# Mechanisms of glucocorticoid responsiveness in the lung during development and inflammation

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*We shall not cease from exploration*

*And the end of all our exploring*

*Will be to arrive where we started*

*And know the place for the first time.*

**– T.S. Eliot**

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## ABSTRACT

Glucocorticoids (GCs) are vital hormones involved in lung development and the regulation of the inflammatory/immune response. High inter-individual variability in GC responsiveness exists among patients using steroids as treatment for inflammatory diseases. Evidence suggests that vitamin D (VitD), another player in lung development, improves GC function. Even though progress has been made in the study of steroid insensitivity, the molecular mechanisms are not completely elucidated and the effects of limited GC response during lung development have not been explored. The first objective of the present thesis was to study mechanisms of steroid responsiveness in asthma using mouse models of the disease. The Balb/c strain demonstrated a steroid insensitive phenotype associated with increased amounts of active p38 MAPK and subsequent inactivation of the GC receptor (GR) following allergen challenge. Additionally, lymphoblast cell lines derived from asthmatic children were used to study mechanisms for variable GC responsiveness and to explore the modulatory role of VitD on GC function. Poor responsiveness to steroids in asthmatic children was associated with limited GR nuclear bioavailability as a consequence of decreased baseline GR expression and faster hormone-induced downregulation. Suggestive evidence for a beneficial effect of VitD in steroid sensitivity is presented. Finally, steroid responsiveness and VitD modulation of GC function was studied in epithelial cells of the developing lung of normoresponsive and atopic rat models. The developmental airway epithelium of the atopic rat appeared to be more sensitive to steroids, possibly making the lung more susceptible to the deleterious effects of GCs, and VitD attenuated the GC response. Overall this thesis highlights the complexity of steroid function and its regulation by

multiple mechanisms ranging from altered expression, reduced activation, abnormal nuclear translocation and increased homologous downregulation of GR.

## RÉSUMÉ

Les glucocorticoïdes (GC) sont des hormones vitales impliquées dans le développement des poumons et de la régulation de la réponse inflammatoire/immunitaire. Une grande variation interindividuelle de la réactivité des GC existe chez les patients utilisant des stéroïdes comme traitement pour les maladies inflammatoires. Les preuves suggèrent que la vitamine D (VitD), une autre molécule impliquée dans le développement des poumons, améliore la fonction des GC. Même si des progrès ont été réalisés dans l'étude de l'insensibilité aux stéroïdes, les mécanismes moléculaires ne sont pas complètement élucidés et les effets de la réponse compromise aux GC au cours du développement pulmonaire n'ont pas été explorés. Le premier objectif de cette thèse est d'étudier les mécanismes de réactivité des stéroïdes dans l'asthme en utilisant des modèles murins de la maladie. La souche Balb/c a démontré un phénotype d'insensibilité aux stéroïdes associé à des quantités accrues de p38 MAPK sous forme active et l'inactivation subséquente du récepteur des GC (GR) après provocation par un allergène. De plus, des lignées cellulaires lymphoblastiques provenant d'enfants asthmatiques ont été utilisées pour étudier les mécanismes de réactivité variable des GC et ont permis d'explorer le rôle modulateur de la VitD sur la fonction des GC. Chez les enfants asthmatiques, une faible réactivité aux stéroïdes a été associée à une biodisponibilité nucléaire limitée du GR à la suite de l'expression basale diminuée du GR et de la rapide régulation négative induite par l'hormone. Des évidences suggérant un effet bénéfique de la VitD sur la sensibilité aux stéroïdes sont présentées. Enfin, la réactivité des stéroïdes et la modulation de la fonction des GC par la VitD ont été étudiées dans les cellules épithéliales du poumon en développement des modèles de rats normaux et atopiques. L'épithélium des voies

respiratoires du rat atopique semble être plus sensible aux stéroïdes, en rendant possiblement les poumons plus susceptibles aux effets néfastes des GC, et la réponse des GC est atténuée par la VitD. Cette thèse met en évidence la complexité de la fonction de stéroïdes et de sa régulation par des mécanismes multiples allant de l'expression altérée, l'activation réduite, la translocation nucléaire anormale et l'augmentation de la régulation négative homologue des GC.

## ABBREVIATIONS

| | |
|---|---|
| 1α-OHase | 25-hydroxyvitamin D-1-alpha-hydroxylase |
| 11β-HSD | 11beta-hydroxysteroid dehydrogenase |
| AA | Arachidonic acid |
| ACTH | Adrenocorticotropic hormone |
| AHR | Airway hyperresponsiveness |
| AP | Activator protein |
| APC | Antigen-presenting cell |
| ARE | Adenylate-uridylate-rich element |
| ASM | Airway smooth muscle |
| BAL | Bronchoalveolar lavage |
| BN | Brown Norway |
| BUD | Budesonide |
| CAMP | Childhood Asthma Management Program |
| CBG | Corticosteroid-binding globulin |
| CBP | CREB-binding protein |
| CCL | Chemokine (C-C motif) ligand |
| CDK | Cyclin-dependent kinase |
| CHI3L1 | Chitinase 3-like 1 |
| Col3a1 | Collagen III alpha 1 chain |
| COPD | Chronic obstructive pulmonary disease |
| COX | Cyclooxygenase |
| CREB | Cyclic AMP response element-binding protein |
| CRH | Corticotrophin releasing hormone |
| CTL | Control |
| DBD | DNA-binding domain |
| DC | Dendritic cell |
| DMSO | Dimethyl sulfoxide |
| E | Embryonic day |
| ERK | Extracellular-regulated kinase |
| FASN | Fatty acid synthase |
| FcεRI | Fc epsilon receptor I |
| $FEV_1$ | Forced expiratory volume in one second |
| FGF | Fibroblast growth factor |
| GC | Glucocorticoid |
| GH | Growth hormone |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GR | Glucocorticoid receptor |
| GRE | Glucocorticoid response element |
| GSK | Glycogen synthase kinase |
| GSR | Good steroid responders |
| HDAC | Histone deacetylase |
| HSP | Heat shock protein |
| IFN-γ | Interferon gamma |

| | |
|---|---|
| Ig | Immunoglobulin |
| IκBα | Inhibitor of NFκB alpha |
| IL | Interleukin |
| IP | Intraperitoneal |
| IPO13 | Importin 13 |
| JNK | c-Jun N-terminal kinase |
| LBD | Ligand binding domain |
| LPS | Lipopolysaccharide |
| Ly-6B | Lymphocyte antigen 6 complex, locus B |
| MAPK | Mitogen-activated protein kinase |
| MC | Mast cell |
| MCh | Methacholine |
| MIF | Macrophage migration inhibitory factor |
| MKP-1 | MAPK phosphatase-1 |
| MMP | Matrix metalloproteinase |
| NFκB | Nuclear factor kappa B |
| NKT | Natural killer T cell |
| NLS | Nuclear localization signal |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NPC | Nuclear pore complex |
| NTD | N-terminal transactivation domain |
| OVA | Ovalbumin |
| PDGF | Platelet derived growth factor |
| PEEP | Positive end-expiratory pressure |
| PGE2 | Prostaglandin E2 |
| P-gp | P-glycoprotein |
| PBMC | Peripheral blood mononuclear cell |
| PN | Postnatal day |
| PSR | Poor steroid responders |
| qRT-PCR | Quantitative real-time reverse transcriptase polymerase chain reaction |
| RANK | Receptor activator of NFκB |
| RXR | Retinoid X receptor |
| SCF | Stem cell factor |
| SD | Steroid-dependent |
| SERPIN | Serine protease inhibitor |
| SP1 | Specificity protein 1 |
| SR | Steroid-resistant |
| SRC | Steroid receptor coactivator |
| SS | Steroid-sensitive |
| STAT | Signal transduction-activated transcription |
| SUMO | Small ubiquitin-related modifier |
| SWI/SNF | Switching/sucrose non-fermenting |
| TADA | Transcriptional activator |
| TBP | TATA box binding protein |
| TCR | T cell receptor |

| | |
|---|---|
| TGF-β | Transforming growth factor beta |
| $T_H$ | T helper cell |
| TIF | Transcriptional intermediary factor |
| TLR | Toll-like receptor |
| TNF-α | Tumor necrosis factor alpha |
| $T_{Reg}$ | Regulatory T cell |
| TSLP | Thymic stromal lymphopoietin |
| TTP | Tristetrapolin |
| UTR | Untranslated region |
| VitD | Vitamin $D_3$ or $1\alpha,25(OH)_2D_3$ |
| VDR | Vitamin D receptor |
| VDRE | Vitamin D response element |
| YY1 | Yin Yang 1 |

## THESIS FORMAT

This thesis is composed of five chapters, three of which are scientific data chapters. Chapter I is a review of the current state of the literature as it pertains to this thesis and provides general background information. It also includes the rationale and specific thesis objectives. Chapters II, III and IV contain original data and are written in manuscript format, including the following sections: abstract, introduction, materials and methodology, results, and discussion. Connecting texts between the data chapters are provided in accordance with McGill University's "Thesis preparation and submission guidelines". Chapter V is a general overview and discussion of this thesis' findings and final conclusions. The last section of this thesis includes a list of all the references used throughout the entire thesis.

## CONTRIBUTION OF AUTHORS

In Chapter II, the candidate performed all the experimental procedures, including the allergen sensitization and challenge of mice, as well as the intranasal instillation of budesonide. Pulmonary function measurements and assessment of lung inflammation, including bronchoalveolar lavage and quantification of mRNA and protein expression, were also conducted by the candidate. The IgE ELISA experiment was carried out with the aid and supervision of Dr. Yufa Wang, from Dr. Christine McCusker's laboratory. All data was analysed by the candidate.

In Chapter III, lymphoblast cell lines derived from asthmatic children were obtained from our collaborators Dr. Scott T. Weiss and Dr. Benjamin A. Raby. The candidate grew the cell lines in culture, treated them with different agents and extracted mRNA, total and nuclear proteins. The candidate also conducted all qRT-PCR, Western blots, immunofluorescence experiments, microscope photography and data analysis.

In Chapter IV, airway cells were isolated from PN14 rat pups with the occasional help from Isabel Mandeville. The candidate grew the cells in culture, conducted all the experimental procedures and data analysis.

<u>In addition to the manuscripts included in this thesis, the candidate contributed to the following manuscripts:</u>

Reduced viability of mice with lung epithelial-specific knockout of glucocorticoid receptor. Manwani N, Gagnon S, Post M, Joza S, Muglia L, **Cornejo S**, Kaplan F, Sweezey NB. Am J Respir Cell Mol Biol. 2010 Nov;43(5):599-606.

Inflammatory cytokines, goblet cell hyperplasia and altered lung mechanics in Lgl1+/- mice. Lan J, Ribeiro L, Mandeville I, Nadeau K, Bao T, **Cornejo S**, Sweezey NB, Kaplan F. Respir Res. 2009 Sep 21;10:83.

## ORIGINALITY

The candidate has made significant distinct original contribution to knowledge. Original data is presented in Chapters II, III and IV. The identification of a murine model of steroid insensitive asthma is a novel finding. The candidate also determined that the limited steroid responsiveness in this model was associated with elevated levels of active p38 MAPK following allergen challenge. The steroid regulation of GR expression in lymphoblasts from asthmatic children is also original as is the correlation found between levels of GR and therapeutic outcome and the mechanism of homologous downregulation of GR for steroid insensitivity. Furthermore, the candidate provides molecular evidence for the modulatory role of VitD on GC response. In the last data chapter, the strain-specific regulation by GC and VitD of GR and IPO13 in developmental epithelial cells is also an original finding. Finally the effects of GC and VitD on VDR expression in airway epithelial cells and the attenuation of GC-induced nuclear translocation by VitD are novel.

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### CHAPTER II

# CHAPTER I

## Literature Review

The "fetal origins hypothesis" argued that specific *in utero* exposures contribute to disease susceptibility in later life [1]. Barker's hypothesis was based on the idea that during critical developmental windows the organism is more "plastic" to its environment. While over time this "plasticity" is lost, the early developmental "programming" can generate permanent changes to organs and systems impacting life-long disease risk [2]. For example, young children who will become asthmatic later in life show pre-existing abnormalities in lung structure and function before the onset of the disease [1]. Important morphological changes in the lung that occur during late phase development are regulated by glucocorticoids (GCs) and vitamin D (VitD). In this thesis I will explore mechanisms of GC action in asthma and lung development and clarify the modulatory role of VitD on GC responsiveness during these processes.

### 1.1 Glucocorticoids

GCs are stress-induced steroid hormones regulated by the hypothalamo–pituitary–adrenal axis and their primary role is to maintain physiological homeostasis [3]. GCs are essential for life and regulate a myriad of biological processes, including growth and development, metabolism, reproduction, behavior, immune function and cell survival [3]. Originally widely studied in the context of the stress response, it was not until 1949 that the potent anti-inflammatory and immunosuppressive actions of GCs were first described [4]. Since then, synthetic GCs (e.g. prednisone, dexamethasone, hydrocortisone, and budesonide) have become a commonly prescribed drug worldwide and are used regularly in the treatment of allergic and chronic inflammatory diseases, including asthma [5].

Cortisol, the most abundant endogenous GC in humans, is secreted by the adrenal cortex in an endocrine response, which is initiated by the secretion of corticotrophin releasing hormone (CRH) from the hypothalamus and subsequent pituitary

adrenocorticotropic hormone (ACTH) release [3]. GC gets distributed to the rest of the body through the circulation and under normal conditions 80-90% of plasma cortisol is bound to corticosteroid-binding globulin (CBG), 10-15% to albumin and 5% is free [6, 7]. Two mechanisms have been described for CBG-mediated GC delivery to target tissues. CBG is a non-inhibitory serine proteinase inhibitor (SERPIN) that serves as a substrate for neutrophil elastase, which is present at high concentrations at inflammatory sites [8]. Following the proteolytic attack by elastase, CBG irreversibly loses its steroid binding site and releases the hormone; therefore, CBG plays a crucial role in targeted release of GC to sites of inflammation [8]. Alternatively, it has been suggested that CBG releases its cargo after binding to its cell-membrane receptor [9]. Once free, the lipophilic GC readily diffuses into the cell cytoplasm and exerts its genomic effects by binding and activating a cytosolic transcription factor, the glucocorticoid receptor (GR).

The access of cortisol to GR is controlled by a pre-receptor enzymatic mechanism. In the cytoplasm, cortisol is inactivated through its conversion to cortisone by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), while 11β-HSD1 catalyses the conversion of cortisone to active cortisol [10]. Thus, the levels of these two enzymes regulate the intracellular bioavailability of cortisol. This is very important, since acute increases in GC levels are adaptive but chronic high levels of the hormone can be detrimental. Furthermore, Andres et al. observed cell-type specific regulation of the 11β-HSD1 promoter [11]. Since GR is ubiquitously expressed and its activation associated to numerous functions, tissue-specific 11β-HSD expression is believed to provide fine-tuning for GC responses [11].

### 1.1.1 Glucocorticoid Signaling Pathway

In the absence of GC, GR is sequestered in the cytoplasm in a heteromeric complex consisting of several proteins, including heat shock protein (HSP)90, HSP70, immunophilins, p23 and others [12]. Ligand binding induces the dissociation of the protein complex and causes a conformational change in GR that exposes its nuclear localization signal (NLS) [13]. GR nuclear translocation occurs through the nuclear pore complex (NPC) and involves different nuclear transport proteins, like importin α/β, importin 7, and importin 13 (IPO13) [14, 15]. Activated GR is able to regulate the expression of genes by directly binding to DNA (cis-regulation) or by interacting with other transcription factors (trans-regulation) (Figure 1.1) [16]. In the nucleus GR homodimerizes and binds to glucocorticoid response elements (GRE) in the promoter region of glucocorticoid responsive genes. Variations in copy number, location within the promoter and sequence of the canonical GRE (5'-GGTACAnnnTGTTCT-3') regulate GC induction or repression [17, 18].

***Regulation of Gene Expression***

After binding to GREs, GR interacts with cyclic AMP response element-binding protein (CREB)-binding protein (CBP) and p300, which acetylate lysine residues in core histones, causing chromatin remodeling [19]. This process is further stimulated by chromatin remodeling complexes, including switching/sucrose non-fermenting (SWI/SNF) and steroid receptor coactivator (SRC)-1, and leads to the subsequent recruitment of the basal transcriptional machinery [19, 20]. In this way, GR induces the transcription of selective genes, including the anti-inflammatory interleukin (IL)-10, inhibitor of nuclear factor kappa B (IκB)α and mitogen-activated protein kinase (MAPK) phosphatase (MKP)-1 [21-23].

GC can also induce gene expression via trans-activation. Ligand-bound GR can physically interact with several members of the signal transduction-activated transcription (STAT) family, such as STAT1, STAT3 and STAT5. In doing so, it can synergistically enhance the expression of STAT regulated genes, such as insulin-like growth factor-1, β-casein, and fibrinogen [24-26]. The molecular mechanism for this synergistic effect is not known but it has been suggested that GR acts as a coactivator and promotes the recruitment of nuclear cofactors to STAT-bound promoters [24, 26]. STAT proteins are mediators of cytokine signaling and the synergistic interaction between GR and STATs might be a mechanism to enhance immune function [27].

On the other hand, GC can also inhibit gene expression and it is this ability that accounts for most of its anti-inflammatory effects. GC gene repression involves at least three different mechanisms: negative GREs (nGREs), promoter interference and trans-repression [16]. nGREs have a more variable sequence (5'-ATYACnnTnTGATCn-3') in comparison to GREs and bind GR with lower affinity [17]. In a few genes, GR can directly bind nGRE in their promoter region and negatively influence their expression by a mechanism called cis-repression (Figure 1.1B, 1.1D). More frequently, promoter regions contain overlapping transcription factor binding sites such that GR binding to nGRE prevents binding of other factors that would normally induce transcription [16].

Finally, trans-repression does not require GR DNA binding but protein-protein interaction between GR and pro-inflammatory transcription factors such as activator protein (AP)-1 and nuclear factor kappa B (NFκB) [28, 29]. For example, GR inhibits AP-1-induced collagenase expression by interacting with the c-Jun subunit of AP-1 and interfering with its transactivation potential [28, 30]. Similarly, GR inhibits NFκB by physically interacting with its p65 subunit. It has been suggested that by binding to p65,

GR inhibits serine-2 phosphorylation of RNA polymerase II preventing transcription initiation [31]. Furthermore, Ito et al. proposed a mechanism by which ligand-bound GR recruits histone deacetylase (HDAC)2 to promoter regions of actively transcribed genes, leading to chromatin condensation and switching off NFκB-induced gene expression [20].

***Regulation of mRNA Stability***

Another powerful mechanism by which GC affects gene expression, independently of gene transcription, is the regulation of mRNA turnover. As expected, mRNA stability is altered during several physiological processes in which GC takes part, such as stress, development, inflammation, and cancer [32]. GC has variable effects, increasing transcript stability of the pituitary growth hormone (GH)1 and fatty acid synthase (FASN) in the fetal lung, while destabilizing the mRNA species of several inflammatory genes, including IL-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, cyclooxygenase (COX)-2 and chemokine (C-C motif) ligand (CCL)11 [32, 33]. The stability of mRNAs is controlled by adenylate-uridylate-rich elements (AREs) in their 3'-untranslated region (UTR). AREs are heterogeneous sequences (AUUUA pentamers and AT-rich stretches) that interact with ARE-binding proteins like tristetrapolin (TTP), which promotes mRNA decay [33]. The amplification of the inflammatory response upon cell activation depends on serine-threonine kinases such as p38 MAPK, which stabilizes the mRNAs of several pro-inflammatory genes by inhibiting TTP binding to their AREs. It has been suggested that glucocorticoids decrease mRNA stability of inflammatory genes, such as COX-2, by inhibiting p38 MAPK activation and inducing the expression of MKP-1, the main repressor of p38 MAPK [32, 33].

***Non-genomic Actions of Glucocorticoids***

Since the early studies of the GC pathway, it became evident that several hormone effects were too fast to be a result of altered gene expression. Some of these effects include changes in calcium influx, neutrophil degranulation, phagocytosis and superoxide anion production by macrophages, activation of endothelial nitric oxide synthase (NOS), inflammatory signaling transduction cascades, cell adhesion, and apoptosis [5, 33]. It has been proposed that three mechanisms mediate the rapid non-genomic effects of GC in a cell-type specific manner: binding to the classical cytosolic GR, binding to a modified membrane-bound GR, and physiochemical interactions with cellular membranes [33]. Although there is evidence supporting each one of these mechanisms, they are very poorly understood.

The cytosolic GR has been implicated in the rapid non-genomic anti-inflammatory effects of GC through its inhibition of arachidonic acid (AA) release [34]. The release of AA from cell membrane phospholipids is essential for the production of inflammatory cytokines. GC binding to its receptor causes the release of Src kinase from the cytoplasmic GR heteromeric protein complex, leading to the activation of lipocortin 1 and the subsequent inhibition of AA release [34]. Some other rapid GC actions are thought to be mediated through a membrane-bound GR, which has been identified in several immune cell types, including T cells. GC binding to these receptors impairs T cell receptor (TCR) signaling by inhibiting the activity of kinases, which are essential for cell migration, proliferation and cytokine synthesis [5].

Finally, the highly lipophilic GC molecule diffuses in plasma and mitochondrial membranes, interfering with the normal function of ion channels and increasing mitochondrial proton leak [33, 34]. It has been suggested that GC inhibition of mucus

hypersecretion is a result of decreased intracellular $Ca^{2+}$ following hormone treatment [33]. Furthermore, since the proper functioning of immune cells depends on intracellular ion balance and coupling of oxidative phosphorylation, these GC effects have been suggested to result in immunosuppression [34].

