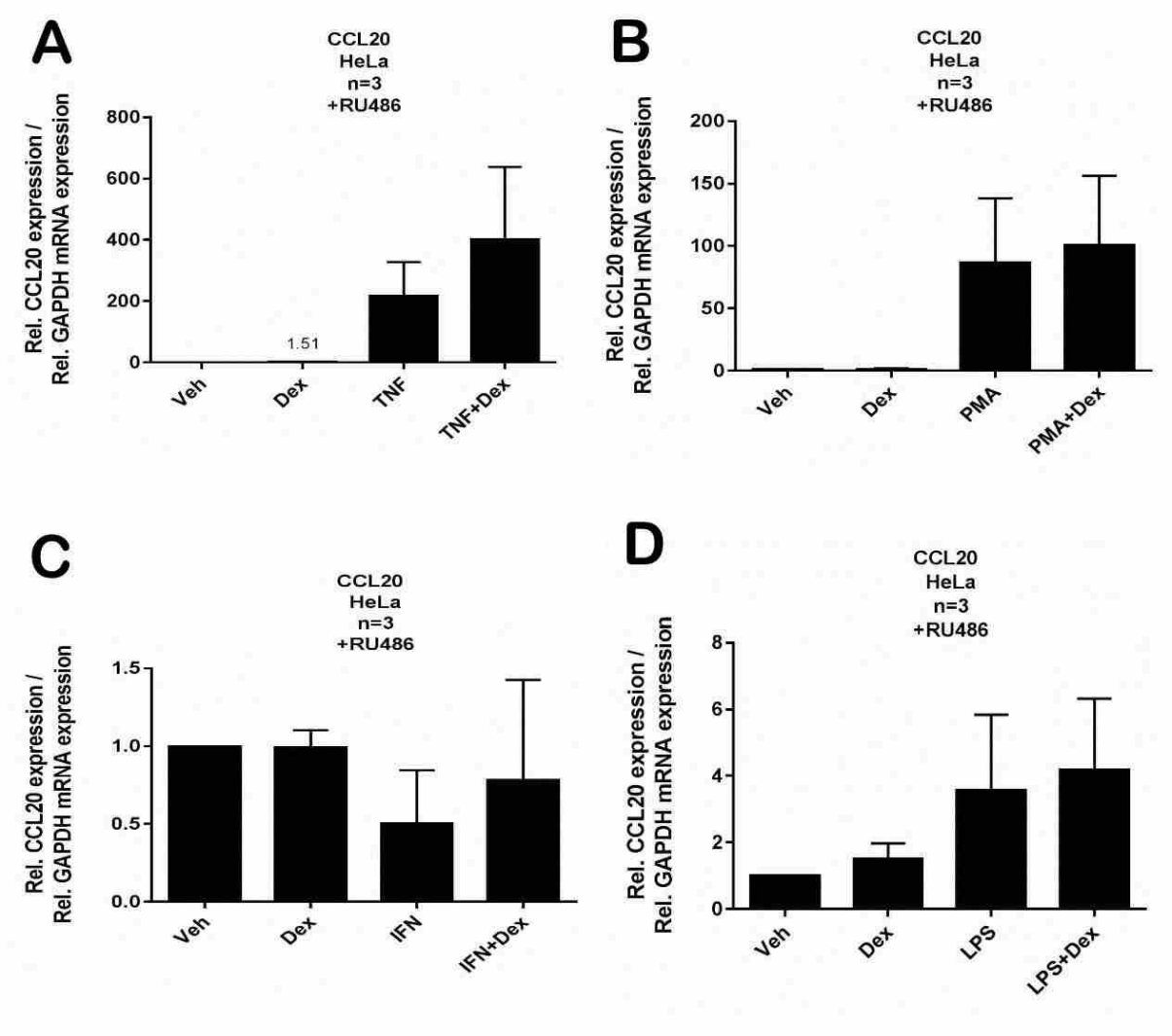
End1 cells were seeded into 12-well plates at a density of 5 x $10^6$ cells/well and incubated for 48 hours. Cells were treated with 100 nM dex, 20 ng/mL TNFα, or a combination of both, as stated, in serum-free DMEM. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. CCL20 mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Relative gene expression was normalized to basal activity

(Vehicle) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean +/- SEM. Statistical analysis was carried out using GraphPad Prism™ software, with different letters representing statistical significance by one-way ANOVA, with Tukey's Multiple Comparison Test. Statistical significance by a t-test is denoted by *, **, *** or **** to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively.



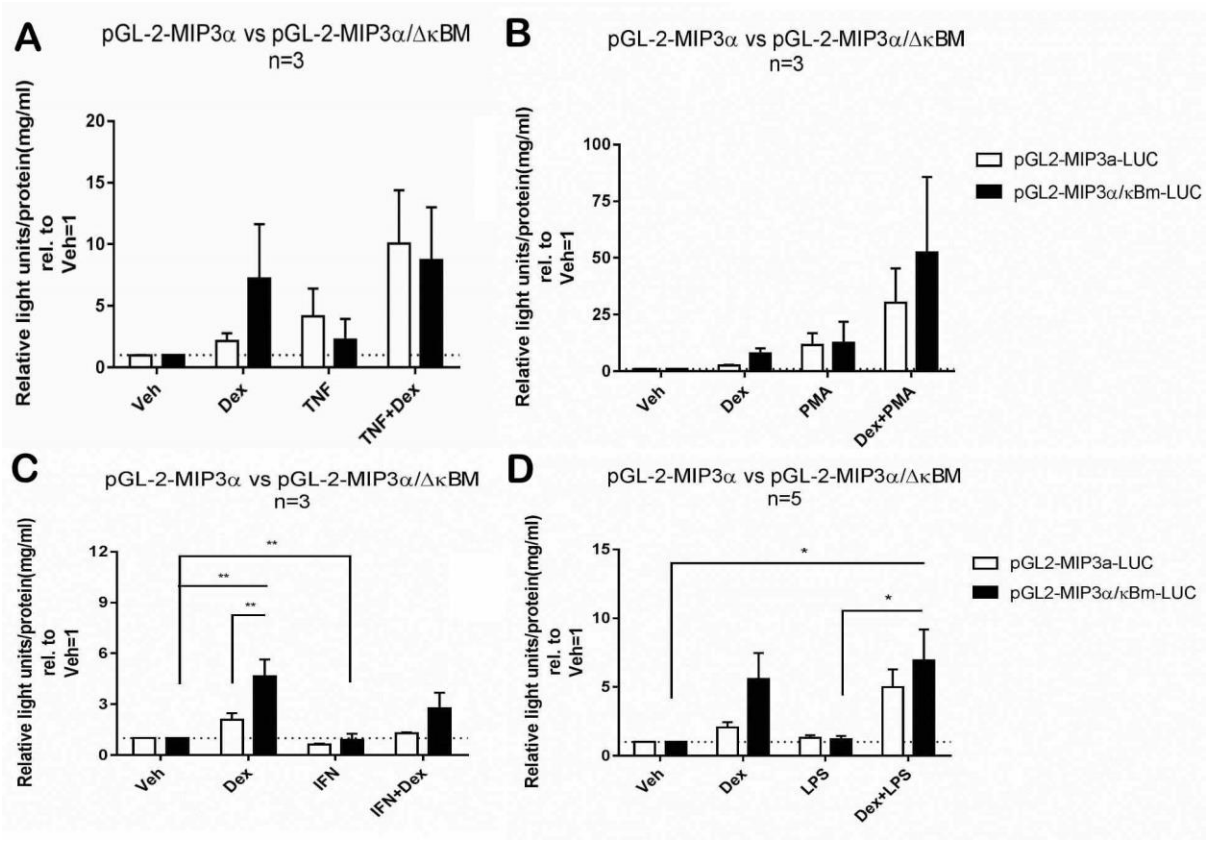

**Supplementary Figure 2. The pro-inflammatory ligands and dex can induce the expression of CCL20 mRNA expression and can modulate the dex response in A549 cells.**