### 1.1.2 Glucocorticoid Receptor Genomic Structure

The human GR cDNA was originally cloned in 1985 and later mapped to chromosome 5 (5q31p) [35, 36]. In 1991, Encio and Detera-Wadleigh reported that the GR genomic structure consisted of ten exons (1-8, 9α and 9β) spanning 110 kb (Figure 1.2) [37]. Two additional exons upstream of exon 1 have been identified, all of which entirely represent the 5'-UTR. The now named exons 1A (981 bp), 1B (77 bp) and 1C (184 bp) are under the control of three different promoters [38]. No TATA or CAAT boxes have been recognized in these promoters but binding sites for a number of transcription factors have been found, including AP-1, AP-2, NFκB, specificity protein 1 (SP1), Yin Yang 1 (YY1), nGRE and a GRE [37-42]. Exon 2 which spans 1197 bp encodes the amino-terminal residues. The DNA-binding domain (DBD) is encoded by exons 3 (167 bp) and 4 (117 bp), while the ligand-biding domain (LBD) and 3'-UTR are formed from the last five exons [37].

#### *mRNA Splice Variants*

When first cloned, two human GR mRNA species were identified: GRα (5.5 kb) and GRβ (4.3 kb). They are generated by alternative splicing and both contain exons 1-8 and either exon 9α or 9β (Figure 1.2) [37]. Consequently, translation results in two protein isoforms, but only GRα is able to bind GC and trans-activate or trans-repress gene transcription. Additionally, exon 1A can also undergo alternative splicing producing exons 1A1 (212 bp), 1A2 (383 bp) and 1A3 (981 bp); therefore, human GR mRNA can

contain five varieties of exon 1 [38]. Even though none of the versions of exon 1 contains protein coding information, it has been suggested that they might be involved in regulating tissue-specific GR gene expression. Breslin et al. detected higher expression levels of GR mRNA species containing exon 1A3 in cancer cell lines of hematopoietic origin (CEM-C7 and IM-9) when compared to other cancer cell lines [38]. Moreover, the effect of GC treatment on 1A3-containing GR transcript expression was cell-type dependent, causing an up-regulation in CEM-C7 cells but a repression in IM-9 cells [38].

Several other variants have been described in cancer cells, for example GR-P, which lacks exons 8 and 9, and GR-A, which lacks exons 5-7 [43]. Another GR mRNA species (GRγ) is generated as a result of the use of an alternative splice donor site, which causes the retention of three bases from the intron between exons 3 and 4 [44]. The extra three bases code for an additional arginine at position 453 in between the two zinc fingers of the DBD. GRγ mRNA is expressed at levels between 3.8 and 8.7% of total GR and is less transcriptionally active than GRα [44].

### 1.1.3 Glucocorticoid Receptor Protein

Similar to other members of the nuclear receptor superfamily, GR contains three functional domains: the N-terminal transactivation domain (NTD), the central DBD and the C-terminal LBD (Figure 1.3) [7]. The NTD is the most poorly conserved region between nuclear hormone receptors, and even among the different GR isoforms [12]. In the standard GR-α, which is 777 amino acids long, the NTD region (aa 1-420) contains a transcriptional activation function (AF)-1. The AF-1 mediates most of GR's transcriptional activity and binds several coregulators and components of the basal transcriptional machinery, including CBP, SRC-1, transcriptional activator (TADA)2, and TATA-box binding protein (TBP) [12]. Furthermore, this region is important in

modulating GR function since it is rich in phosphorylation sites. GR phosphorylation has been shown to regulate receptor stability, subcellular trafficking, cofactor binding, transcriptional activation, and turnover [45].

The central DBD (aa 421-486) is highly conserved among nuclear hormone receptors and contains a single globular domain with two zinc finger motifs [7]. Within the first zinc finger, three amino acids form the proximal (P)-box, which is responsible for the binding to the GRE on DNA and confers receptor specificity [12]. The second zinc finger stabilizes the GRE binding and plays an important role in receptor dimerization at the GRE through the five amino acids that make the distal (D)-box. The DBD also contains one of the two NLSs found in GR (NLS1) [13].

A small flexible hinge region connects the DBD to the C-terminal LBD (aa 527-777). The LBD contains twelve α helices and four small β strands that fold into a three-layer hydrophobic helical sandwich for the binding of GC [7]. The last C-terminal helix contains a second trans-activation domain (AF-2), which upon ligand binding undergoes a conformational change, stabilizing the receptor and promoting the binding of coactivators containing LXXLL motifs, such as SRC-1 and transcriptional intermediary factor-2 (TIF-2) [46]. The LBD also contains a second nuclear localization motif (NLS2) and sequences important for the interaction with HSPs, ligand-binding specificity, and receptor dimerization [13, 46].

***Translational Isoforms***

Alternative translation initiation of GR mRNA has been suggested as a mechanism to increase diversity in GRα protein expression. Lu and Cidlowski identified several internal AUG codons in exon 2 that serve as translation start sites and that generate eight GRα isoforms that have been designated GRα-A to D (A, B, C1, C2, C3,

D1, D2, and D3) (Figure 1.4) [47]. They demonstrated that these isoforms were generated from a single GRα mRNA species by means of leaky 5' ribosomal scanning and/or ribosomal shunting. The relative expression levels of these GRα isoforms vary significantly among different tissues, suggesting that they provide a mechanism for tissue-specific modulation of GC action [47].

The translational isoforms are only distinguished by the length of their NTD, which contains one of the two transactivation domains (AF-1). Surprisingly, all GRα isoforms have similar affinities to GC, bind to GREs following GC-induced nuclear translocation and are able to trans-repress pro-inflammatory genes [47, 48]. The exceptions are GRα-D isoforms, which localize to the nucleus even in the absence of hormone and have the lowest transcriptional activity [47, 48]. Interestingly, the expression of individual isoforms in osteosarcoma cells altered their sensitivity to GC-induced apoptosis; while GRα-D expression correlated with resistance, the expression of the most transcriptionally active isoform (GRα-C3) was linked to greater sensitivity [48].

It has been suggested that the cellular composition of the GRα isoforms regulate GC signalling and this composition is not static. Polymorphisms of the GR gene, heterogeneity in the 5'-UTR of GRα mRNA, and post-translational modifications in the subtypes of GRα protein, have been suggested as possible mechanisms to regulate the relative expression of these isoforms [13]. Further studies are necessary to clarify the exact role these translational isoforms play in modulating GC function.

***Post-translational Modifications of Glucocorticoid Receptor***

Post-translational modifications of GR isoforms provide an additional level of modulation of receptor function. These modifications, including phosphorylation, ubiquitination, sumoylation, and acetylation, have been shown to alter GR stability,

nuclear shuttling and transcriptional activity (Figure 1.4). Human GRα contains at least six serine residues that are sites for phosphorylation, such as S113, S141, S203, S211, S226, and S404 [45]. Some of these residues are constitutively phosphorylated and get hyperphosphorylated upon GC binding, while the phosphorylation of others is strictly ligand-dependent [49]. Several kinases have been implicated in GRα phosphorylation, including MAPKs, cyclin-dependent kinases (CDK), and glycogen synthase kinase (GSK)-3 [45]. Experimental evidence suggests that phosphorylation at S203 renders the human GRα transcriptionally inactive. Blind and Garabedian used chromatin immunoprecipitation and GR phosphorylation-site specific antibodies to show that phosphorylation at S203 caused a lack of GRα nuclear accumulation and no receptor binding to GREs [49]. Furthermore, inhibition of extracellular-regulated kinase (ERK) MAPK decreases phosphorylation at S203 and promotes nuclear translocation of GRα and subsequent transcriptional activity [50]. In contrast, phosphorylation of S221 following GC binding is associated with GRα recruitment to GREs and increased transcriptional activity [49].

Phosphorylation of certain receptor residues is also thought to regulate GR turnover by functioning as recognition signals for E2 ubiquitin-conjugating and/or E3 ubiquitin ligase enzymes [51]. Wang and DeFranco identified an E3 enzyme whose expression modulates GRα levels and transactivation activity [52]. Ligand binding promotes the ubiquitination of lysine 419 located in the PEST (Pro, Glu, Ser, and Thr) degradation motif at the end of the receptor's NTD, which targets GR for proteasomal degradation [53].

GRα can also be modified by the covalent attachment of small ubiquitin-related modifier (SUMO) peptides to three of its lysine residues (K277, K293, and K703) [13].

GR sumoylation has been shown to alter receptor stability and transcriptional activation in a promoter- and cell-specific manner [13]. Finally, the acetylation status of GRα has been recognized as an important modulator of receptor function. GRα gets acetylated at lysine residues K494 and K495 after GC binding and before nuclear translocation [54]. In the nucleus, HDAC2 deacetylates GRα, and this step is a prerequisite for GRα binding to the NF-κB complex and trans-repression of pro-inflammatory genes [54].

### 1.1.4 Mechanisms of Glucocorticoid Resistance

GCs are the most effective treatment for inflammatory and autoimmune diseases; however, 5-10% of asthmatics and up to 30% of patients with rheumatoid arthritis, systemic lupus erythematous, and severe ulcerative colitis do not fully respond to this medication [55, 56]. Individuals with complete resistance are rare but the spectrum of relative GC responsiveness is composed of patients who require high doses of the drug, which can cause many adverse side effects. Steroid resistance has been mostly studied in chronic asthma, where it is defined as a lack of improvement in pulmonary function after GC therapy [57]. Several molecular mechanisms that are not mutually exclusive have been proposed for GC insensitivity, including decreased GR ligand affinity, altered nuclear translocation of GR, reduced GR DNA binding and increased activation of pro-inflammatory transcription factors [55]. Great advances have been made in our understanding of these mechanisms in the context of inflammation; however, little is known about how relative steroid insensitivity could affect normal development. It is reasonable to speculate that these same mechanisms could render the developing lung less GC responsive, affecting lung growth and maturation and perhaps leading to an organ more susceptible to respiratory disorders.

***Genetic Predisposition***

Familial glucocorticoid resistance is a very rare inherited syndrome which results from mutations in the GR gene. The mutations found in the few cases reported have been shown to reduce receptor binding affinity to cortisol and DNA, reduce GR expression, and increase receptor thermolability [58]. On the other hand, several GR polymorphisms have been identified but few are thought to be functionally relevant. The ER22/23EK polymorphism is located in the NTD region of GR and consists of two linked single nucleotide mutations in codons 22 and 23, resulting in an amino acid change GAG AGG (GluArg) → GAA AAG (GluLys) [59]. ER22/23EK has been associated with relative systemic GC resistance and carriers have a more favorable metabolic profile, making them less susceptible to develop type-2 diabetes or cardiovascular disease [59]. Russcher et al. found an association between ER22/23EK and decreased GR trans-activating capacity but no trans-repressional activity *in vitro* [60]. Interestingly, the ER22/23EK polymorphism has been shown to promote the expression of the less transcriptionally active GRα-A in comparison to GRα-B. It has been suggested that this decreased relative expression of GRα-B could explain the GC insensitivity observed in individuals having the ER22/23EK polymorphism [60]. However, no associations have been found with inflammatory bowel disease, rheumatoid arthritis or glucocorticoid resistance in children with acute lymphoblastic leukemia [59, 61]. A second polymorphism consisting of an A to G substitution located in the 3'-UTR of exon 9β results in increased expression and stability of GRβ *in vivo* [59]. Some reports associate this 9β polymorphism with reduced immunosuppression. While there are conflicting reports of associations with rheumatoid arthritis, carriers of this polymorphism have a lower risk of persistent *Straphylococcus*

*aureus* nasal carriage [59]. Furthermore, this 9β polymorphism has not associated with glucocorticoid resistance in acute lymphoblastic leukemia [61].

***Defects in Ligand Binding***

The inflammatory milieu is believed to be a modulator of GR function and the expression of certain cytokines has been shown to reduce the ligand binding affinity of GR. IL-2 and IL-4 are overexpressed in bronchial biopsies of steroid-resistant (SR) asthmatics and in bronchoalveolar lavage (BAL) of severe asthmatics, which tend to be less GC responsive [58, 62]. Interestingly, GC inhibits the expression of these two cytokines in lymphocytes of steroid-sensitive (SS) but not SR rheumatoid arthritis patients [56]. Sher et al. showed that peripheral blood mononuclear cells (PBMCs) from a subset of SR asthmatics had decreased GR binding affinity, which normalized when cells were in culture for 48 hours [57]. However, this defect was mimicked by the incubation with IL-2 and IL-4 or IL-13 alone and blocked by a p38 MAPK inhibitor [57, 63]. It has been suggested that the elevated levels of these cytokines in GC insensitive asthmatics leads to the activation of p38 MAPK and subsequent phosphorylation of serine residues in GR, altering the receptor's function [63, 64]. Further evidence for the involvement of stress-activated protein kinases in steroid resistance, comes from studies that have found increased activation of c-Jun N-terminal kinase (JNK) and p38 MAPK in epithelial cells of patients with SR Crohn's disease [65]. Also, alveolar macrophages of GC insensitive asthmatics show increased activation of p38 MAPK [66].

A similar decrease in ligand binding has also been observed after nitrosylation of GR both *in vitro* and *in vivo* [67, 68]. During inflammation NOS is induced, leading to the production of nitric oxide (NO). Interestingly, septic shock is characterized by high

amounts of NO and GC fails to exert its anti-inflammatory effects in this condition [68]. Whether GR nitrosylation plays an important role in other SR conditions is not known.

### *Altered Glucocorticoid Receptor Nuclear Translocation and DNA Binding*

Studies using mouse T cells have demonstrated that steroid resistance can be induced by IL-2 through a mechanism by which STAT5 forms a heterodimer with GR and inhibits its nuclear translocation [69]. Interestingly, IL-2-induced GC resistance was blocked by a p38 MAPK inhibitor, suggesting that the GR-STAT5 interaction occurs through one of the phosphoserine residues of GR [69]. In BAL cells from GC-insensitive asthmatics, GRα fails to translocate into the nucleus in response to dexamethasone [70]. Similar results have been reported by Matthews et al. in PBMCs of a subset of SR and steroid-dependent (SD) asthmatics [71]. It is believed that this defective nuclear shuttling might be a result of GR phosphorylation by MAPK and subsequent interaction with importins. Furthermore, a dysregulation of nuclear transport proteins has been proposed as a mechanism for altered GC sensitivity. Work from the Kaplan group demonstrated that inhibition of IPO13 synthesis in rat lung epithelial cells prevented nuclear translocation of GR and abrogated the GC-mediated silencing of inflammatory cytokine production [15]. More recently, genetic variation in IPO13 was associated with improved lung function in asthmatic children who were not using GCs, suggesting that IPO13 variation may improve nuclear bioavailability of endogenous glucocorticoids [72]. However, at the moment there are no reports of direct links between altered function of importins and steroid resistance.

GCs have a very limited beneficial effect in patients with chronic obstructive pulmonary disease (COPD). In this condition, as in SR asthma, there is a marked increase in oxidative stress, which has been suggested to inhibit nuclear import of GR by

impairing the GC-dependent dissociation of HSP90 from the receptor [73, 74]. Reduced GR binding to GREs in response to dexamethasone has also been observed in PBMCs of SR asthmatics [75]. This decrease in GR-DNA binding has been associated with: elevated levels of AP-1, increased c-Fos transcription rates, and increased phosphorylation of JNK in response to inflammatory stimuli [76-78].

In a subset of SR asthmatics, a normal GC-induced nuclear translocation of GR was observed but no acetylation of histone 4, suggesting that GCs were not able to activate the transcription of anti-inflammatory genes [71]. In fact, alveolar macrophages from GC insensitive asthmatics show reduced MKP-1 expression after GC treatment, which correlates with increased p38 MAPK activity [66].

***Increased Expression of Glucocorticoid Receptor β***

The involvement of GRβ in steroid resistance is a controversial issue since there is evidence supporting and disproving this hypothesis. Most reports agree that human GRβ is not to able to bind GC and has no transcriptional activity [79]. Nevertheless, it has an intact DBD and when overexpressed *in vitro* it represses the transcriptional activity of GRα; thus, it might act as a dominant-negative inhibitor of GC by preventing GRα binding to DNA [79]. Alternatively, it has been proposed that GRβ's inhibitor role results from its ability to form a heterodimer with GRα, decreasing its transcriptional activity [80]. Some studies have reported increased expression of GRβ in cells from SR patients with inflammatory conditions, including asthma, rheumatoid arthritis and inflammatory bowel disease [56, 81, 82]. However, a similar number of studies do not confirm these findings [83-86]. Certain pro-inflammatory cytokines have been shown to induce the expression of GRβ and block GC mediated functions [42]. Microbial superantigens have a similar effect on GRβ expression, through the induction of IL-2 and IL-4, possibly

explaining GC resistance to microbial colonization in chronic rhinosinusitis [87]. Yet the extremely low expression of GRβ in comparison to GRα has raised doubts on the physiological relevance of this isoform in steroid resistance or insensitivity [79]. Furthermore, Torrego et al. demonstrated that the IL-2 and IL-4-induced GC-insensitivity was not a result of GRβ up-regulation in PBMCs of healthy and asthmatic individuals [88].

Studies of the nuclear localization of GRβ have also provided conflicting results. While some authors find GRβ constitutively in the nucleus, others find it in both nucleus and cytoplasm [79]. Moreover, it has been suggested that cytoplasmic GRβ interferes with the nuclear translocation of GRα, providing another mechanism for GC insensitivity [70]. GRβ was always thought to be transcriptionally inactive, but a study found that even though it cannot bind GCs, it can bind a GR antagonist (mifepristone), translocate into the nucleus and regulate gene expression [89]. However, an endogenous ligand for GRβ has not yet been identified.

***Alternative Mechanisms***

Increased expression, activation or altered GC-modulation of transcription factors could contribute to a reduction in GC sensitivity. As mentioned before, PBMCs from SR asthmatics show increased activation of AP-1, which prevents GR-DNA binding [76]. SR asthmatics also have increased JNK phosphorylation, which is not reversed by GCs [78]. Similarly, PBMCs from SR rheumatoid arthritis patients show increased levels of activated NFκB, which GC fails to inhibit *in vitro* [56]. Finally, since STAT5 and GR form complexes, overexpression of activated STAT5 could reduce the induction of GC responsive genes, contributing to steroid insensitivity [56, 69].

Acetylation of histones is a key step in GR regulation of anti-inflammatory genes and a subset of SR asthmatics show a lack of histone acetylation in response to GC [71]. Furthermore, GCs repress the expression of pro-inflammatory genes by recruiting HDAC2 to their promoter regions [20]. Interestingly, HDAC2 expression and activity is reduced in certain GC insensitive conditions. Macrophages, bronchial and peripheral lung biopsies of COPD patients show decreased HDAC2 activity and expression [90]. GC insensitivity in severe asthma has also been associated with diminished HDAC activity [91].

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine that counteracts GC function by preventing the induction of MKP-1 [92]. Ishiguro et al. showed that colonic mononuclear cells from patients with SR ulcerative colitis have increased MIF expression and p38 MAPK activity [93]. Interestingly, cell treatment with a MIF antibody decreased the levels of activated p38 MAPK and restored the anti-inflammatory effects of GC [93]. Polymorphisms in the MIF gene have been associated with GC resistance in children with nephrotic syndrome, juvenile rheumatoid arthritis and Crohn's disease [94-96]. Also, MIF has been implicated in steroid resistance in systemic lupus erythematosus, acute respiratory distress syndrome and severe asthma [97].

P-glycoprotein (P-gp) is a member of the ATP-binding cassette transporter superfamily that is responsible for the efflux of certain drugs, including GCs, from cells. Since overexpression of P-gp leads to decreased intracellular levels of GCs, it has been suggested as a possible mechanism for GC insensitivity in inflammatory diseases [98]. In support of this hypothesis, high levels of P-gp have been found in lymphocytes and epithelial cells from inflammatory bowel disease patients who did not respond to GC therapy and in lymphocytes of SR rheumatoid arthritis patients [98, 99]. Although

conflicting reports exist, some studies have found an association between polymorphisms in the MDR1 gene, which codes for P-gp, and SR Crohn's disease, ulcerative colitis and rheumatoid arthritis [100, 101].

IL-10 is a potent anti-inflammatory and immunosuppressive cytokine that is induced by GCs. T cells from SR asthmatics fail to induce IL-10 synthesis following GC stimulation, an effect that is reversed by calcitriol (vitamin $D_3$) [102]. Furthermore, a mutation in the IL-10 promoter, which induces the production of high levels of the cytokine, has been associated with favorable prednisone response in children with acute lymphoblastic leukemia and rheumatoid arthritis patients [103, 104].

**1.2 Vitamin D**

Vitamin $D_3$ (VitD, $1\alpha,25(OH)_2D_3$), initially characterized for its role in calcium and bone homeostasis, is now recognized as a modulator of fundamental biological processes, such as immunity, inflammation, cell proliferation and development. Adequate levels of VitD are required for proper health; however, deficiency is believed to affect a large proportion of the population. In fact, 30-50% of Canadians are believed to be VitD deficient and pregnant women, lactating mothers and newborns are at specially high risk [105]. Recently, several studies have suggested an enhancive role of VitD in steroid responsiveness during inflammatory processes (see section 1.4.3 Vitamin D in Asthma). In light of these findings, it is reasonable to speculate that VitD could have similar modulatory effects on GC function during lung development.

VitD, also known as cholecalciferol, can be ingested in the diet but is mainly synthesised by a multistep reaction that starts in the skin with the UVB-induced conversion of 7-dehydrocholesterol (pro-vitamin $D_3$) into pre-vitamin $D_3$ (Figure 1.5) [106]. Pre-vitamin $D_3$ rapidly gets converted to vitamin $D_3$ in a heat-dependent process

and enters the circulation bound to vitamin D binding protein. In a mechanism to prevent vitamin $D_3$ toxicity, extra UVB degrades excessive pre-vitamin $D_3$ and vitamin $D_3$ into inactive photoproducts. To become hormonally active, vitamin $D_3$ needs to be hydroxylated twice: the first time in the liver by vitamin D-25-hydroxylase to produce $25(OH)D_3$, the major circulating form of VitD; and a second time by 25-hydroxyvitamin D-1-alpha-hydroxylase (1α-OHase) primarily in the kidney. Ultimately, $1\alpha,25(OH)_2D_3$ (VitD) exerts its genomic effects by binding to the vitamin D receptor (VDR) in the cytoplasm of its target cells (Figure 1.5) [107].

Ligand binding causes the dimerization of VDR to an unoccupied retinoid X receptor (RXR) which allows the heteromeric complex to recognize positive and negative vitamin D response elements (VDREs) on the promoter region of VitD-regulated genes [106]. VDREs typically consist of either direct repeats of two PuG(G/T)TCA motifs separated by three nucleotides (DR3) or everted repeats separated by six nucleotides (ER6) [108]. Variations in the VDRE sequence are thought to induce differential conformational changes in the VDR/RXR complex that determine the recruitment of either coactivators or corepressors to the promoter region of genes, thus regulating the induction or repression of gene transcription [108]. VitD increases calcium absorption, bone mineralization and turnover by regulating the expression of genes such as calbindin9k, epithelial calcium channel, osteocalcin, receptor activator of NFκB (RANK), and osteoprotegerin [108].

### 1.2.1 Vitamin D as an Immunomodulator

The importance of VitD as an immunomodulator became apparent when it was discovered that VDR and the VitD metabolic enzymes were expressed in many immune cells, including macrophages, lymphocytes, dendritic and epithelial cells (Figure 1.5)

[107]. It has been suggested that VitD enhances the antimicrobial activity of macrophages. Liu et al. demonstrated that *Mycobacterium tuberculosis* stimulates toll-like receptors (TLRs) in human macrophages and this induces the expression of VDR and 1α-OHase, which enhances the VitD-dependent production of the antimicrobial peptide cathelicidin [109].

VitD inhibits the proliferation of T cells and alters their cytokine expression profile, repressing the production of the T helper cell type ($T_H$)1 cytokines IL-2 and interferon (IFN)-γ, while inducing the expression of $T_H$2 cytokines, such as IL-5 and IL-10 [110]. It has been suggested that VitD promotes $T_H$2 cell development through the stimulation of IL-4 production, nevertheless some studies have shown that it inhibits the expression of this cytokine [110, 111]. The observed contradictory effects of VitD on the expression of $T_H$2 cytokines reflects the complexity of VitD's role in the immune response and could also possibly be a result of experimental differences, such as timing and/or dose of exposure as well as target cells [112]. Due to its ability to repress $T_H$1 responses, VitD has been proposed as a possible treatment for autoimmune disorders. In fact, VitD has proven beneficial in animal models of systemic lupus erythematosus, autoimmune encephalomyelitis, type-1 diabetes mellitus, collagen-induced arthritis, and inflammatory bowel disease [107]. It has also been suggested that VitD suppresses autoimmunity by inhibiting $T_H$17 responses. In an experimental model of colitis, VitD reduced the severity of the disease by decreasing the expression of IL-17 as well as IL-6 and IL-23, two cytokines that induce $T_H$17 cell differentiation and IL-17 production [113]. Also, VitD stimulates the generation of regulatory T cells ($T_{Regs}$), which have suppressor rather than effector functions [114]. Interestingly, there is a positive correlation between circulating levels of 25(OH)$D_3$ and the ability of $T_{Regs}$ to suppress T

cell proliferation [115]. Along with its effects on T cells, VitD also inhibits B cell proliferation, differentiation, and immunoglobulin (Ig) production [116].

Similarly, VitD inhibits the maturation and differentiation of dendritic cells (DCs) by enhancing their IL-10 secretion while suppressing IL-12 synthesis [117]. Furthermore, VitD interferes with the NFκB signalling pathway at multiple levels: prevents the degradation of IκBα, inhibits the nuclear translocation of NFκB, and promotes VDR interaction with NFκB blocking its binding to DNA [107].

### 1.3 Lung Development

The development of the mammalian lung involves several processes including branching morphogenesis, cell differentiation, vasculogenesis and alveolarization. These processes are tightly regulated by a wide range of molecules, including transcription and growth factors, microRNAs, extracellular signaling proteins and hormones, such as GC and VitD [118]. Precise expression of these factors in time and location is required to produce a normal functional lung. This is of crucial importance, since it has been suggested that altered lung development could be a susceptibility factor for respiratory diseases such as asthma. Lung organogenesis is fairly conserved among mammals but tends to show temporal variation. The development and maturation of the lung has been divided histologically into 5 stages: embryonic, pseudoglandular, canalicular, saccular, and alveolar (Figure 1.6) [119].

The embryonic stage (embryonic days (E)12-13 in rats and weeks 3-7 in humans), starts with the formation of a ventral lung bud on the foregut endodermal tube [120]. The primitive trachea separates from the primitive esophagus, leading to the development of the two primary bronchi. During the pseudoglandular stage (E13-18 in rats and weeks 5-17 in humans) these bronchi extend into the surrounding mesenchyme dividing

dichotomously until ultimately all the airways of the mature lung are formed. The early stages of lung bud formation and branching are dependent mainly on fibroblast growth factor (FGF) signalling [121]. During this time, pluripotent cells located at the distal tip of the bronchial epithelium leave descendent cells on the airways that start to differentiate into neuroendocrine, Clara, ciliated and goblet cells [121]. Similarly, multipotent mesodermal progenitors begin to differentiate into airway smooth muscle (ASM) cells that envelop the endodermal tube or vascular smooth muscle cells which become incorporated into vessels [121]. Nerve and cartilage formation are also observed at this time of development [120].

During the canalicular stage (E18-20 in rats and weeks 16-26 in humans) further expansion and maturation of the respiratory tree occurs giving rise to the respiratory bronchioles, alveolar ducts and sacs [118]. This stage is also characterized by increased innervation and vascularization along the airways and by the beginning of the differentiation of the epithelium into alveolar type I and type II cells [119-121].

The dramatic thinning of the interstitial tissue and expansion of the airspaces which mark the saccular stage (E20-postnatal day (PN)5 in rats and weeks 24-38 in humans), bring the capillary network closer to the epithelium [120]. At this time, alveolar cells are more clearly differentiated into mature squamous type I, which serve as a blood-air barrier, and round secretory type II, which are responsible for surfactant production [118]. These are crucial steps in pulmonary development and by the end of this stage the fetal lung is able to sustain gas exchange in premature babies [118].

In humans, alveolarization starts a few weeks (gestational week 38) before birth and continues until at least 7 years of age. On the other hand, in rodents the alveolar stage is exclusively postnatal (PN5-30 in rats) [118]. Even though this is a long phase, most

alveolar formation occurs early and in rats “bulk alveolarization” finishes by PN14. During this period, alveolar sacs are subdivided by the growth of ridges which are termed secondary septa and produce most of the gas exchange surface area [118]. As alveoli mature, the initial double layer capillaries remodel into a single layer, forming a very efficient unit for gas exchange [121].

### 1.3.1 Glucocorticoids in Lung Development

There is a surge in circulating levels of cortisol in late gestation which is essential for the transition to extra-uterine life. These high levels of GC play a crucial role in fetal lung maturation and surfactant production. In addition to increased secretion of GC from the fetal adrenal glands, altered activity of the HSD enzymes provide higher local levels of active hormone during this time period [122]. Throughout gestation, the human fetal lung expresses exclusively 11β-HSD2, which inactivates cortisol; however, there is a marked decrease in expression of this enzyme near term [122, 123]. In contrast, rodent developmental lungs only express 11β-HSD1, which catalyzes the conversion of 11-dehydrocorticosterone to active corticosterone, the most abundant endogenous GC in rodents [124]. Interestingly, 11β-HSD1 activity increases significantly during late gestation, amplifying the GC-driven lung maturation [124].

In line with the importance of GC in lung organogenesis, GR is highly expressed in fetal pulmonary tissue [122]. Additionally, its expression significantly increases over time, reaching a peak right before birth and making the lung more GC-sensitive [125]. Further evidence for the crucial role of GC during lung development comes from studies of mutant mice. *Gr* null mice have collapsed lungs at birth and die a few hours later due to respiratory failure [126]. These mice have lungs that resemble those of E15.5 embryos, as well as elevated plasma corticosterone, increased lung weight, thicker septal walls, altered

epithelial cell differentiation and reduced mRNA expression of surfactants [126, 127]. Furthermore, the lungs of *Gr* knock-out mice also show decrease expression of one of the epithelial sodium channel subunits and aquaporin-1, two membrane channels implicated in the withdrawal of liquid from the lungs at birth [127]. A very similar pulmonary phenotype is observed in *Crh*-null mice, which are glucocorticoid insufficient [128].

It is believed that GC stimulates alveolar type II cell differentiation by inhibiting DNA synthesis and subsequent cell proliferation [129]. Type II cells are responsible for the production of pulmonary surfactant, which reduces surface tension at the alveolar air-liquid interface, preventing lung collapse at end expiration and facilitating breathing [130]. GC up-regulates the levels of surfactant proteins by increasing their expression and/or mRNA stability [131-134]. Furthermore, GC induces the expression of FASN, enzyme that catalyzes the synthesis of fatty acids, which are the main substrate for phospholipids [135]. Similarly, GC also increases the levels of saturated phosphatidylcholine, the major phospholipid component of surfactant, stimulates the appearance of lamellar bodies in alveolar type II cells and enhances their response to surfactant secretagogues [134, 136, 137].

Finally, GC is responsible for prompting important structural changes required for the development of a functional lung. It has been suggested that GC mediates thinning of the alveolar septa by inducing programmed cell death of interstitial fibroblasts and type II epithelial cells [138]. Additionally, GC promotes microvascular maturation, defined as the conversion from a double to a single layer capillary network in the alveolar septa, by capillary fusion and differential growth [139].

### 1.3.2 Vitamin D in Lung Development

Evidence for VitD involvement in lung development first came from studies that associated respiratory distress with VitD deficiency in pre-term infants [140]. Later, Gaultier et al. showed that rat pups born of VitD-deficient mothers had altered lung mechanics, possibly due to abnormal alveolar formation and connective tissue development [141]. Since then, data of *in vitro* and animal studies have supported the idea that VitD contributes to late phase lung organogenesis. More recently, a study in mice confirmed that VitD deficiency during pregnancy leads to altered lung structure and decreased pulmonary function [142]. It is possible that VitD exerts both direct and indirect effects on the maternal inflammatory environment and on gene expression in the developing lung through its modulatory role of GC action. Therefore, VitD deficiency might contribute to asthma risk in susceptible individuals (Figure 1.7).

VDR is expressed in fetal alveolar type II cells and its expression is dependent on cell maturation status during late gestation, a time characterized by epithelial differentiation and surfactant production [143]. Furthermore, VitD treatment induces the maturation, proliferation and DNA synthesis of these cells [144, 145]. VitD has also been shown to increase surfactant production and secretion [144, 146]. Alveolar type II cells are known to be able to metabolize VitD to its C-3-epimer, which has decreased affinity to VDR but higher metabolic stability and is able to induce surfactant phospholipid and protein synthesis [147]. Singh and coworkers demonstrated that new-borns have significant circulating levels of this VitD metabolite which decrease with age [148]. It has been suggested that VitD and its C-3-epimer regulate lung maturation by modulating epithelial-mesenchymal interactions and lipofibroblast survival [149].

Nguyen et al. showed that fetal lung fibroblasts have the capacity to carry out the last hydroxylation step in the synthesis of active VitD [150]. Even though the authors were not able to detect VDR in these cells, others have identified the presence of VDR in a human fetal fibroblast cell line [150, 151]. VitD treatment of these cells, altered their metabolism, decreased their numbers and DNA synthesis [152]. On the other hand, in rat immature lung fibroblasts VitD acts synergistically with retinoic acid to induce proliferation through a platelet derived growth factor (PDGF)-mediated autocrine mechanism [153]. Various VitD analogues have also been shown to possess mitogenic activity and activate the VDRE in the PDGF-A gene, which is essential for normal alveolarization [154]. Sakurai et al. proposed a mechanism of alveolar maturation by which VitD, through a specific temporal and spatial expression and function, modulates septal thinning as well as fibroblast and alveolar type II cell differentiation and proliferation [149].

**1.4 Asthma**

Asthma is a chronic respiratory disease characterized by persistent airway inflammation, reversible airflow obstruction and enhanced airway hyperresponsiveness (AHR) [155]. It is estimated that it affects 300 million people worldwide and approximately 250 thousand die of it every year [155]. In Canada alone by 2010 almost 2.5 million people over the age of 12 had been diagnosed with asthma [156]. Furthermore, it is the most common chronic disease in children, affecting 15.6% of the Canadian pediatric population of ages 4-11 [157]. The origins of asthma are complex and multifactorial, but epidemiological studies have identified several risk factors for the development of the disease, including genetic predisposition, sensitization to allergens,

exposure to microbes and pollutants, maternal diet during pregnancy or lactation, and psychosocial factors [158].

Airway inflammation is the major component of asthma and the complex interaction between immune cells, structural cells and their mediators is responsible for the onset of the pathophysiological characteristics of the disease. It is believed that allergic inflammation is promoted by an imbalance between $T_H1$ and $T_H2$ cytokines, leading to a $T_H2$-skewed immune response [159]. In allergic asthma, an inhaled antigen is recognized in the lung by antigen-presenting cells (APCs), most commonly DCs but also macrophages and B cells (Figure 1.8) [160]. APCs process the antigen and present it to naïve T cells, triggering their differentiation and clonal expansion. Subsequent antigen exposure activates $T_H2$ cells and recruits them to the airways, where they produce cytokines like IL-4, IL-5 and IL-13 [160]. These cytokines activate B cells and induce the synthesis and secretion of IgE [159]. Circulating IgE quickly binds to high-affinity IgE Fc epsilon receptor I (FcεRI) on the surface of mast cells (MCs) and basophils, sensitizing them to the specific allergen [161]. Antigen binding to the IgE–FcεRI complexes triggers the "early phase" of the allergic reaction which consists in the release of granules containing histamine, eicosanoids, free radicals and cytokines [159, 161]. These factors are responsible for the manifestation of the main features of asthma, including ASM contraction, mucus hypersecretion and vasodilation [159]. The secretion of chemokines and inflammatory cytokines leads to the "late phase" reaction, characterized by the recruitment and activation of eosinophils, $T_H2$ cells, macrophages and neutrophils [159]. $T_H17$ cells are also believed to be important players in this phase, recruiting neutrophils and amplifying the $T_H2$ cell-mediated eosinophilic inflammation [162]. Interestingly, $T_H17$ cells seem to be particularly important in neutrophilic and SR asthma [162].

Chronic airway inflammation, primarily mediated by eosinophils, causes continuous tissue injury and repair and eventually leads to structural changes in the airways [162]. These morphological changes, termed airway remodeling, include epithelial shedding, goblet cell hyperplasia, loss of ciliated cells, vessels dilation and edema, increased numbers of activated fibroblasts, as well as ASM hyperplasia and hypertrophy [163]. Interestingly, many factors involved in the epithelial-mesenchymal interactions during remodeling of the asthmatic lung, also control normal lung remodeling during development [164]. It is believed that in chronic asthma, communication between the injured epithelium and the underlying fibroblasts leads to the reactivation of these developmental growth factors which stimulate mesenchymal cell proliferation and airway remodeling (Figure 1.9).

### 1.4.1 Inflammatory Cells in Asthma

#### *Dendritic cells*

DCs are present in higher numbers in the bronchial mucosa and BAL of atopic asthmatics than in those of healthy controls, and they further increase after allergen challenge [165-167]. The main role of DCs in asthma is to act as potent APCs and promote a $T_H2$ response; however, they might also be important in T cell recruitment to the airways [160]. DCs can be activated directly by allergens or indirectly through mediators secreted by lung epithelial cells, and once activated they travel to the lymph nodes where they stimulate $T_H2$ or $T_H17$ responses [168]. In asthma, DCs produce high amounts of prostaglandin E2 (PGE2), which inhibits their ability to synthesize IL-12 and induces the production of CCL17 and CCL22 [160]. These two chemokines are responsible for the recruitment of CD4+ $T_H2$ lymphocytes to the airways, where they get

re-activated by DCs [168]. The persistent stimulation of CD4+ $T_H2$ cells by DCs is believed to contribute to the chronic inflammation observed in asthmatics [168].

***T lymphocytes***

Asthmatics have increased numbers of CD4+ $T_H2$ cells in their airways that express IL-4, IL-5 and IL-13 [169]. CD4+ $T_H2$ cells are believed to be able to initiate and perpetuate the disease, through the synthesis of these key pro-inflammatory cytokines. For example, IL-4 is important for B cell switching to IgE production, MC development, and eosinophil and basophil activation, while IL-5 is essential for airway eosinophilia [162, 170]. IL-13, the other major $T_H2$ cytokine, also induces IgE synthesis, MC development, and eosinophilia, as well as AHR and mucus hypersecretion [170]. Asthma is more complex and heterogeneous than previously appreciated and different T cell subsets other than $T_H2$ cells have been identified as players in the pathophysiology of the disease [162].

Increasing evidence suggests that CD4+ $T_H17$ cells play an important role in allergic inflammation. The combination of transforming growth factor (TGF)-β and IL-6, two cytokines that have been associated with severe asthma, promotes the differentiation of $T_H17$ cells [159, 163]. IL-17, one of the major products of these cells, induces airway neutrophilia and stimulates the production of pro-inflammatory cytokines by fibroblast, macrophages, epithelial and endothelial cells [170]. Interestingly, IL-17 is increased in the sputum, BAL and sera of asthmatic patients, and its expression correlates with asthma severity [162, 171].

Other subsets of T lymphocytes that are believed to take part in the induction and modulation of the allergic immune response include CD8+ T cells, γδ T cells, and natural killer T (NKT) cells. Bronchial biopsies of asthmatics contain CD8+ T cells that produce

IL-4 and IL-5 [170]. Furthermore, results from studies in animal models of asthma suggest that CD8+ T cells induce AHR and eosinophilia, worsening asthma symptoms [162]. Similarly, γδ T cells are overrepresented in BAL from asthmatics; however, mouse data has shown that two classes of γδ T cells exist, one that expresses IL-5 and IL-13 and promote AHR, and a second one that produces IL-17A and has opposite effects [162, 170]. Finally, in chronic inflammatory lung disease, NKT cells produce IL-13 and induce the secretion of this $T_H2$ cytokine from macrophages [172]. These cells also synthesize IFN-γ and promote IgE production, AHR and eosinophilia [162].

***Mast Cells and Basophils***

MCs are involved in both, the early and late phase asthmatic reaction. They originate in the bone marrow where they start their differentiation and proliferation in response to several cytokines including stem cell factor (SCF), IL-3, IL-4, IL-9 and IL-10 [173]. Normally, upon stimulation MC progenitors leave the bone marrow and settle in different tissues, including the lamina propria adjacent to airway blood vessels [159, 173]. However, asthmatics have additionally elevated numbers of MCs in the bronchial epithelium and in close proximity to ASM bundles and mucous glands [159]. Furthermore, there is a greater accumulation and activation of MCs in the bronchi of allergic asthmatics when compared to non-atopic asthmatics [173]. As previously stated, the binding of allergen to IgE–FcεRI complexes on mast cells causes their degranulation, which amplifies the allergic response [161]. In addition to producing histamine, proteases, leukotrienes, and prostaglandins, MCs also synthesize TNF-α, IL-4, IL-5, IL-6, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [173]. Studies have shown that MCs are crucial players in airway remodeling, inducing mucus hypersecretion, ASM hypertrophy and basement membrane thickening [173].

Basophils, another type of granule-containing cells, are present in large numbers in the bronchi of patients with allergic and fatal asthma [174]. Even though basophils represent a different cell lineage, they share many morphological and functional characteristics with MCs, including the expression of FcεRI and the production of similar effector molecules [174]. However, recent studies have suggested that basophils are more than effector cells and in fact they might act in the early phase of inflammation as APCs, inducing $T_H2$ cell differentiation in response to allergens [175].

***Eosinophils***

Eosinophils are considered central effector cells in the late phase of the asthmatic reaction. Usually eosinophils fully differentiate in the bone marrow; however, there is some evidence that these cells can also undergo local differentiation at inflammatory sites [159]. Increased numbers of eosinophils are observed in peripheral blood, bronchial mucosa, BAL and sputum of asthmatics and have been linked to disease severity [159, 176]. Several $T_H2$ cytokines like IL-3, GM-CSF but particularly IL-5, promote maturation, differentiation, survival and activation of eosinophils, leading to airway accumulation of these cells [159]. In addition to perpetuating airway inflammation through the secretion of pro-inflammatory cytokines, chemokines, and oxygen radicals, eosinophils might also be directly involved in airway remodeling due to their ability to synthesize fibrogenic cytokines such as TGF-β, IL-11, IL-17, and IL-25 [163]. Furthermore, they release cytotoxic proteins, such as eosinophil cationic protein, eosinophil peroxidase, and major basic protein, which have been found elevated in the sputum of asthmatics and are associated with the epithelial damage observed in these patients [159]. A number of studies have demonstrated that asthmatics with eosinophilia

have increased thickness of the subepithelial membrane zone when compared to non-eosinophilic asthmatics and healthy individuals [176].