A549 cells were seeded into 12-well plates at a density of 5 x $10^5$ cells/well and incubated for 48 hours. Cells were treated with 100 nM dex, and/or 5 ng/mL PMA (A), 20 ng/mL IFNγ (B), 5 ng/mL LPS (C), or a combination of both, as stated, in serum-free DMEM. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. CCL20 mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Relative gene expression was normalized to basal activity (Vehicle) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean +/- SEM. Statistical analysis was carried out using GraphPad Prism™ software, with different letters representing statistical significance by one-way ANOVA, with Tukey's Multiple Comparison Test. Statistical significance by a t-test is denoted by *, **, *** or **** to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively.




**Supplementary Figure 3. The GR is required for the dex response on CCL20 mRNA, as the GR inhibitor RU486 abolishes the response and the dex modulation of TNFα, PMA, IFNγ and LPS.**

HeLa cells were seeded into 12-well plates at a density of 5 x 105 cells/well and incubated for 48 hours. Cells were treated HeLa cells were seeded into 12-well plates at a density of 5 x 105 cells/well and incubated for 48 hours. Cells were treated with 1 nM RU486, 100 nM dex 20 ng/mL and/or TNFα (A), and/or 5 ng/mL PMA (B), 20 ng/mL IFNγ (C), 5 ng/mL LPS (D), as stated in the presence or absence of 100 nM dex, or 100nM dex alone as stated, in serum-free DMEM for 24 hours. Thereafter, the cells were washed twice with PBS and harvested for total RNA with TRIzol® and 500 ng RNA was reverse-transcribed. CCL20 mRNA expression was measured by qRT-PCR and normalised to GAPDH mRNA expression. Relative gene expression was normalized to basal activity (Vehicle) in order to obtain relative fold expression. Graphs represent pooled results of at least three independent experiments and are plotted as mean +/- SEM. Statistical analysis was carried out using GraphPad Prism™ software, with Statistical significance by one-way ANOVA, with Tukey's Multiple Comparison Test denoted by #, ##, ###, or #### to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively. Statistical significance by a t-test is denoted by *, **, *** or **** to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively.

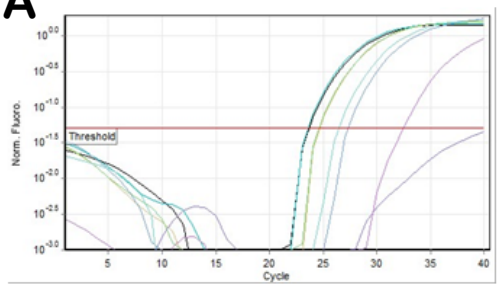



Supplementary Figure 4. Mutation of the NFκB binding site on the CCL20 promoter-reporter construct causes a reduction of activation by the pro-inflammatory ligands and dex compared to the wild-type promoter.

HeLa cells were seeded into 48-well plates at a density of 5 x $10^5$ cells/well. The following day, the cells were transiently transfected with either 250 ng pGL2-MIP3α-LUC or pGL2-MIP3α/κBm-LUC DNA. Cells were treated with either 20 ng/mL TNFα (A), 20 ng/mL PMA (B) 5 ng/mL LPS (C), or 20 ng/mL IFNγ (D), in the presence or absence of 100 nM dex, as stated, in serum-free DMEM. Total cells were harvested, and RLUs measured via a luminometer and normalised to protein content, determined with a Bradford assay. Graphs represent pooled results of at least three independent experiments and are plotted as mean +/- SEM. Statistical analysis was carried out using GraphPad Prism™ software, with Statistical significance by one-way ANOVA, with Tukey's Multiple Comparison Test denoted by *, **, *** or **** to indicate P< 0.05, P< 0.001, P<0.0005 or P< 0.0001, respectively.