***Neutrophils***

Neutrophils, cells of the innate immune system that are the first line of defense against pathogens, have also been implicated in asthma. They have been proposed as important players in the disease due to their ability to produce cytokines, lipid mediators, reactive oxygen species and proteases [159]. Patients with mild or moderate asthma tend to have normal numbers of neutrophils in BAL and induced sputum [176]. However, neutrophilia has been found during asthma exacerbations, in chronic severe asthma, sudden-onset fatal asthma, nocturnal asthma, and in non-eosinophilic asthmatics that respond poorly to GC treatment [159, 177]. The expression of IL-8, a major chemotactic factor for neutrophils, is elevated in BAL, sputum and tracheal suction of neutrophilic asthmatics [176, 178]. Furthermore, these patients have blood neutrophils that secrete more IL-8 *in vitro* and exhibit systemic inflammation with increased levels of C-reactive protein and IL-6, which are associated with worse clinical outcomes [178]. Interestingly, systemic GC treatment has been linked to increased neutrophilia, possibly by an inhibition of apoptosis [176, 179]. Finally, the observation that neutrophils remain elevated during the recovery period from an acute asthma exacerbation and a near fatal asthma episode, has led some researchers to propose that neutrophils might be not only important in the initiation but also in the resolution of the attack [176].

***Macrophages***

Even though macrophages are known to be important mediators in chronic inflammation, their exact role in asthma is still not completely understood. Pulmonary macrophages can induce ASM contraction and might play a role in AHR through the

production of histamine and by modifying cholinergic and β-adrenergic responses [97]. Also, macrophages in BAL of severe asthmatics have a dysfunctional activation and defective phagocytosis of apoptotic cells, as well as increased expression of IL-13 [172, 180]. Circulating monocytes differentiate into macrophages that can be divided into "classically activated" (M1), which are induced by IFN-γ and lipopolysaccharide (LPS), or "alternatively activated" (M2), induced by IL-4 and IL-13 [97]. Due to the high levels of $T_H2$ cytokines in asthma it is not surprising that M2 cell numbers are increased in BAL and airway wall tissue of asthmatics [172, 181]. M2 cells produce chemokines and other pro-inflammatory mediators that could be involved in exacerbations and remodeling, such as matrix metalloproteinase (MMP)-12 and chitinase 3-like 1 (CHI3L1), both of which have been linked to asthma and disease severity [97]. On the other hand, Goleva et al. showed that BAL macrophages of SR asthmatics exhibit an M1 expression profile, including elevated production of IL-6, IL-8, and TNF-α [182]. In the lung also exists a population of regulatory macrophages that expresses high levels of IL-10, an anti-inflammatory cytokine that inhibits lung eosinophilia, IgE synthesis, APC function as well as superoxide and NO release from inflammatory cells [159]. Interestingly, pulmonary macrophages from asthmatics synthesize less IL-10 than those of healthy individuals [22].

***Structural cells***

In asthma, pulmonary epithelial and endothelial cells, ASM cells, and fibroblasts are not only target but also effector cells. The lung epithelium of asthmatics in addition to exhibiting alterations such as shedding, loss of ciliated cells, goblet cell hyperplasia and disrupted tight junctions, produces high levels of cytokines, chemokines, and growth factors [163]. These factors include IL-16, CCL5, CCL11, CCL13 and CCL17, all of

which promote the recruitment and activation of T cells, eosinophils and basophils [183]. Furthermore, it has been suggested that epithelial cells could also play a role in the recruitment and maturation of DCs by secreting CCL2, CCL20, β-defensins, IL-1, GM-CSF and thymic stromal lymphopoietin (TSLP) [168]. Similarly, endothelial cells from bronchial biopsies of asthmatic patients produce significant amounts of chemokines that bring inflammatory cells to the airways, such as CCL5, CCL7, CCL11, CCL13, and CCL24 [183].

Asthmatic bronchi exhibit ASM hyperplasia and hypertrophy, which constitute the main cause of airway obstruction and contribute to AHR [163]. ASM cells express TLRs and receptors for cytokines and chemokines as well as cellular adhesion molecules that might play a role in regulating their interactions with inflammatory cells [163]. Moreover, the stimulation of ASM cells by IL-1β, TNF-α and IFN-γ causes the production of mediators that contribute to additional airway inflammation [183]. ASM cells also produce the profibrotic cytokine IL-11 and IL-6, which could induce ASM remodeling, mucus hypersecretion and IgE production [183].

Finally, during the inflammatory process lung fibroblasts are activated and secrete a large array of pro-inflammatory factors, as well as collagens I, III and V, the proteoglycans lumican and biglycan and the glycoproteins fibronectin and tenascin [163]. Additionally, myofibroblasts might be active players in the development of lung eosinophilia by synthesizing GM-CSF and SCF, which promote the differentiation, activation and survival of these inflammatory cells [183].

### 1.4.2 Glucocorticoids in Asthma

Inhaled corticosteroids are potent anti-inflammatory drugs and are the most effective medication in the treatment of asthma. The lipophilic inhaled GCs readily

diffuse into airway cells and act through molecular mechanisms previously described (see section 1.1.1 Glucocorticoid Signaling Pathway). Even though they do not cure the disease, they improve quality of life, lung function, and reduce overall asthma symptoms, as well as airway inflammation, exacerbations, and AHR [155, 184]. More importantly, regular use of inhaled GCs reduces hospitalizations by up to 80% and decrease asthma mortality [184, 185].

GCs can have direct inhibitory effects on cells involved in asthmatic inflammation. They significantly reduce the numbers of T cells, eosinophils, DCs and MCs in the airways of asthmatic patients [165, 186]. It is believed that this effect is a result of induced apoptosis as well as synthesis inhibition of chemotactic factors by epithelial and inflammatory cells [160, 187]. GCs also reduce alveolar macrophage expression of pro-inflammatory mediators while inducing IL-10 production [22]. Similarly, they inhibit T lymphocyte activation and cytokine synthesis but enhance $T_{Reg}$ differentiation and function [162, 170]. Furthermore, GCs reduce endothelial cell leakage and goblet cell mucus secretion [187].

Overall, inhaled GCs have proven to be a very effective medication in the control of asthma. However, as mentioned before, a significant proportion of asthmatics do not completely respond to this treatment or require higher GC doses. This is particularly worrisome since the use of high doses of corticosteroids could have systemic side effects, including reduction in growth velocity and bone turnover, diminished adrenal cortical function, as well as easy bruising, cataracts and glaucoma [155, 188]. Additionally, patients receiving normally prescribed doses could also show local adverse effects such as oral candidiasis, dysphonia, and hoarseness. In fact, it has been reported that more than

30% of asthmatics on inhaled GCs experience some kind of side effect, which significantly affects treatment adherence [189].

Several molecular mechanisms are believed to regulate steroid sensitivity and resistance in asthma and other inflammatory diseases (see section 1.1.4 Mechanisms of Glucocorticoid Resistance). A better understanding of these mechanisms and development of alternative therapies could be extremely useful, particularly for severe asthmatics, which tend to be less GC responsive.

### 1.4.3 Vitamin D in Asthma

The recognition of VitD's immunomodulatory role led several researchers suggest a possible involvement of this secosteroid in asthma. Gene association studies have linked genetic variants in VDR as well as in genes involved in the VitD pathway with asthma and atopy [190-192]. However, others have not reproduced these results in a different population [193, 194]. Furthermore, no consensus has been reached about the beneficial or detrimental effects of VitD on the asthmatic phenotype. While some claim that VitD deficiency is to blame for the asthma pandemic, others have suggested that VitD supplementation and food fortification, especially in industrialized countries, is responsible for the increase in asthma prevalence [112, 195]. Animal and human studies have produced conflicting results, and have given support to both sides of the controversy. For example, in an asthma mouse model, VitD inhibits airway inflammation by decreasing IL-4 production and affecting T cell homing [196]. Similarly, UVB irradiation, which should elevate systemic VitD levels, protects antigen-sensitized mice from allergic airway disease by decreasing inflammation and AHR [197]. On the other hand, Wittke and colleagues demonstrated that mice lacking VDR do not develop AHR and airway inflammation in spite of exhibiting elevated levels of $T_H2$ cytokines and IgE

[198]. To make matters more complex, experiments from the same lab show that VitD-deficient and *Vdr* knock-out mice have different phenotypes and VitD supplementation does not lead to more severe disease [198, 199].

The important role of VitD in the development of the lung and immune system has prompted several groups to investigate the correlation between VitD levels during pregnancy or early childhood and risk of developing asthma. These studies have also produced inconsistent results, probably due to differences in study design, population sizes and the evaluation of VitD status. In a Finnish cohort study, VitD supplementation during the first year of life was associated with increased risk of atopy, allergic rhinitis, and asthma during adulthood [200]. Nevertheless, that study lacked direct measurements of VitD levels in both mothers and children [200]. In another study, high circulating VitD levels in pregnant mothers was associated with increased risk to develop eczema and asthma at 9 years of age; however, the study had inadequate follow-up of children, losing a great proportion of them during the progression of the study [201]. Also, Camargo et al. showed that even though cord-blood VitD levels inversely correlated with risk for respiratory infections and wheezing, they did not associate with incident asthma [202]. On the other hand, several interventional studies with large cohort sizes have shown a protective effect of high maternal VitD intake on childhood wheezing, allergic rhinitis and asthma [203-206]. Recently it was shown in an observational study that children from an Australian cohort, especially boys, with low VitD levels were at increased risk of developing atopy, AHR, and asthma [207]. Interestingly, it was also found that insufficient VitD levels at 6 years of age were predictors of the development of this respiratory disease later in life [207].

More evidence for a beneficial role of VitD in asthma comes from a case-control study in which a positive correlation was found between VitD insufficiency or deficiency and asthma in African American children and young adults [208]. In other studies, authors have examined the effects of VitD on disease morbidity and response to treatment. Chinellato and coworkers reported that children with well-controlled asthma had higher serum VitD levels than children with partially controlled or non-controlled disease [209]. Low VitD levels in asthmatics have also been associated with decreased lung function, increased AHR and corticosteroid use, as well as decreased responsiveness to GCs *in vitro* [210, 211]. Similarly, in a Costa Rican cohort of asthmatic children, insufficient VitD levels were associated with increased chance of hospitalization for asthma, elevated total IgE and eosinophil counts, as well as increased use of anti-inflammatory drugs and AHR [212]. Moreover, serum VitD levels inversely correlated with severe asthma exacerbations in a similar study in North American children with mild-to-moderate disease [213]. Interestingly, the observed beneficial effects of VitD were greater among children who received inhaled GCs, which is consistent with a role of VitD on enhancing GC responsiveness [213]. It has been suggested that VitD exerts this positive effect by increasing the GC-induced secretion of IL-10 from $T_{Regs}$ [102].

Finally, further evidence of beneficial effects of VitD comes from a clinical study in which VitD supplementation of Polish asthmatic children significantly prevented exacerbations triggered by acute respiratory tract infections [214]. Although not always consistent, the overall results from the different studies suggest a positive effect of VitD on asthma and disease morbidity, probably by enhancing GC responsiveness and preventing infections. However, more experimental work needs to be done to confirm these findings and to put an end to the VitD controversy.

### 1.4.4 Animal Models of Asthma

The use of experimental models has proven effective in unveiling some of the molecular mechanisms that govern the pathophysiology of complex diseases such as asthma. Even though animals, with the exception of cats and horses, do not naturally develop an asthma-like condition, $T_H2$ airway inflammation has been experimentally induced in many species including guinea pigs, mice, rats, sheep, dogs and rabbits [215]. Since asthma is multifaceted, no single animal model features all the characteristics of the disease. Mice and rats have been by far the most widely used because they are relatively inexpensive to maintain and it is feasible to assess their airway pulmonary function [215, 216]. Interestingly, studies in these animals have demonstrated the existence of innate strain variations in the susceptibility to develop the different asthmatic features [215, 217, 218].

Models of allergic asthma are generated by first sensitizing the animals via an intraperitoneal (IP) injection of an antigen, usually ovalbumin (OVA), along with an adjuvant like aluminum hydroxide. The animals are then given time for their immune system to react and are subsequently challenged with the antigen either by aerosol or nasal instillation. Even though there is strain variability in the outcome measures, generally the antigen sensitization/challenge results in lung inflammation consisting of eosinophilia, epithelial wall thickening and AHR [215].

Two mouse strains commonly used in the study of asthma are the Balb/c and C57BL/6. Even though there are some discrepancies in the literature about the exact response of these two strains to OVA sensitization/challenge, it is widely known that the Balb/c and C57BL/6 strains show very distinct phenotypes. For example, the Balb/c strain develops more severe features of the disease following OVA challenge, including

increased Ig production, AHR correlated to IL-4 synthesis, and accumulation of eosinophils in the peribronchial and peripheral lung [217, 218]. In contrast, OVA challenge in C57BL/6 mice in spite of causing BAL eosinophilia, only leads to very modest increases in serum Ig and AHR [217, 218]. Furthermore, in these mice eosinophils primarily localize to the peripheral lung tissue and are absent from the airways [217]. Due to these phenotypic differences, the Balb/c and C57BL/6 asthma mouse models were used in this thesis to identify molecular mechanisms responsible for variability in GC responsiveness.

Rats are also commonly employed in the study of asthma and as in mice there are marked phenotypic differences between strains. The naturally atopic Brown Norway (BN) rat produces very high IgE levels following allergen sensitization, and after allergen challenge develops an early and late allergic response consisting of T-cell mediated eosinophilic airway inflammation and AHR [216]. Other strains of rat used are the innately hyperresponsive Fisher, which possesses more ASM with greater contractile properties, and the Lewis, which is usually used as a control [216]. Interestingly, it was recently shown by the Kaplan group that these rat models have distinct developmental respiratory phenotypes, which are associated with highly diverse transcriptome signatures [219]. In this thesis, I used airway epithelial cells derived from PN14 rats to study GC responsive traits during development and to expand our knowledge of the modulatory role of VitD on GC responsiveness during this time period.

### 1.4.5 The Childhood Asthma Management Program

Several asthma cohort studies have been carried out in order to better understand the epidemiology and pathophysiology of the disease. Since asthma is a major disorder in children and it is believed to affect lung growth and function in the long-term, many

cohort studies have been focused in the pediatric form of the disease. In the early 1990s the National Heart, Lung, and Blood Institute established the Childhood Asthma Management Program (CAMP), a randomized, double blind clinical trial testing for the safety and efficacy of long-term use of anti-inflammatory drugs in the treatment of asthma [220]. Children 5-12 years of age with mild-to-moderate asthma were recruited and received either an inhaled GC (budesonide), a non-steroidal anti-inflammatory drug (nedocromil), or placebo twice daily [220]. All patients used an inhaled short-acting β-agonist bronchodilator (albuterol) to control asthma symptoms and were treated for five to six years. Over this time period, children were monitored to determine how the three treatments affected pulmonary function, physical and psychological growth, as well as morbidity and mortality [220].

Results showed that there were no significant differences between the three groups in terms of lung growth and change in forced expiratory volume in one second ($FEV_1$) after bronchodilator administration [184]. However, children receiving budesonide had lower AHR to methacholine (Mch), fewer hospitalizations and emergency visits, and less use of albuterol. Furthermore, the side effects of long-term use of this inhaled GC were very limited and consisted mainly of a small, transient decrease in growth velocity [184].

Also, during the clinical visits, blood samples were obtained from these patients and among other things, lymphoblasts were isolated and immortalised for later use in cell culture experiments. These cells have been used in this thesis to identify molecular differences between asthmatics that show good or poor response to GC treatment and to elucidate the effects of VitD on innate GC responsiveness.

## 1.5 Thesis Rationale and Aims

It is known that organisms, including humans, exhibit a level of plasticity during their developmental period and alterations of the normal developmental program can lead to permanent changes in organ structure and function [2]. Several studies have suggested that early life events having disruptive effects in lung organogenesis could pre-dispose to the manifestation of asthma and other respiratory diseases later in life [1]. As indicated in this introduction, GCs play a crucial role in the development of a functional lung. Clinical and experimental evidence have demonstrated that steroid responsiveness in the context of asthma and inflammation is highly variable; however, little is known about how inter-individual differences in GC response affect the course of normal lung development. VitD, a secosteroid involved in the development of the immune and respiratory systems, acts during the same time window as GC and has been recently proposed as a modulator for steroid action in asthma.

The objective of the present thesis is to study mechanisms of steroid responsiveness in the processes of lung development and asthma. A combination of molecular and cell culture techniques, as well as animal models and immortalized cell lines derived from asthmatic children with different steroid responsive phenotypes, were used to determine molecular differences that controlled GC responsiveness. Furthermore, experiments were conducted to try to clarify the interaction between GC and VitD and the effects of this latter agent on GC function.

The specific objectives in this thesis are:

1. To investigate GC response in two inbred mouse strains with different asthma susceptibility phenotypes.

2. To investigate mechanisms of GC responsiveness in lymphoblast cell lines established from asthmatic children in the Childhood Asthma Management Program (CAMP).

3. To investigate GC response in epithelial cells of the developing lung in normoresponsive and atopic rat models.

**Figure 1.1 Schematic of genomic actions of the glucocorticoid receptor.** In the absence of GC, GR is sequestered in the cytoplasm by chaperone proteins, including HSP90. GC binding to GR leads to the dissociation of these proteins, causing the activation of the receptor. Importins (IPO) then transport the activated GR into the nucleus through the nuclear pore complex (NPC). In the nucleus, GR affects gene expression by cis-regulation (A-D), which involves direct binding to positive (GRE) or negative (nGRE) response elements, or by trans-regulation (E-F), which requires interaction with other transcription factors (TFs). PI-TF = pro-inflammatory TF; TFRE = TF response element.

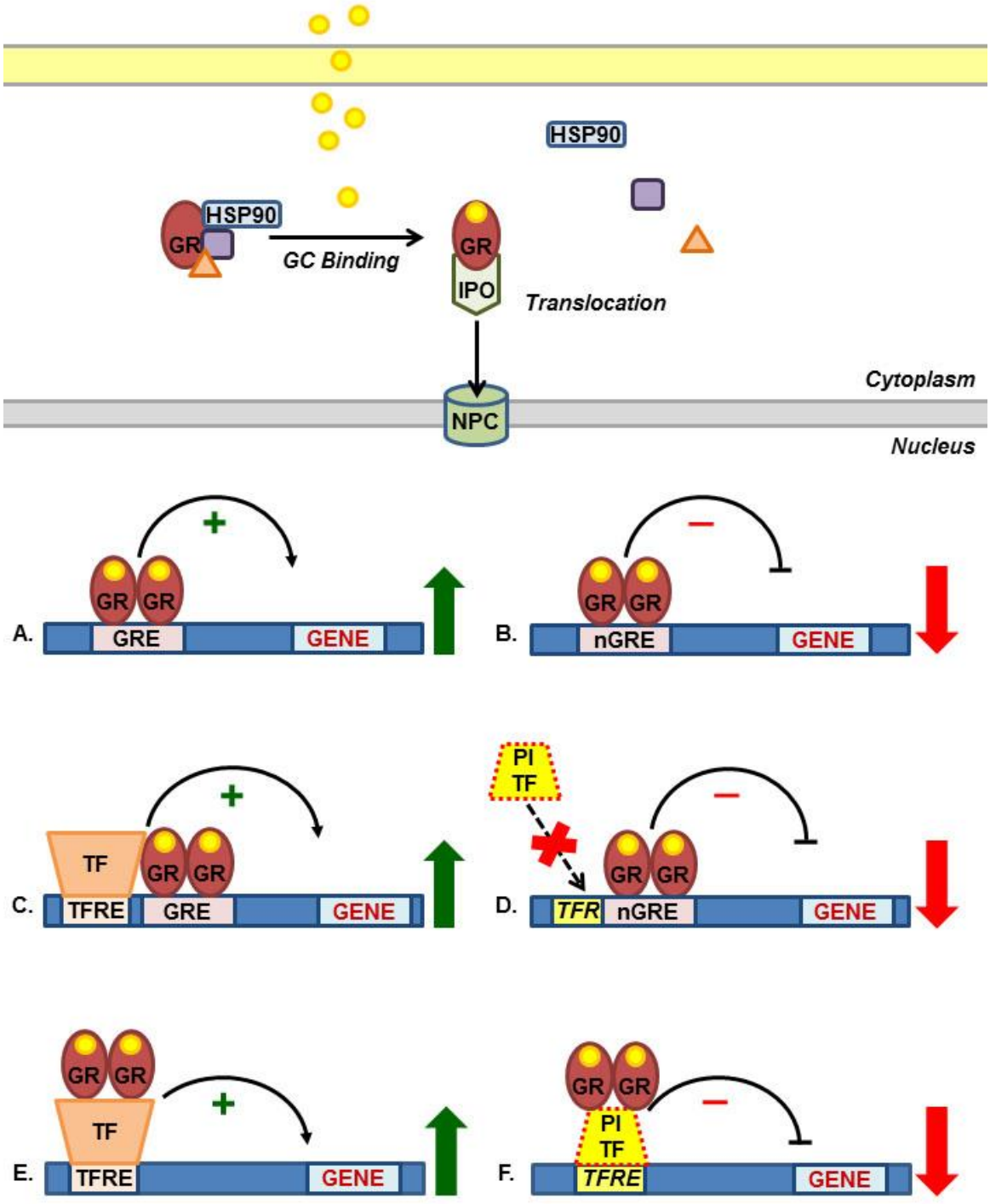

HSP90
HSP90
GR
GC Binding
GR
IPO
Translocation
Cytoplasm
NPC
Nucleus
A.
GR GR
GRE
GENE
B.
GR GR
nGRE
GENE
C.
TF
GR GR
TFRE
GRE
GENE
D.
PI
TF
GR GR
TFR
nGRE
GENE
E.
GR GR
TF
TFRE
GENE
F.
GR GR
PI
TF
TFRE
GENE

**Figure 1.2 Glucocorticoid receptor mRNA splice variants.** Alternative splicing of the GR primary transcript can generate at least five different GR isoforms. GRα and GRβ contain both exons 1-8 and either exon 9α or 9β. GRγ has an additional arginine in the DBD region as a result of the use of an alternative splice donor site. GR-A lacks exons 5-7 and failure to splice out the intron between exons 7 and 8 generates GR-P. Adapted from Oakley and Cidlowski, 2011 [13].

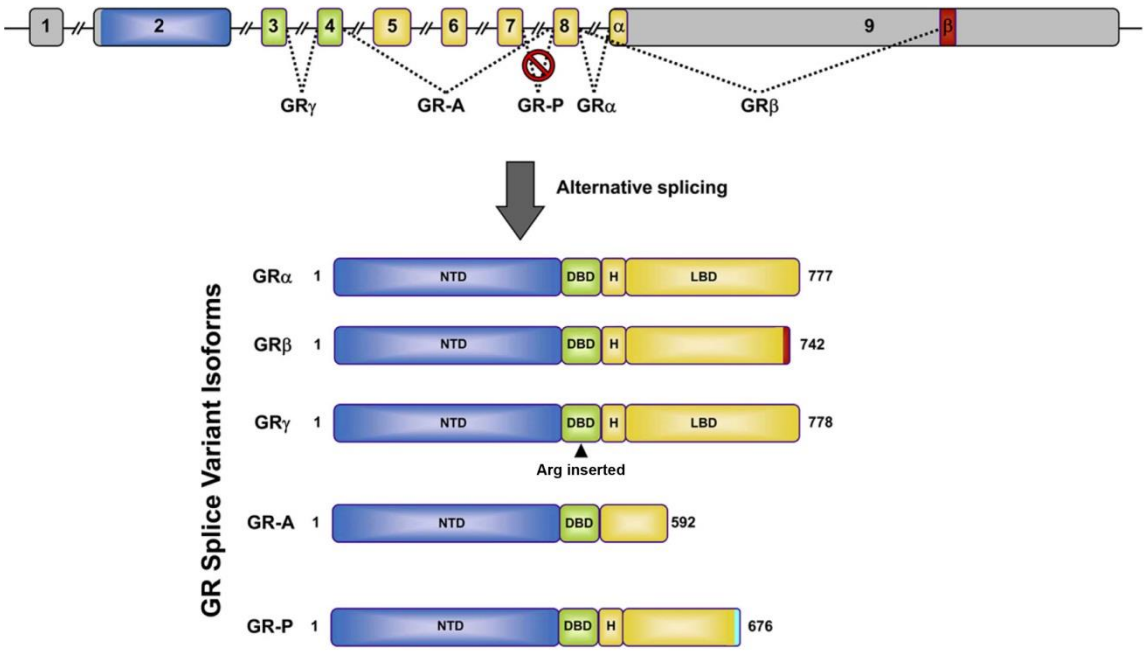

1
2
3
4
5
6
7
8
α
9
β
GRγ
GR-A
GR-P
GRα
GRβ
Alternative splicing
GR Splice Variant Isoforms
GRα 1 NTD DBD H LBD 777
GRβ 1 NTD DBD H 742
GRγ 1 NTD DBD H LBD 778
Arg inserted
GR-A 1 NTD DBD 592
GR-P 1 NTD DBD H 676

**Figure 1.3 Structure of the human glucocorticoid receptor protein.** GR contains an NTD, DBD, a small flexible hinge (H) region and a C-terminal LBD. Red bars indicate areas involved in transcriptional activation (AF-1 and AF-2), dimerization, nuclear translocation (NLS), and binding of chaperone proteins, coactivators and ligand.

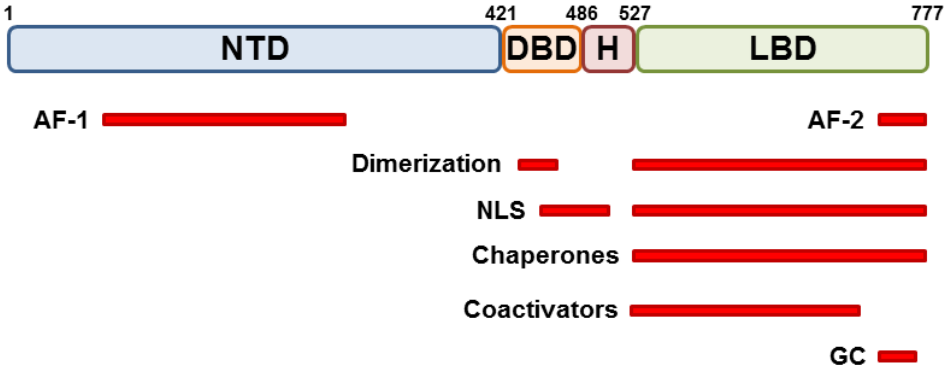

1
421
486
527
777
NTD
DBD
H
LBD
AF-1
AF-2
Dimerization
NLS
Chaperones
Coactivators
GC

**Figure 1.4 GRα translational isoforms and post-translational modification sites.** Alternative translation initiation from different AUG codons (indicated by asterisks) generates eight GRα isoforms. Residues that are sites for phosphorylation (P), sumoylation (S), ubiquitination (U), and acetylation (A) are indicated. Adapted from Oakley and Cidlowski, 2011 [13].

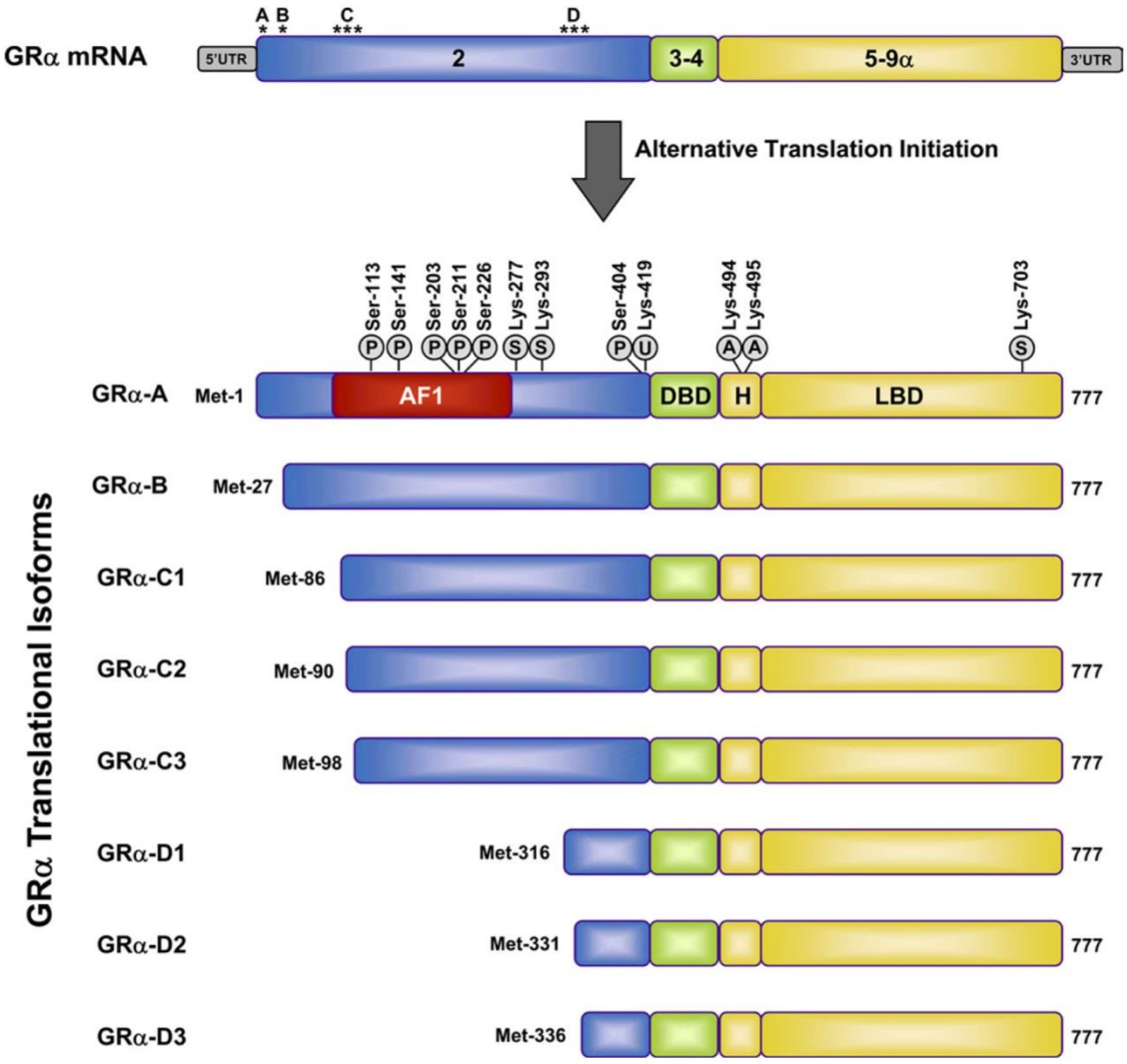

GRα mRNA
A B C D
5'UTR
2
3-4
5-9α
3'UTR
Alternative Translation Initiation
Ser-113 Ser-141 Ser-203 Ser-211 Ser-226 Lys-277 Lys-293 Ser-404 Lys-419 Lys-494 Lys-495 Lys-703
P P P P P S S P U A A S
GRα-A
Met-1
AF1
DBD
H
LBD
777
GRα-B
Met-27
GRα-C1
Met-86
GRα-C2
Met-90
GRα-C3
Met-98
GRα-D1
Met-316
GRα-D2
Met-331
GRα-D3
Met-336
GRα Translational Isoforms

**Figure 1.5 Schematic of vitamin D metabolism.** Vitamin D ($VD_3$) is produced in the skin or ingested in the diet. To become hormonally active it normally needs to be hydroxylated twice, first in the liver by vitamin D-25-hydroxylase (CYP27A1) and a second time in the kidneys by 1α-OHase (CYP27B1). However, immune cells, including macrophages, DCs, T and B cells also express these enzymes and provide an alternative mechanism of activation. In these cells vitamin D acts in an autocrine or paracrine fashion by binding to the vitamin D receptor (VDR) and regulating the expression of vitamin D responsive genes. Finally, vitamin D hydroxylation by CYP24A1 produces calcitroic acid, which is excreted in the bile. Adapted from Mora et al. 2008 [106].

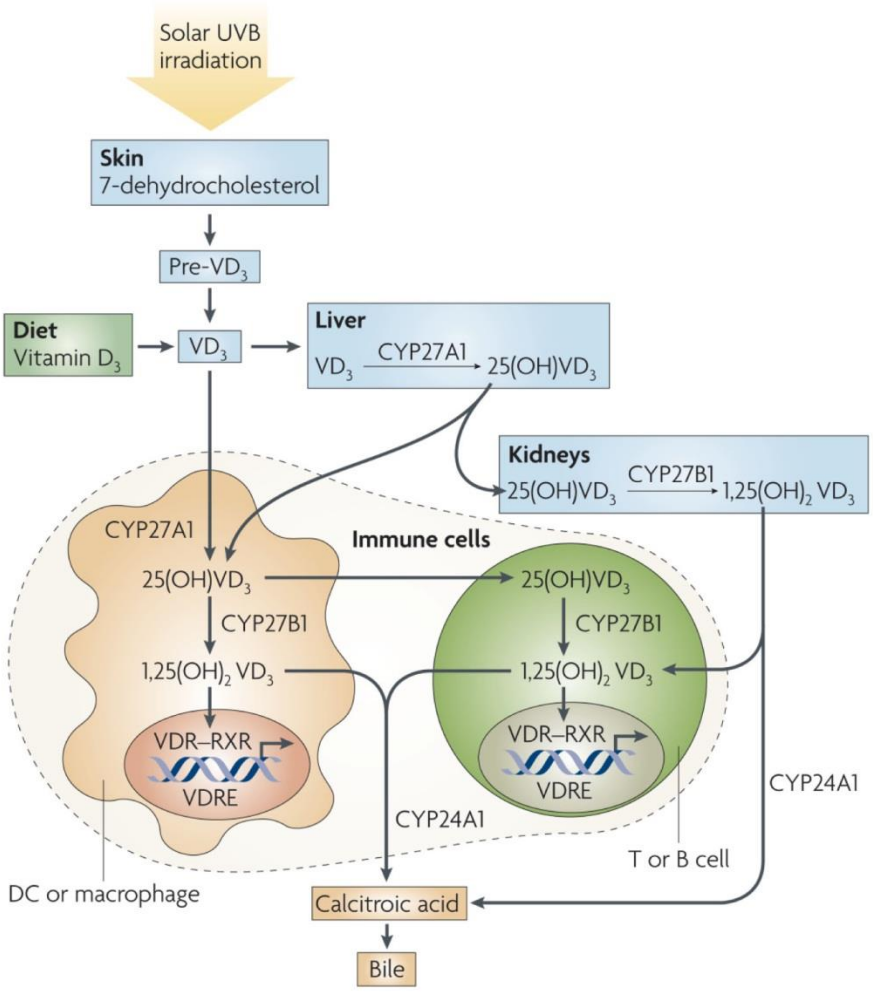

Solar UVB irradiation
Skin
7-dehydrocholesterol
Pre-VD3
Diet
Vitamin D3
VD3
Liver
VD3 CYP27A1 25(OH)VD3
Kidneys
25(OH)VD3 CYP27B1 1,25(OH)2 VD3
Immune cells
CYP27A1
25(OH)VD3
CYP27B1
1,25(OH)2 VD3
VDR–RXR
VDRE
25(OH)VD3
CYP27B1
1,25(OH)2 VD3
VDR–RXR
VDRE
CYP24A1
CYP24A1
DC or macrophage
T or B cell
Calcitroic acid
Bile

**Figure 1.6 Cartoon depicting the airways during the different stages of lung development.** Lung organogenesis starts with the appearance of a lung bud during the embryonic stage. This is followed by the formation of major bronchi and the division of the tracheal-esophageal tube. The primitive airways continue dividing dichotomously during the pseudoglandular and canalicular stages, giving rise to the respiratory bronchioles and alveolar ducts. The saccular stage is characterized by a dramatic expansion of the airspaces, the formation of alveolar sacs and a clear differentiation of the epithelium into alveolar type I and type II cells. During the alveolar stage alveolar sacs are subdivided forming a very efficient unit for gas exchange. E = embryonic day; H = humans; PN = postnatal day; R = rats; W = weeks. Adapted from Hislop 2005 [119].

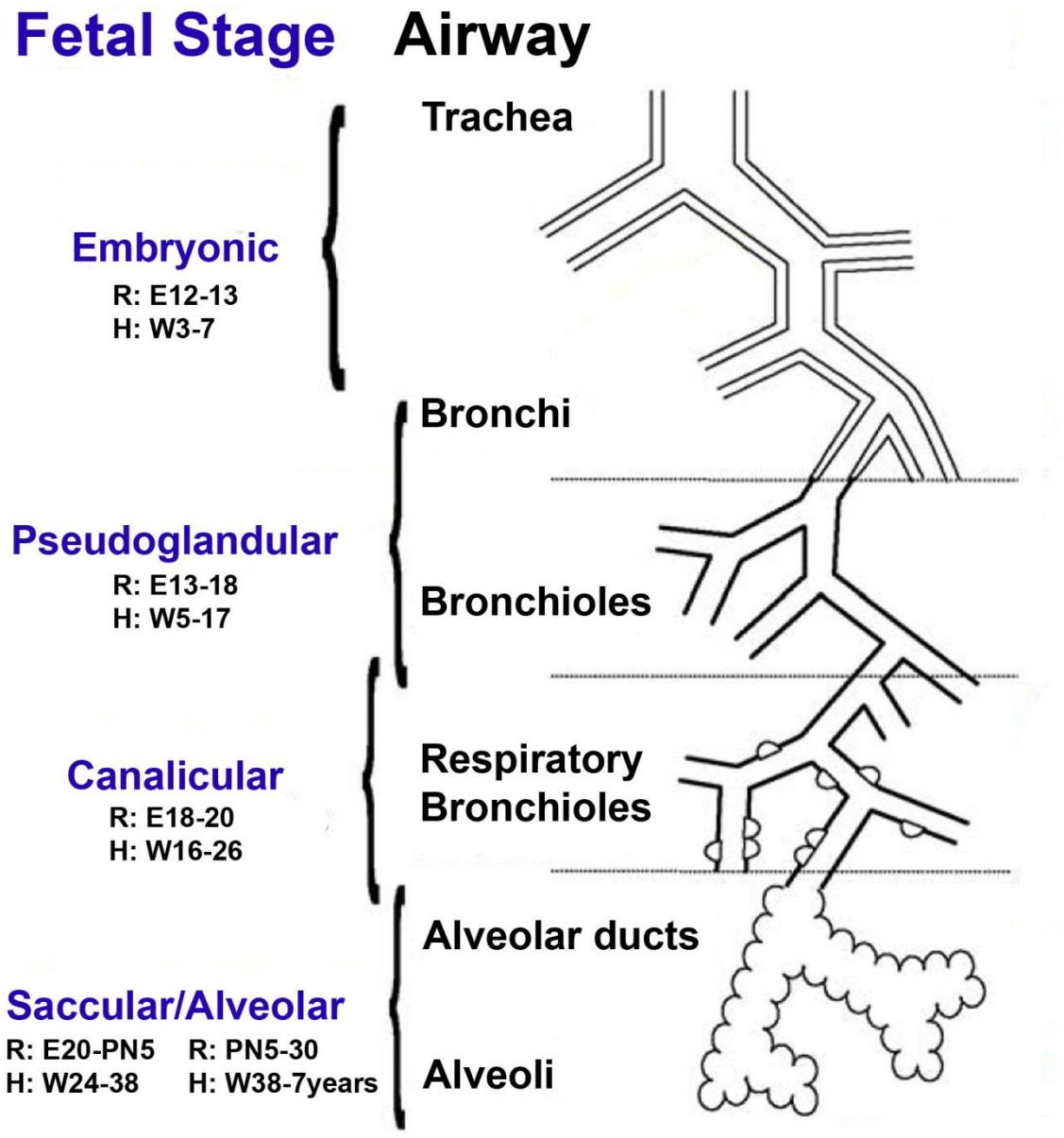

Fetal Stage
Airway
Trachea
Embryonic
R: E12-13
H: W3-7
Bronchi
Pseudoglandular
R: E13-18
H: W5-17
Bronchioles
Canalicular
R: E18-20
H: W16-26
Respiratory Bronchioles
Alveolar ducts
Saccular/Alveolar
R: E20-PN5
H: W24-38
R: PN5-30
H: W38-7years
Alveoli

**Figure 1.7 Cartoon illustrating the possible effects of vitamin D deficiency on lung development.** Vitamin D deficiency may impact respiratory and immune development increasing asthma risk both directly via gene-environment interactions with developmentally regulated genes or indirectly via the inflammatory milieu of the fetus in children born to mothers predisposed to asthma.

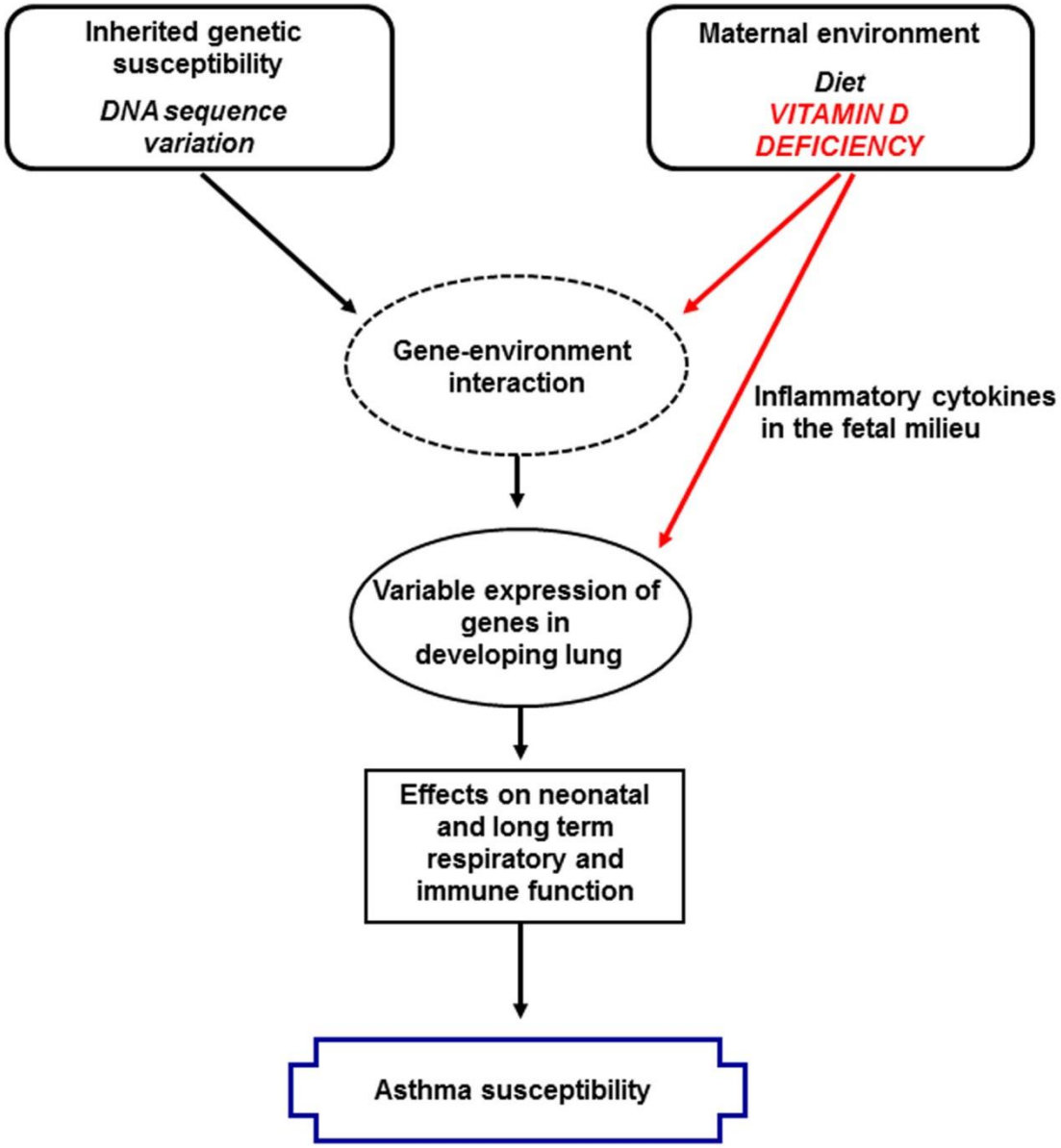

Inherited genetic susceptibility
DNA sequence variation
Maternal environment
Diet
VITAMIN D DEFICIENCY
Gene-environment interaction
Inflammatory cytokines in the fetal milieu
Variable expression of genes in developing lung
Effects on neonatal and long term respiratory and immune function
Asthma susceptibility

**Figure 1.8 Schematic of the pathogenesis of asthma and the pivotal role of $T_H2$ lymphocytes.** In the asthmatic lung, APCs recognize common inhaled antigens and present them to naïve T cells, triggering their activation, differentiation to $T_H2$ and clonal expansion. Subsequent allergen exposure activates $T_H2$ cells and recruits them to the lung, where they produce pro-inflammatory cytokines like IL-4, IL-5 and IL-13. IL-5 plays an important role in lung eosinophilia, while IL-4 and IL-13 induce B cell activation and IgE secretion. Chronic inflammation leads to morphological changes in the airways, which include goblet cell hyperplasia and ASM hypertrophy. Adapted from Bosnjak et al. 2011 [160].