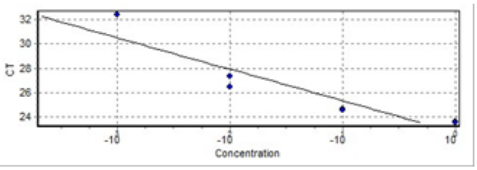
CCL20 (500nM)

A




B

Eff=2.444

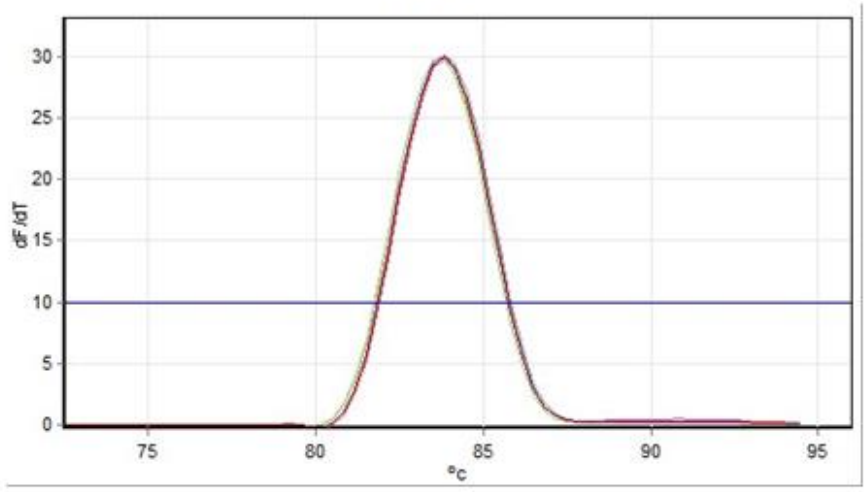


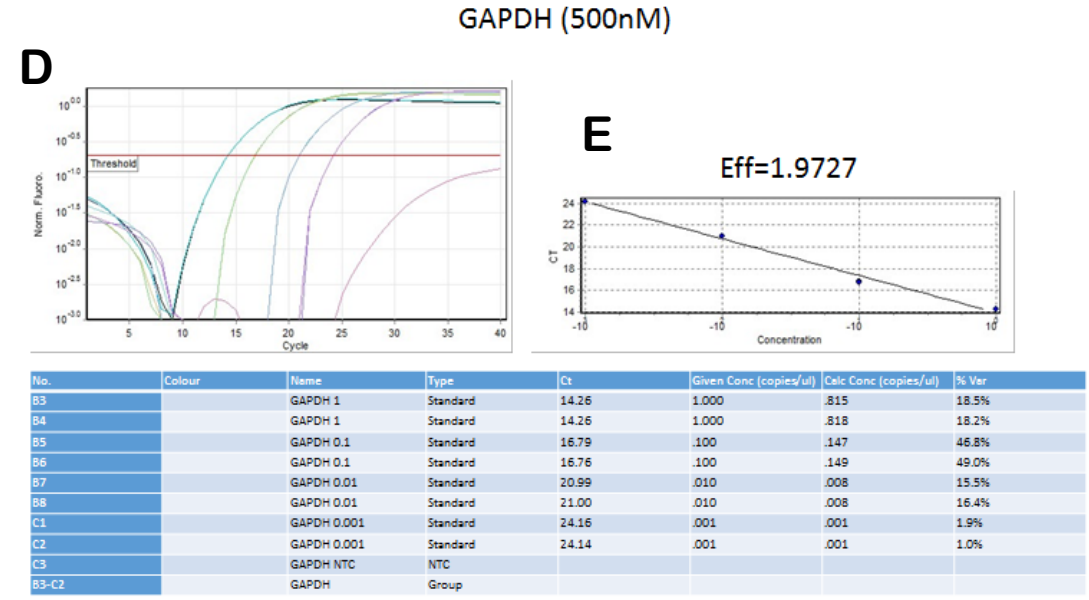

| No. | Colour | Name | Type | Ct | Given Conc (copies/ul) | Calc Conc (copies/ul) | % Var |
|---|---|---|---|---|---|---|---|
| B3 | | CCL20 1 | Standard | 23.61 | 1.000 | .452 | 54.8% |
| B4 | | CCL20 1 | Standard | 23.51 | 1.000 | .494 | 50.6% |
| B5 | | CCL20 0.1 | Standard | 24.61 | .100 | .186 | 86.2% |
| B6 | | CCL20 0.1 | Standard | 24.53 | .100 | .200 | 99.8% |
| B7 | | CCL20 0.01 | Standard | 26.42 | .010 | .037 | 274.9% |
| B8 | | CCL20 0.01 | Standard | 27.30 | .010 | .017 | 73.2% |
| C1 | | CCL20 0.001 | Standard | | .001 | | |
| C2 | | CCL20 0.001 | Standard | 32.42 | .001 | .000 | 81.5% |

Standard Curve

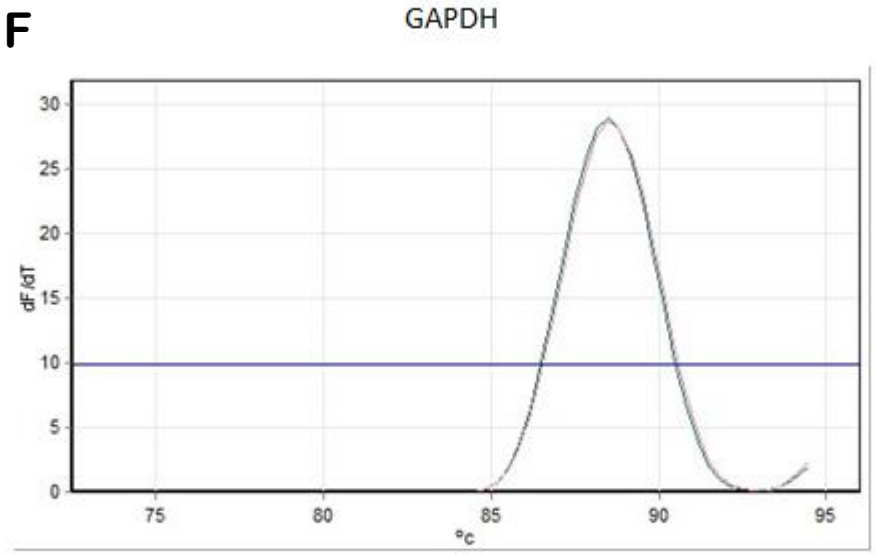
C

CCL20






| No. | Colour | Name | Type | Ct | Given Conc (copies/ul) | Calc Conc (copies/ul) | % Var |
|---|---|---|---|---|---|---|---|
| B3 | | GAPDH 1 | Standard | 14.26 | 1.000 | .815 | 18.5% |
| B4 | | GAPDH 1 | Standard | 14.26 | 1.000 | .818 | 18.2% |
| B5 | | GAPDH 0.1 | Standard | 16.79 | .100 | .147 | 46.8% |
| B6 | | GAPDH 0.1 | Standard | 16.76 | .100 | .149 | 49.0% |
| B7 | | GAPDH 0.01 | Standard | 20.99 | .010 | .008 | 15.5% |
| B8 | | GAPDH 0.01 | Standard | 21.00 | .010 | .008 | 16.4% |
| C1 | | GAPDH 0.001 | Standard | 24.16 | .001 | .001 | 1.9% |
| C2 | | GAPDH 0.001 | Standard | 24.14 | .001 | .001 | 1.0% |
| C3 | | GAPDH NTC | NTC | | | | |
| B3-C2 | | GAPDH | Group | | | | |




**Supplementary Figure 5. Standard curves generated to determine the efficiency of the CCL20 and GAPDH primer sets.**

Primer efficiencies were used to determine relative transcript levels by the method described by Pfaffl et al. 2002. The Ct values were determined using the Rotogene software for CCL20 and GAPDH (A and D) and primer efficiencies calculated for decreasing amounts of primers used (B and E) (Pfaffl *et al.*, 2002).

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