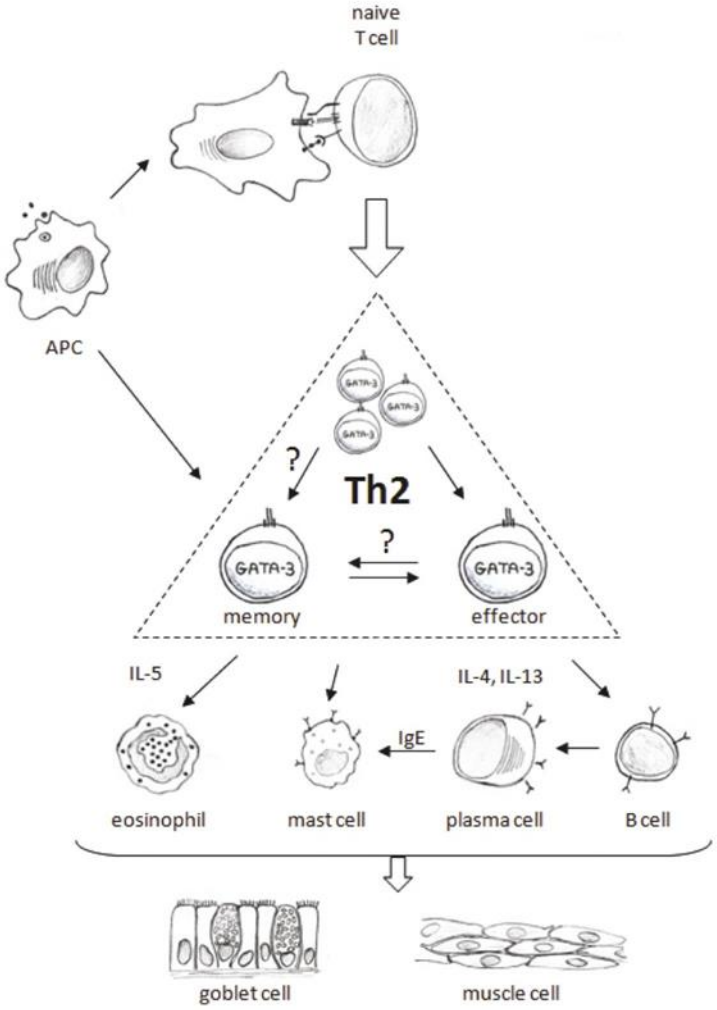
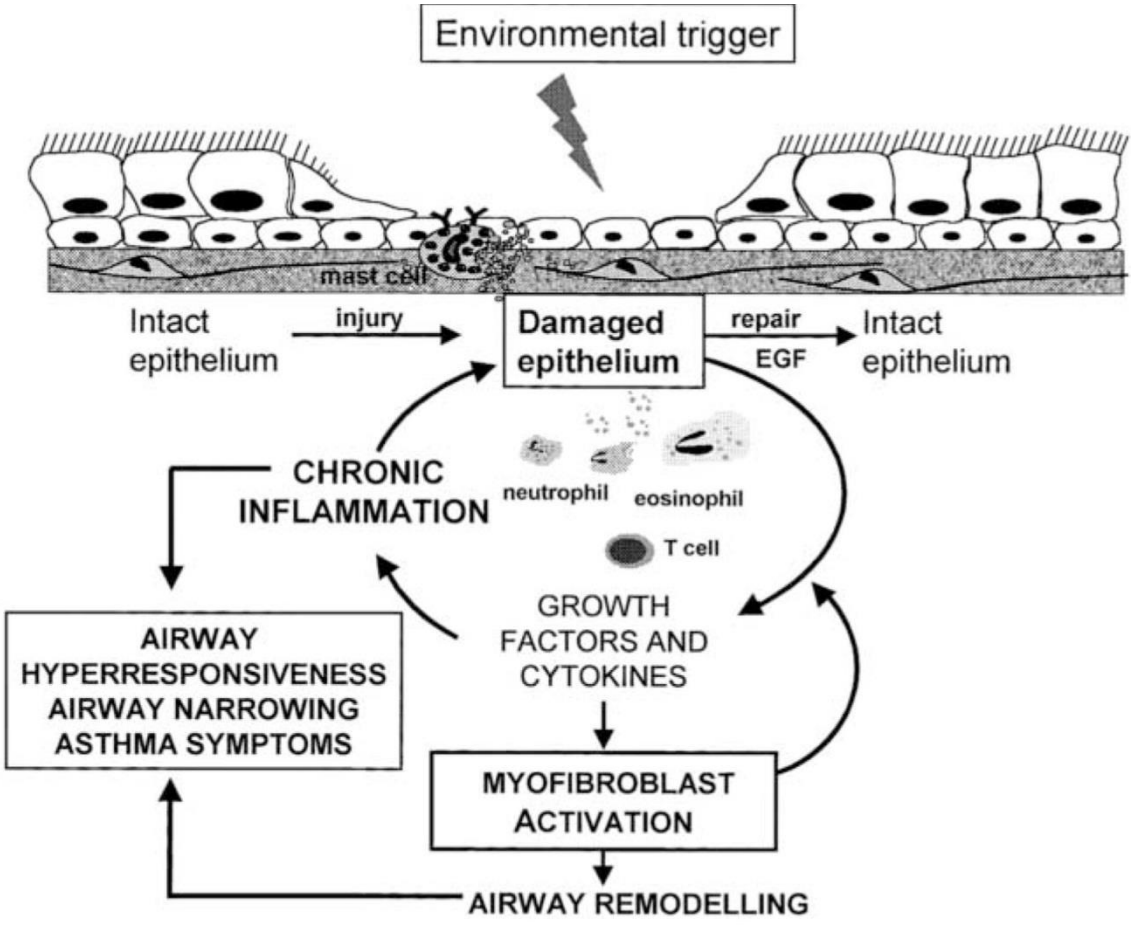

naive
T cell
APC
GATA-3
GATA-3
GATA-3
?
Th2
?
GATA-3
GATA-3
memory
effector
IL-5
IL-4, IL-13
IgE
eosinophil
mast cell
plasma cell
B cell
goblet cell
muscle cell

**Figure 1.9 Model illustrating the activation of the epithelial-mesenchymal interactions in chronic asthma.** Communication between the damaged epithelium and underlying fibroblasts leads to reactivation of developmental growth factors which induce cell proliferation and lung remodeling in asthma. $T_H2$ cytokines, such as IL-4 and IL-13, may augment the remodeling process and help perpetuate chronic inflammation. Adapted from Holgate et al. 2004 [164].


Environmental trigger
mast cell
Intact epithelium
injury
Damaged epithelium
repair
EGF
Intact epithelium
CHRONIC INFLAMMATION
neutrophil
eosinophil
T cell
GROWTH FACTORS AND CYTOKINES
AIRWAY HYPERRESPONSIVENESS
AIRWAY NARROWING
ASTHMA SYMPTOMS
MYOFIBROBLAST ACTIVATION
AIRWAY REMODELLING

*Epidemiological studies demonstrate that in asthmatic patients steroid insensitivity correlates with disease severity [221]. The first objective of this thesis was to investigate molecular mechanisms responsible for variable GC responsiveness in asthma. Two mouse strains with different "asthmatic" phenotypes were used to determine if variability in steroid responsiveness exists in murine models of the disease and experiments were conducted to identify the possible molecular mechanisms responsible for GC insensitivity. The results of these experiments are presented in the following chapter.*

# CHAPTER II

## The Balb/c Mouse Models a Steroid Resistant Asthma Phenotype

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## 2.1 Abstract

A significant number of asthmatics do not respond to inhaled glucocorticoids (GC), the most effective anti-inflammatory therapy. A substantial proportion of variability in GC response is due to genetic factors. Clarification of these genetic factors and of their mechanism of action may be used to predict the probability that a patient will have a beneficial vs. a poor response to GC therapy for asthma. The objective of this study was to investigate steroid responsiveness in two mouse strains with different asthma susceptibility phenotypes.

Following allergen challenge, with or without budesonide intervention, Balb/c and C57BL/6 mice were assessed for airway hyperresponsiveness, inflammatory cytokines, and expression of the GC receptor (GR), phosphorylated-p38 MAP kinase and importin 13 (IPO13, mediates nuclear entry of GR).

Both strains exhibited elevated respiratory resistance, increased expression of inflammatory cytokines and reduced expression of GR and IPO13 following allergen challenge. Budesonide improved pulmonary function, reduced cytokine levels and normalized expression of GR and IPO13 in C57BL/6 but not in Balb/c mice. Allergen induced reduction of nuclear GR and activation of p38 MAPK (inhibits GR function) was suppressed by budesonide in C57BL/6 but not in Balb/c mice.

Balb/c mice, which display features of a severe asthma phenotype following allergen challenge, also show a limited response to steroid intervention and may serve as a model to investigate therapeutic strategies to enhance steroid responsiveness.

## 2.2 Introduction

Inhaled glucocorticoids (GCs) are the most effective and commonly used treatment for the long-term control of asthma. In asthmatic patients, the use of GCs

reduces airway hyperresponsiveness (AHR) and airway inflammation [155]. Overall, GCs effectively reduce asthma symptoms, prevent exacerbations, and decrease asthma-associated mortality [155, 184].

Despite their overall effectiveness, there is considerable interindividual variation in therapeutic response to GCs [222]. Up to one third of asthmatics show little improvement in lung function following GC therapy [222]. Moreover, while some patients do respond to higher doses than normally prescribed, administration of high doses of GCs for prolonged periods can have marked adverse effects [155, 186]. This variation is multifactorial, but it is clear that it has a strong genetic component [223].

GCs exert their effects through the GC receptor (GR), a ligand binding transcription factor, which in the unbound state is sequestered in the cytoplasm by molecular chaperones [12]. Upon binding of its ligand, the GR translocates into the nucleus, where it can regulate transcription via binding to positive and negative GC response elements (GRE) in the promoter region of genes and via protein-protein interactions [224]. Multiple mechanisms act to generate a host of GR proteins from a single GR gene (Reviewed in [225]). In humans, alternative splicing results in the generation of the two most prominent GR species GRα and GRβ, proteins that have different carboxyl termini and transcriptional activities [70, 226]. The anti-inflammatory effects of GC are mediated through GRα. Increased expression of GRβ has been associated with dominant negative inhibition of GRα binding to GREs and is also believed to impede nuclear translocation of GRα [70, 227].

We showed previously that the nuclear transport receptor importin 13 (IPO13) promotes nuclear entry of GR and strongly potentiates the anti-inflammatory effects of GCs in airway epithelial cells [15]. More recently, we demonstrated that IPO13 genetic

variation is associated with an improvement in airway responsiveness in childhood asthma resembling that observed following long-term inhaled corticosteroid therapy [72]. These findings suggested that IPO13 affects nuclear bioavailability of endogenous corticosteroids and that impaired IPO13 function may contribute to GC insensitivity in a subset of asthmatics.

While hormone binding is an essential proximal event for GR activation, GR function is also regulated by post-translational phosphorylation. Allergen induced inflammatory cytokines activate a kinase cascade which eventually leads to the phosphorylation and activation of p38 mitogen activated protein kinase (MAPK) [63]. Phosphorylation of GR by phospho-p38 MAPK inhibits GR-mediated transcriptional activation and impairs GR nuclear import [63, 228]. Thus, variant cellular levels of phospho-p38 MAPK could lead to impaired GC sensitivity via a mechanism involving phosphorylation of GR.

The molecular mechanisms that contribute to GC-resistance remain incompletely understood. Murine models of allergic asthma have provided useful information on the mechanisms of allergic inflammation and immune response. Allergen-induced airway disease is mouse strain dependent [218] and there is considerable interbred variability in the susceptibility to develop an “asthma-like” phenotype [229, 230]. Less information is available, however, on interstrain variability in GC responsiveness. The objective of the present study was to investigate how two strains of mice, characterized by different asthma susceptibility phenotypes, differ in handling and response to steroids in the ovalbumin (OVA)-induced acute asthma model. Following allergen exposure, the Balb/c and C57BL/6 strains display different asthma-related phenotypes, including AHR, serum IgE and bronchoalveolar lavage (BAL) cell infiltrate [231]. Since asthma severity is

believed to correlate with GC responsiveness [221], we hypothesized that the Balb/c mouse, would show diminished GC responsiveness compared to the C57BL/6 mouse, following allergen challenge.

## 2.3 Materials and Methodology

### *Animals*

Balb/c and C57BL/6 inbred male mice, 8-10 week old (Charles River Laboratories, Saint-Constant, QC, Canada), were housed in the Montreal Children's Hospital Animal Facility. All procedures were in accordance with the Canadian Council on Animal Care and approved by the Animal Care Committee of the McGill University Health Centre.

### *Allergen sensitization and challenge*

Mice were sensitized and challenged essentially as previously described with the following modifications [232]. On Days 0 and 7, animals were sensitized by an intraperitoneal injection (i.p.) of 10 μg OVA (grade V; Sigma Chemical Co, St Louis, MO) in 0.2ml of a 5mg/ml suspension of $Al(OH)_3$. On Days 14, 18, and 19 animals were challenged by intranasal instillation under light anaesthesia, with 10μg OVA in 50μl of sterile saline. Control mice were sensitized and challenged with saline (Fig 1A).

### *Budesonide Treatment*

Micronized dry powder of budesonide (Astra Zeneca, Lund, Sweden) was dissolved in 2% Dimethyl sulfoxide (DMSO; Sigma Chemical Co, St Louis, MO) in sterile saline. A standard dose of budesonide (3mg/kg) [233, 234] was given by intranasal instillation under light anaesthesia on Days 17, 18, 19 and 20. The control groups (saline sensitized and challenged) received either saline or 2% DMSO instead of budesonide.

***Airway Responsiveness***

Airway responsiveness was measured 48 hours after the last OVA-challenge using a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, QC, Canada). Mice were ventilated quasisinusoidally at a frequency of 150 breaths/min and tidal volume of 10 ml/kg at positive end-expiratory pressure (PEEP) level of 3 cm of $H_2O$. After a standard volume history, a "standardized breath" type signal at 150 breaths/min was applied to measure the baseline respiratory system resistance (R). Increasing doses of methacholine (MCh, 6.25, 12.5, 25 and 50mg/ml) were aerosolized and maximum R was recorded.

***OVA-specific IgE ELISA***

Serum OVA-specific IgE levels were assessed using a modified ELISA (Pharmigen OPTEIA IgE Kit, San Diego, CA) protocol as described [235]. Serum was incubated overnight at 4°C with protein G sepharose beads. Plates were coated with OVA before adding serum.

***Bronchoalveolar Lavage (BAL)***

BAL was performed by instilling the lungs four times with 1ml cold phosphate-buffer saline through a tracheal cannula. Lavage fluid was centrifuged and pellets were resuspended in 0.5 ml cold saline. For differential cell counts, cytospin slides (Cytospin 4; Shandon, Pittsburgh, PA) were stained and counted (> 200 cells/slide).

***Isolation of Total Lung RNA***

Total RNA, isolated from 1/2 lung using Trizol reagent (Invitrogen, Burlington, ON, Canada) and total RNA was isolated following manufacturer's protocol. RNA was resuspended in RNASecure reagent (Ambion, Austin, TX, USA) and traces of DNA were removed using the Turbo DNase-free kit (Ambion, Austin, TX, USA).

***Quantitative real-time reverse transcriptase-PCR (qRT-PCR)***

Quantitative real-time RT-PCR was performed on the Mx4000 QPCR system (Stratagene, La Jolla, CA) using the Quantitect One-Step Probe RT-PCR Kit (Qiagen Mississauga, ON, Canada). Gene-specific primers and FAM-labelled probes for mouse glucocorticoid receptor (GR), importin 13 (IPO13), interleukin 4 (IL-4) and interleukin 13 (IL-13) were designed (QuantiProbe Design Software, Qiagen, Mississauga, ON, Canada; Table 2.1). Quantitect Gene Expression Assay for mouse 18S (Qiagen Mississauga, ON, Canada) was used to control for input RNA. Results were analyzed according to the standard curve method. One-step real-time qRT-PCR reactions were performed in 25 µL volumes for 45 cycles, using 10 ng of total RNA for GR and IPO13; 20 ng for IL-13 and IL-4; and 50 pg for 18S. Individual runs were performed in triplicates.

***Protein isolation and Western Blots***

Total protein was isolated from 1/2 lung using $KPO_4$ buffer and western blotted for p38 MAPK (phosphoY182 + T180; Abcam, Cambridge, MA) as described [228] except that membranes were blocked overnight with 10% donkey serum in TBS-T.

For nucleocytoplasmic separation, fresh 1/4 lungs were dounce homogenized using buffers provided in NE-PER® kit (Pierce Biotechnology, Rockford, IL) and proteins isolated following manufacturer's instructions. Fifteen µg of total protein was electrophoresed on 8% SDS-polyacrylamide gels, transferred to PVDF membrane (Biorad, Hercules, CA) and probed with anti-GR and anti-Lamin B (a nuclear marker; Santa Cruz Biotech., Santa Cruz, CA). The ECL Plus Western Blotting Detection System was used for protein detection (GE Healthcare, Buckinghamshire, UK).

***Statistical Analysis***

All data are presented as mean ± SEM. Statistical significance was determined by two-way Analysis of Variance (ANOVA). Pair-wise group comparisons were then assessed using Student-Neuman-Keuls test. Significance was defined as $p<0.05$.

## 2.4 Results

***Budesonide intervention prevents allergen-induced AHR in C57BL6 but not Balb/c mice***

In order to compare the effects of acute OVA sensitization/challenge on AHR in Balb/c and C57BL/6 mice, we measured pulmonary resistance using the flexiVent small animal ventilator. OVA treatment was associated with a significant increase in airway responsiveness in both mouse strains (Fig 2.1B, $p<0.05$). Intranasal budesonide intervention prevented the development of OVA-induced AHR in C57BL/6 but not in Balb/c mice (Fig 2.1B). No significant difference in AHR was observed in control mice (saline sensitized and challenged) treated with saline or 2% DMSO (data not shown).

***Effects of budesonide on allergic response in C57BL6 and Balb/c mice***

To evaluate the effect of budesonide intervention on the OVA-specific allergic response, IgE serum levels and cell infiltrates in BAL were assessed (Figure 2.2 and Table 2.2). As shown in Fig 2, all OVA-treated animals developed OVA-specific IgE when compared to respective controls ($p<0.05$). Moreover, IgE levels were significantly higher in OVA-treated Balb/c mice when compared to C57BL/6 mice. Budesonide treatment prevented the OVA-induced IgE response in the C57BL/6 but not in the Balb/c mice.

The inflammatory cell infiltrates were assessed in BAL fluid. Total cell counts increased after allergen challenge in both strains (Table 2.2). Significant BAL

eosinophilia and neutrophilia was observed in C57BL/6 ($p<0.005$) and Balb/c ($p<0.05$) mice. No significant change in lymphocyte numbers was observed. Budesonide intervention prevented BAL eosinophilia and neutrophilia in the C57BL/6 but not in the Balb/c strain.

To further evaluate the effects of GC treatment on allergic response, we quantified the mRNA levels of two $T_H2$ cytokines, IL-4 and IL-13, in whole lung tissue 48 hours after the last OVA challenge. OVA treatment led to induction of IL-4 mRNA expression which was not detectible by QRT-PCR in saline controls of either strain (Figure 2.3A, $p<0.00005$). Induction of IL-4 mRNA was suppressed by budesonide in the C57BL/6 but not in Balb/c mice. OVA caused an increase in the levels of IL-13 mRNA in the lungs of both C57BL/6 ($p<0.00005$) and Balb/c ($p<0.03$) mice compared to their controls. Budesonide prevented this increase in C57BL/6 but not in Balb/c mice.

***Budesonide restores allergen suppressed levels of GR mRNA levels in C57BL/6 but not in Balb/c mice***

Having established that following acute allergen challenge Balb/c mouse exhibit features of a steroid insensitive phenotype, we next wished to determine the mechanism whereby GCs fail to attenuate inflammation in this strain, initially focusing on the most proximal molecule, GR. The expression of GR mRNA was assessed by QRT-PCR using total RNA obtained from lung tissue 48 hours after the last OVA challenge. Baseline levels of GR mRNA were significantly higher in the Balb/c compared to C57BL/6 mice (Figure 2.4A, $p<0.05$). Allergen challenge caused a significant decrease in the levels of GR in both strains ($p<0.05$). Budesonide intervention completely prevented suppression of GR in the C57BL/6 strain. Budesonide treated Balb/c mice tended to show an increase in GR mRNA over that observed in the presence of OVA alone. This increase did not

reach significance (p=0.09). GR levels, however, did remain significantly below that observed in untreated control Balb/c mice. Changes in the pattern of GR protein expression, as assessed by western blot, were consistent with those observed for GR mRNA as illustrated in the representative western blot (Figure 2.4B).

***Allergen-induced levels of activated phospho-p38 MAPK are not suppressed by budesonide intervention in Balb/c mice***

An increase in inflammatory cytokines in the airways, including IL-13, activates p38 MAPK, which can in turn alter GR function via phosphorylation of serine residues (possibly Ser226 or Ser211) [63, 228, 236]. Given the persistent elevation of IL-13 in Balb/c mice, we asked whether high cytokine levels would induce activation of p38 MAPK. We analyzed the proportion of total p38 MAPK in the lung that was activated in control, OVA treated and OVA/budesonide treated mice using phospho-p38 MAPK-specific antibodies on western blots (Figure 2.5A, B). Baseline levels of phospho-p38 MAPK were similar in both strains (p=0.59). OVA challenge led to a 6-fold increase ($p<0.05$) in the proportion of phosphorylated p38 MAPK in lungs of Balb/c mice, an effect that was not attenuated by budesonide intervention. By contrast, budesonide significantly reduced the percentage of phospho-p38 MAPK in lungs of C57BL/6 mice ($p<0.001$). A reduction in activated p38 MAPK would be expected to lead to an increase in active GR and enhanced GC responsiveness in cells.

***Allergen challenge is associated with altered nucleocytoplasmic distribution of GR and reduced IPO13 levels in Balb/c mice***

Phospho-p38 MAPK mediated phosphorylation of GR has been associated with impaired GR nuclear import [63]. Having established that Balb/c mice had a higher proportion of activated p38 MAPK in the lung, we next wished to address whether there

was any interstrain variability in nuclear localization of GR. To this end, we analyzed the nucleocytoplasmic distribution of GR on western blots (Figure 2.6A-C). Following allergen challenge, both C57BL/6 and Balb/c mice had a significant decrease in the proportion of GR that localized to the nucleus ($p<0.05$). Budesonide intervention prevented this change in C57BL/6 mice. Budesonide treated Balb/c mice continued to have a significantly lower proportion of GR localized to the nucleus compared to controls.

In order to determine whether variant IPO13 levels influenced changes in nucleocytoplasmic distribution of GR in our model, we assessed IPO13 mRNA levels by QRT-PCR. IPO13 mRNA levels were significantly reduced after OVA challenge in both the C57BL/6 and Balb/c strains ($p<0.005$) (Figure 2.7). Budesonide intervention prevented suppression of IPO13 mRNA levels in C57BL/6 mice. Budesonide treated Balb/c mice had IPO13 mRNA levels that were significantly higher than in the OVA treated group ($p<0.05$); but were still significantly lower than that of untreated Balb/c controls ($p<0.05$).

**2.5 Discussion**

Inhaled corticosteroids are the mainstay and first line of treatment for most asthmatics. However, glucocorticoid insensitivity and steroid resistance (non-response to high doses of oral corticosteroid) are a major barrier to treatment of a significant number of patients. Steroid insensitive asthma is associated with more severe disease [221]. In the present study we exploited the inbred mouse system to demonstrate that Balb/c mice, which display features of a severe asthma phenotype following allergen challenge, also show a limited response to steroid intervention as assessed by effects on AHR, eosinophilia and inflammatory cytokine levels. This relative steroid-insensitivity was

associated with a reduction in GR expression, a decrease in the proportion of GR that localized to the nucleus, and an increase in lung levels of phospho-p38 MAPK. To our knowledge this is the first report to demonstrate a significant steroid insensitive phenotype in the Balb/c mouse.

Other investigators have demonstrated variable GC effects on inflammation and AHR in OVA-induced asthma in Balb/c mice [237, 238]. Conflicting reports on steroid responsive phenotypes may reflect intrastrain variability as well as diversity of protocols for sensitization/challenge; route and dose of exposure; and assessment for AHR (reviewed in [239]).

GC sensitivity clearly describes a spectrum of pharmaco-responsive phenotypes. The Balb/c acute OVA challenge model displays features of GC insensitivity following an intranasal budesonide intervention that resembles inhaled corticosteroid treatment in humans. It was therefore of interest to establish whether the molecular mechanisms that account for relative steroid insensitivity in this model resemble the spectrum of human corticosteroid non-responsiveness.

Naïve Balb/c mice had significantly higher GR mRNA levels than their C57BL/6 counterparts. These findings are interesting in light of studies showing that steroid insensitive asthmatics have high GR numbers [221], and that children with asthma express greater quantities of GR mRNA than healthy children [240]. Increased expression of GR may improve ability to regulate cytokine responses. An increase in the number of the GR receptors may be one mechanism to compensate for steroid insensitivity in patients and/or in the Balb/c mouse.

mRNA levels of IL-4 and IL-13 remained elevated in the lungs of Balb/c mice following GC treatment. An association between elevated expression of these cytokines

(together with IL-2) and reduced GR ligand-binding affinity has been demonstrated in GC-resistant patients [57, 241]. The persistence of elevated levels of allergen-induced cytokines can lead to an increase in phosphorylation of p38 MAPK. Phosphorylation of GR by phospho-p38 MAPK limits ligand binding [63] as well as nuclear import [64] and transactivation potential [228]. Our demonstration that the allergen induced increase in phospho-p38 MAPK in Balb/c mice is refractory to budesonide intervention was consistent with a cytokine-induced mechanism of GR inactivation as contributory to relative GC insensitivity in this model. By contrast, GC intervention in C57BL/6 mice was associated with reduced cytokine levels and reduced levels of phospho-p38 MAPK, effects which would be expected to enhance GC responsiveness.

Consistent with the finding of an increase in inactive GR, allergen challenge led to a significant reduction in the percentage of total GR in the nucleus in lungs of Balb/c mice, an effect that was not prevented by budesonide intervention. Indeed, a subset of steroid resistant asthmatics demonstrate impaired nuclear translocation of GR in response to high doses of GCs [71] and it has been proposed that this effect may reflect, at least in part, altered interaction with importin proteins subsequent to GR phosphorylation by p38 MAPKs [242].

We described IPO13, a GC-inducible nuclear import carrier of GR [15]. More recently, we showed that genetic variation in IPO13 is associated with improved AHR in children [72]. The degree of this improvement was similar to that observed with long-term inhaled corticosteroid treatment suggesting that IPO13 variation effects nuclear bioavailability of endogenous corticosteroids. Increased levels of active p38 MAPK, which limit GC regulated transcription, could be expected to affect cellular IPO13 levels. We noted that IPO13 expression, which was suppressed by allergen challenge, remained

below control levels in the Balb/c mouse following budesonide intervention. This effect was limited, and we suspect that it would be likely to be a small contributing factor in the global GC insensitive phenotype of Balb/c mice. An additional effect that is likely to result from MAPK suppression of GR activity is reduction in the levels of MKP-1, a MAPK phosphatase, which is rapidly induced by GR and potently inactivates p38 MAPK.

The molecular mechanisms that contribute to GC-resistance in humans remain incompletely understood. Existing evidence suggests that increased activation of pro-inflammatory transcription factors, reduced GR numbers, limited affinity of GR for its ligand and/or DNA recognition element, and impaired nuclear translocation of GR, contribute to GC resistance [71, 224, 242]. In a subset of human GC-resistant asthmatics, increased GRβ acts as a dominant negative inhibitor of GRα and impedes its nuclear translocation [70, 227]. Little is known about the contribution of translational GR isoforms (present in humans and rodents) to GC insensitivity. Our combined findings suggest that in Balb/c mice gene-environment interactions involving allergen challenge and subsequent high levels of inflammatory cytokines lead to increased quantities of activated p38 MAPK, which potentiates the inflammatory response. Additionally, p38 MAPK can suppress transcription of its own repressor (MKP-1) via the inactivation of GR. We speculate that dysregulation of this pathway in Balb/c mice, via effects on molecules upstream of p38 MAPK, contributes to the relative GC insensitive phenotype.

This study has a number of limitations. We analyzed the relative steroid insensitivity following a standard dose of budesonide of 3mg/kg. Considering an average human weight of 70kg, this dose would be equivalent to more than 100X the maximal prescribed dose (1600ug/day) for human asthmatics [243]. While higher doses of steroid

may have resulted in a more GC responsive phenotype, the use of such doses would not be relevant to the human situation. While budesonide is the standard for treatment, it would be of interest to analyze the effects of other corticosteroids in our model. In our study, budesonide was given to animals for 4 consecutive days, beginning 3 days after the initial allergen challenge and then before or during the $2^{nd}$ and $3^{rd}$ challenge. Since in humans, chronic disease is generally well established before steroid administration, it will be of interest to examine the effects on steroid responsiveness (which may be expected to be even more pronounced) if steroid were administered just prior to the last allergen challenge.

The prevalence of and mortality due to asthma have almost doubled in the last 20 years. A significant number of asthmatics do not respond to GCs, the most effective class of anti-inflammatory asthma therapy. The Balb/c mouse shows many of the manifestations of the GC insensitive phenotype. This relative steroid-insensitive phenotype, associated with reduced expression, suppressed activation and impaired nuclear localization of GR, resembles steroid insensitive phenotypes observed in a subset of human patients. As such, the Balb/c mouse may serve in the long-term as a model to investigate therapeutic strategies to enhance steroid responsiveness. Clarification of genetic factors that contribute to steroid insensitivity will also assist physicians to tailor therapy to the individual patient, maximizing efficacy and minimizing adverse effects.

**Table 2.1 Gene-specific primers and FAM-labelled probes.**

| GENE | PRIMERS | PROBE |
|---|---|---|
| **IL-4** | 5’-GAGATCATCGGCATTTTG-3’<br>5’-TCACTCTCTGTGGTGTTCTTC-3’ | 5’CGGAGATGGATGTGCCAA-3’ |
| **IL-13** | 5’-CATCACACAAGACCAGACTC-3’<br>5’-GAATCCAGGGCTACACAGAA-3’ | 5’-CAACGGCAGCATGGTA-3’ |
| **GR** | 5’-CTTGGGGGCTATGAACTT-3’<br>5’-GGTCTCATTCCAGGGCTT-3’ | 5’-CCGCTCAGTGTTTTCTA-3’ |
| **IPO13** | 5’-AAGCAGATTCACAAGACAAG-3’<br>5’-TGTAGGGAGAGATGAGGGAGT-3’ | 5’-GCAGGTGGAGGAGATC-3’ |

**Table 2.2 Comparison of BAL eosinophilia and neutrophilia between C57BL/6 and Balb/c strains.**

| STRAIN | GROUP | TCC x $10^4$ / BAL | % BAL Eosinophils | Neutrophils |
|---|---|---|---|---|
| **C57BL/6** | **CTL** | 10.9 ± 3.3 | N.D. | N.D. |
| | **OVA** | 18.3 ± 0.3 * | 66.04 ± 0.53 * | 17.04 ± 1.12 * |
| | **OVA + BUD** | 7.7 ± 2.3 | 0.16 ± 0.16 | N.D. |
| **Balb/c** | **CTL** | 13.6 ± 1.3 | N.D. | N.D. |
| | **OVA** | 22.25 ± 1.47 ‡ | 16.54 ± 2.65 ‡ | 7.43 ± 1.61 ‡ |
| | **OVA + BUD** | 17.4 ± 5.7 | 30.82 ± 5.34 ‡ | 16.91 ± 4.25 ‡ |

Data are means ± SEMs ($n \geq 3$). CTL = control; OVA = ovalbumin; BUD = budesonide; N.D. = not detectable. *$p<0.005$ as compared to C57BL/6 CTL. ‡$p<0.05$ as compared to Balb/c CTL.

**Figure 2.1 (A) Schematic of ovalbumin (OVA) sensitization and challenge protocol and therapeutic administration of budesonide (BUD).** Balb/c and C57BL/6 male mice were sensitized and challenged as described in the "Methods" section. Control mice were sensitized and challenged with saline. Budesonide or diluent only were given by intranasal instillation on days 17-20. Outcomes were measured on day 21. **(B)** Airway responsiveness to a medium-dose (12.5 mg/ml) of aerosolized methacholine after 4 days of budesonide or diluent treatment and 48 hours after the last saline or OVA challenge. Response measured as respiratory system resistance (R). Means (n=4-7) are presented ± SEMs. *$p<0.05$ as compared to respective control (CTL).

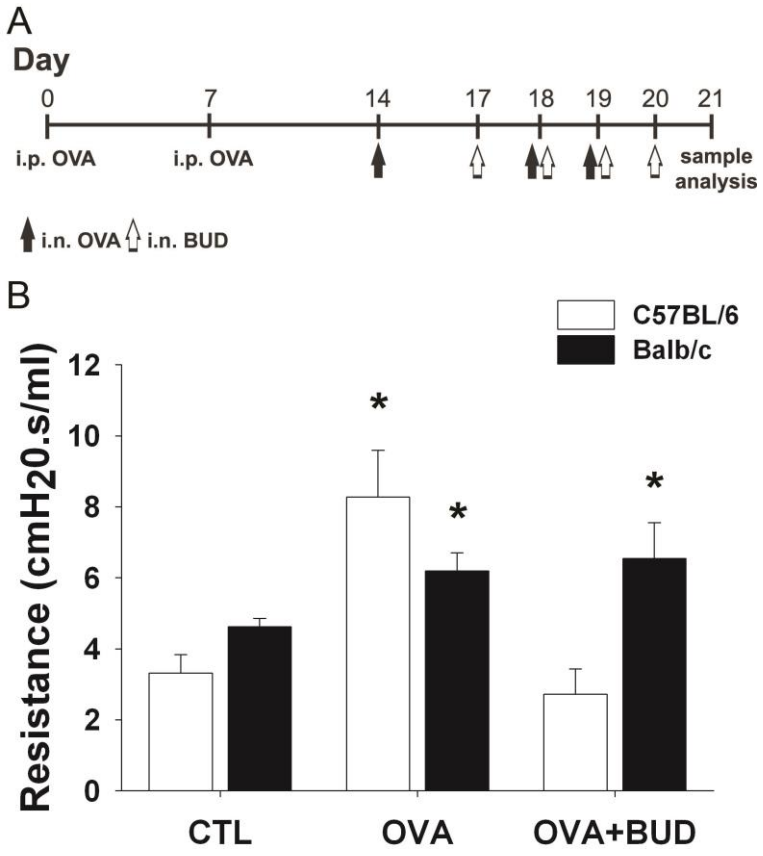

A
Day
0
7
14
17
18
19
20
21
i.p. OVA
i.p. OVA
sample analysis
i.n. OVA
i.n. BUD
B
C57BL/6
Balb/c
Resistance (cmH20.s/ml)
12
10
8
6
4
2
0
*
*
*
CTL
OVA
OVA+BUD

**Figure 2.2** Serum OVA-specific IgE levels after 4 days of budesonide or diluent treatment and 48 hours after the last saline or OVA challenge. Means (n=3-6) are presented ± SEMs. *$p<0.05$ as compared to respective control (CTL).

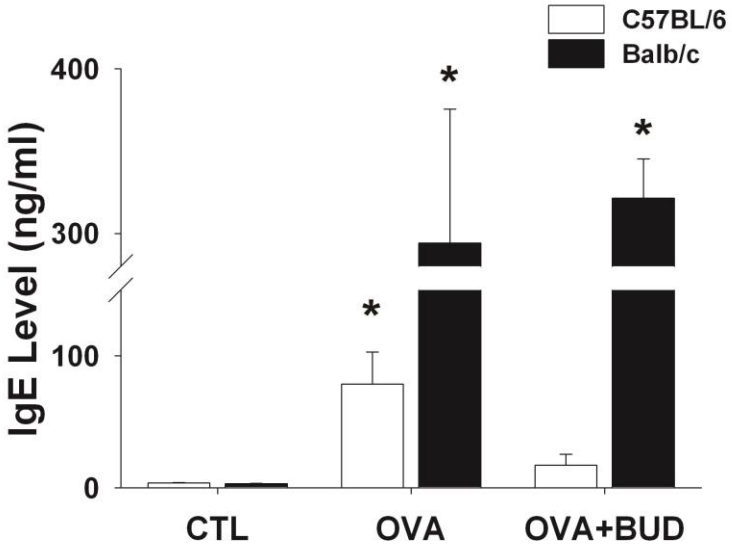

C57BL/6
Balb/c
IgE Level (ng/ml)
400
300
100
0
*
*
*
CTL
OVA
OVA+BUD

**Figure 2.3 Cytokine mRNA levels in lung tissue after 4 days of budesonide or diluent treatment and 48 hours after the last saline or OVA challenge, as assessed by QRT-PCR. (A)** IL-4 levels (n=3-8) ± SEMs. N/D = not detectable. **(B)** IL-13 (n=3-8) ± SEMs. *$p<0.00005$ as compared to respective control (CTL). ‡$p<0.03$ as compared to respective control (CTL).

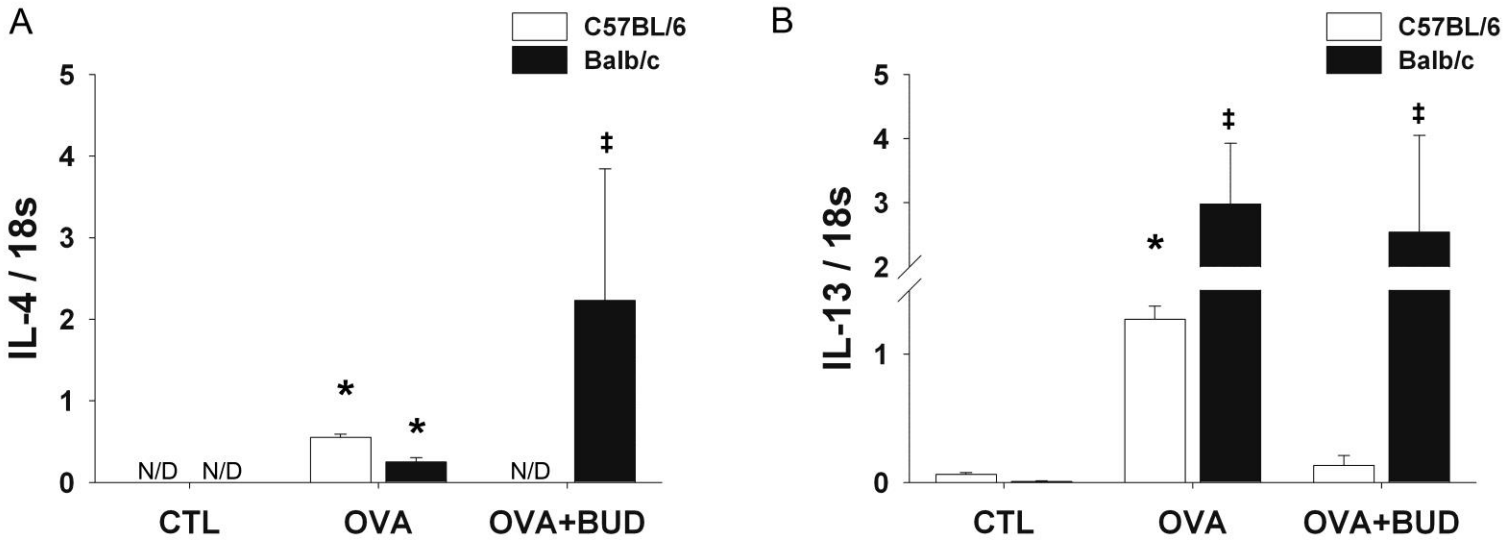

A
C57BL/6
Balb/c
IL-4 / 18s
5
4
3
2
1
0
N/D
N/D
N/D
*
*
‡
CTL
OVA
OVA+BUD
B
C57BL/6
Balb/c
IL-13 / 18s
5
4
3
2
1
0
*
‡
‡
CTL
OVA
OVA+BUD

**Figure 2.4** Glucocorticoid receptor expression in lung tissue after 4 days of budesonide or diluent treatment and 48 hours after the last saline or OVA challenge. Means (n=4) are presented ± SEMs. **(A)** GR mRNA expression in whole lung tissue, as assessed by qRT-PCR. ‡$p<0.05$ Balb/c compared to C57BL/6, *$p<0.05$ as compared to respective control (CTL). **(B)** Representative western blot showing changes in GR protein following OVA or OVA plus budesonide treatment.

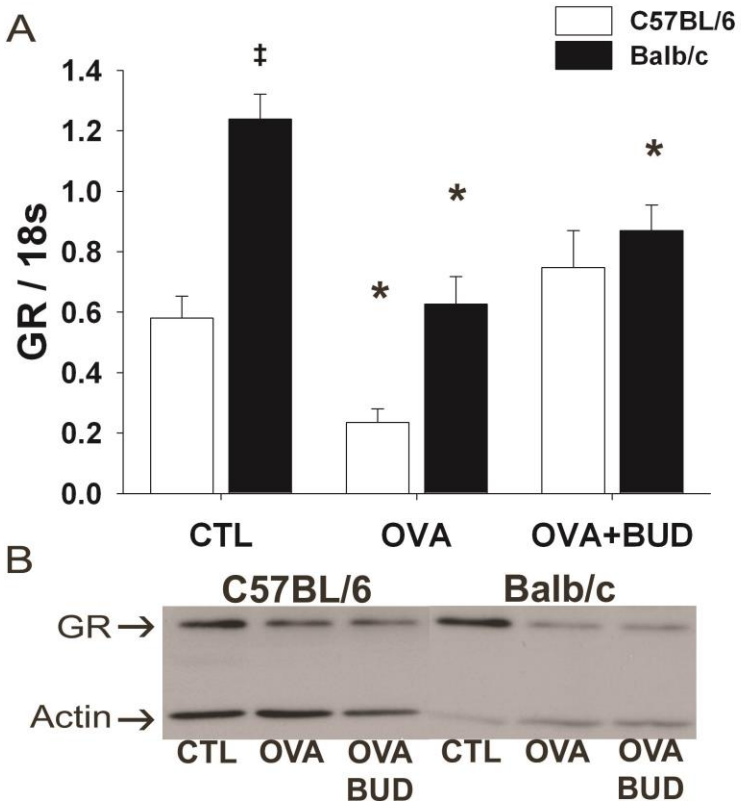

A
C57BL/6
Balb/c
GR / 18s
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
‡
*
*
*
CTL
OVA
OVA+BUD
B
C57BL/6
Balb/c
GR
Actin
CTL
OVA
OVA BUD
CTL
OVA
OVA BUD

**Figure 2.5 (A)** Levels of phospho-p38 MAPK (P-p38) relative to total p38 MAPK in whole lung tissue of control (CTL) mice. **(B)** Fold change in levels of P-p38 relative to total p38 MAPK with respect to CTL mice, following 4 days of budesonide or diluent treatment and 48 hours after the last saline or OVA challenge. A representative western blot illustrating fold change in levels of P-p38 MAPK is shown; lanes 1,4 control, lanes 2,5, OVA treated, lanes 4,6 OVA plus budesonide. Means (n=4) are presented ± SEMs. ‡$p<0.01$ as compared to C57BL/6 CTL. *$p<0.05$ as compared to Balb/c control (CTL).

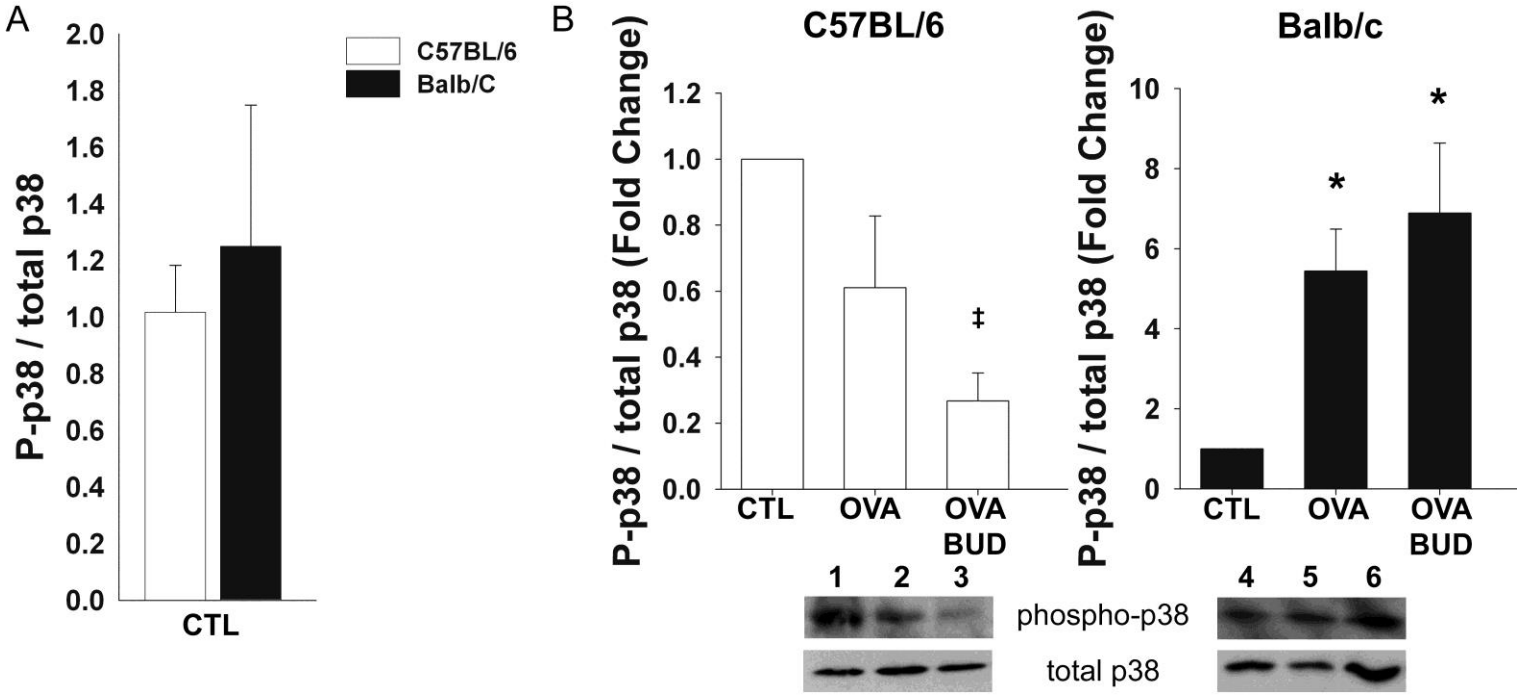

A
P-p38 / total p38
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CTL
C57BL/6
Balb/C
B
C57BL/6
P-p38 / total p38 (Fold Change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
‡
CTL
OVA
OVA BUD
Balb/c
P-p38 / total p38 (Fold Change)
10
8
6
4
2
0
*
*
CTL
OVA
OVA BUD
1 2 3
4 5 6
phospho-p38
total p38

**Figure 2.6** Nuclear and cytoplasmic differential localization of GR protein in lung tissue of **(A)** C57BL/6 or **(B)** Balb/c mice after 4 days of budesonide or diluent treatment and 48 hours after the last saline or OVA challenge.*$p<0.05$ as compared to respective control (CTL) **(C)** Representative western blot for GR illustrating nucleo-cytoplasmic distribution of GR protein following OVA or OVA plus budesonide treatment.

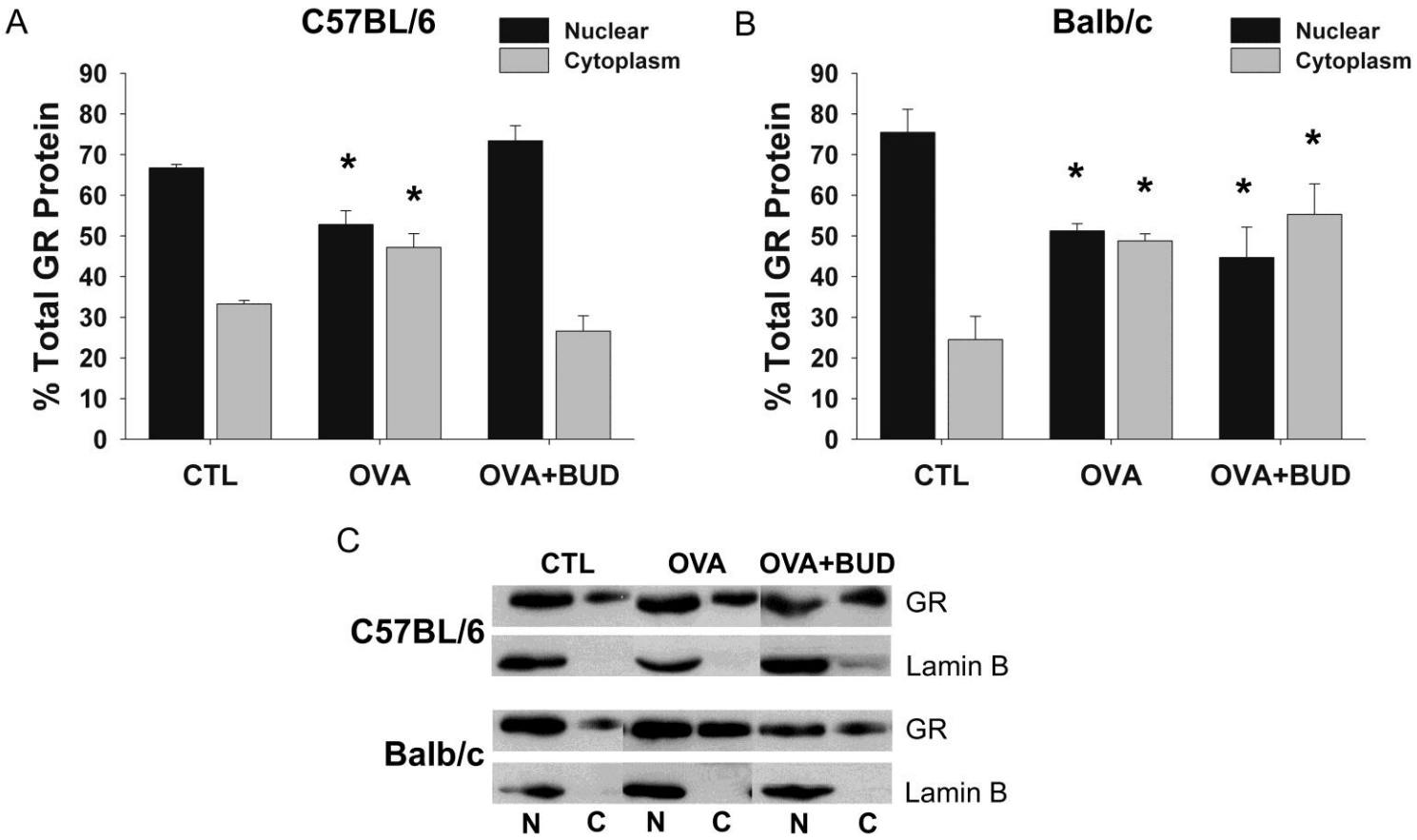
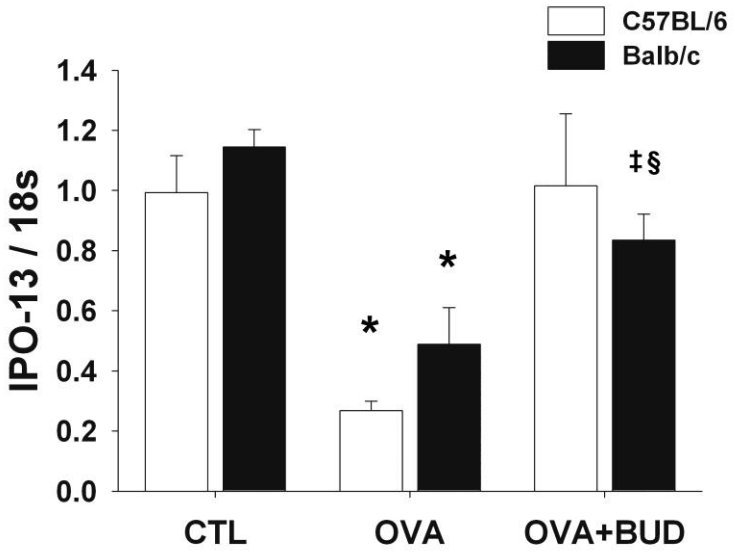

A
C57BL/6
Nuclear
Cytoplasm
% Total GR Protein
90
80
70
60
50
40
30
20
10
0
CTL
OVA
OVA+BUD
B
Balb/c
Nuclear
Cytoplasm
% Total GR Protein
CTL
OVA
OVA+BUD
C
CTL
OVA
OVA+BUD
C57BL/6
GR
Lamin B
Balb/c
GR
Lamin B
N
C
N
C
N
C

**Figure 2.7 Importin 13 mRNA levels.** Means (n=4) are presented ± SEMs. *$p<0.005$ as compared to respective control (CTL). ‡$p<0.05$ as compared to Balb/c CTL. §$p<0.05$ as compared to Balb/c OVA treated group.


C57BL/6
Balb/c
IPO-13 / 18s
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
‡§
CTL
OVA
OVA+BUD

*Asthma is a complex and variable pathology. Interestingly, there is evidence of phenotypic differences between the adult and pediatric forms of the disease and these variations are more apparent among severe asthmatics, which tend to be less GC responsive [244]. In Chapter II of this thesis steroid insensitivity in a murine model of adult asthma was associated with several molecular mechanisms, including altered expression and impaired nuclear localization of GR. The next step of the project was to determine if these mechanisms were also implicated in limiting steroid response in asthmatic children. I used cell lines derived from patients in a pediatric asthma cohort to investigate mechanisms of steroid responsiveness during childhood. Furthermore, cell culture experiments were conducted to explore the possible modulatory role of VitD in GC function. The results of these experiments are presented in the following chapter.*

# CHAPTER III

## Nuclear Bioavailability of the Glucocorticoid Receptor in a Pediatric Asthma Cohort with Variable Steroid Responsiveness

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### 3.1 Abstract

Despite the overall effectiveness of glucocorticoids (GCs) in the treatment of asthma, a large proportion of patients do not fully respond to this medication. The objective of the present study was to investigate the potential molecular mechanisms responsible for steroid insensitivity in pediatric asthma and to evaluate the possible modulatory role of vitamin D (VitD) on steroid function.

Asthmatic children were classified as good (GSR) or poor steroid responders (PSR) based on changes in pulmonary function following GC treatment. Lymphoblasts derived from patients at two ends of the spectrum of GC responsiveness (5 each) were grown in culture and treated with hydrocortisone ($10^{-6}$M) and/or 1α,25-Dihydroxyvitamin D3 ($10^{-8}$M). Baseline and temporal changes in GC receptor (GR) mRNA and protein were evaluated by qRT-PCR and Western blot respectively. The effects of the different treatments on GR nuclear levels were assessed by Western blots. VitD receptor (VDR) mRNA expression was measured by qRT-PCR.

Cells derived from PSR asthmatics displayed increased GR mRNA and decreased GR protein levels when compared to GSR lymphoblasts. In PSR cells GC-induced nuclear translocation of GR was short-lived and homologous downregulation of GR mRNA and protein was faster than in GSR lymphoblasts. GC induced the expression of VDR only in GSR lymphoblasts. In this set of PSR cells, VitD seemed to modify all these responses.

GC insensitivity in some asthmatic children could result from limited GR nuclear bioavailability as a consequence of decreased baseline GR expression and faster hormone-induced downregulation. Despite our limited sample size, our results support a beneficial role of VitD in steroid sensitivity.

## 3.2 Introduction

Asthma is the most prevalent chronic disease in children, affecting around 7.1 million Americans under the age of 18 and more than 15% of the Canadian pediatric population of ages 4-11 [157, 245]. It is characterized by persistent airway inflammation, reversible airflow obstruction and enhanced airway hyperresponsiveness (AHR) [155]. Due to their potent anti-inflammatory effects, inhaled glucocorticoids (GCs) are the most commonly used drug in the treatment of asthma [246]. Overall, GCs reduce symptoms, prevent exacerbations, and decrease asthma-associated mortality [155]. However, there is significant variability in GC responsiveness and up to 40% of asthmatics fail to show improved lung function following inhaled steroid treatment [222]. While some GC insensitive patients do respond to higher doses than normally prescribed, the administration of these doses for prolonged periods can have marked adverse effects. Moreover, poor clinical responses have been observed even among asthmatics receiving high doses of systemic steroids [247]. The economic burden of asthma on healthcare systems is significant, particularly due to the mismanagement of severe asthmatics [55, 155]. Therefore, a better understanding of the molecular mechanisms responsible for GC insensitivity is necessary in order to tailor patient-specific treatment, increasing effectiveness and avoiding side effects.

Most of the anti-inflammatory effects of GCs result from transcriptional regulation of target genes though the glucocorticoid receptor (GR), which in the absence of ligand is sequestered in the cytoplasm [246]. Once activated, GR translocates into the nucleus with the aid of nuclear transport receptors, including importin 13 (IPO13) [15]. Multiple mechanisms have been proposed to contribute to variable steroid responsiveness, including altered expression and dysfunctional nuclear translocation of

GR (Reviewed in [55]). Additionally, since steroids induce GR downregulation, it is reasonable to speculate that differences in temporal activation of this negative feedback mechanism could also add to variations in steroid sensitivity [79].

Vitamin D (VitD) has been suggested to have beneficial effects in asthma management. In murine models of the disease, VitD ameliorates airway inflammation and alters immunoglobulin (Ig)E levels as well as the cytokine expression profile [196, 248]. In asthmatic children, VitD insufficiency has been linked to elevated inflammatory markers, AHR and exacerbations [212, 213]. Interestingly, the observed beneficial effects of VitD were greater among patients receiving inhaled steroids, suggesting that VitD enhances GC responsiveness [213].

To explore the potential molecular mechanisms responsible for GC insensitivity in pediatric asthma, lymphoblast cell lines were derived from asthmatic children classified as good (GSR) or poor steroid responders (PSR). Cell lines derived from patients at two ends of the spectrum of GC responsiveness were grown in culture and treated with GCs. Despite this selection bias and given the increasing amount of evidence suggesting that VitD enhances steroid responsiveness, we also wished to study the possible modulatory role of VitD in GC function at the cellular level. Therefore, lymphoblasts were also co-treated with VitD. We hypothesized that PSR cells would present decreased expression of genes involved in the GC pathway and/or altered nuclear translocation of GR in response to GC treatment and that VitD would positively modify steroid function. Herein we report that lymphoblasts derived from PSR children showed diminished GR protein expression at baseline as well as faster homologous downregulation and nuclear translocation of GR in response to steroids when compared to GSR lymphoblasts. Moreover, VitD seemed to

modify the observed defects in this set of PSR cells, supporting a possible therapeutic use of VitD in pediatric asthma by enhancing steroid response.

## 3.3 Materials and Methodology

### *CAMP lymphoblast cell lines*

The Childhood Asthma Management Program (CAMP) is a multicentered, randomized, double-blind clinical trial, testing the safety and efficacy of long-term anti-inflammatory drugs in the treatment of asthma. The design and methodology of the trial as well as the analysis of the primary outcomes have been published elsewhere [184, 220]. Approval was obtained from the institutional review boards at each of the CAMP study centers. Informed assent and consent was obtained from participants and their parents or guardians.

Patients were classified as good (GSR) or poor steroid responders (PSR) if they showed a positive or negative change in $FEV_1$ following 12 months of budesonide treatment respectively. Patients at two ends of the spectrum of GC responsiveness (5 each) were selected to conduct this study. All selected patients were Caucasians and both groups had similar distribution based on sex and age. Blood was collected in a 10ml "Yellow top" ACD vacutainer vial during a normal follow-up visit during the CAMP Continuing Studies 2 (CAMP CS/2) cohort study. Lymphoblasts were isolated and transformed by Epstein-Barr virus at the Partners HealthCare Center for Personalized Genetic Medicine (Harvard Medical School, Boston, MA).

### *Cell culture and treatment*

Lymphoblasts were plated at 200,000 cells/ml and grown in RPMI (Invitrogen, Burlington, ON, Canada) containing 10% FBS (HyClone, Logan, UT), penicillin (100units/mL) and streptomycin (100µg/mL) (Invitrogen) until day 4. Cells were then

serum-starved for 24 h in RPMI containing 10% charcoal-stripped fetal bovine serum (FBS), penicillin (100units/mL) and streptomycin (100µg/mL) before being treated with hydrocortisone ($10^{-6}$M), 1α,25-Dihydroxyvitamin $D_3$ ($10^{-8}$M) or both for 0.25, 0.5, 2 and 24 h.

***RNA Isolation***

Cells were pelleted down and lysed using 1ml Trizol reagent (Invitrogen) and total RNA was isolated following manufacturer's protocol. RNA was resuspended in RNASecure reagent and traces of DNA were removed using the Turbo DNase-free kit (Ambion, Austin, TX).

***Quantitative Real-Time RT-PCR***

Quantitative real-time RT-PCR (qRT-PCR) was performed on the Mx4000 QPCR system (Stratagene, La Jolla, CA) with the QuantiTect SYBR green RT-PCR kit (Qiagen, Mississauga, ON, Canada) as previously described [219]. Gene-specific primers for SYBR green detection of the GR, IPO13, vitamin $D_3$ receptor (VDR), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed. Synthesis of cDNA was prepared from an initial 200 ng/ml of RNA. RNA was incubated at 65°C for 5 min with 1 mg/ml of random primers and 10 mM dNTPs. To this, 5X first-strand buffer, 1 mM DTT, 1 ml of RNase OUT and 1 ml of Superscript II (Invitrogen) was added and incubated at 42°C for 1 h and at 70°C for 15 min. qRT-PCR was performed in 25 ml reactions for 40 cycles with 1 µl of cDNA. Results were analyzed using the delta-delta cycle threshold method.

***Protein Isolation and Western Blots***

Total protein was isolated using KPO4 buffer and nuclear and cytoplasmic fractions were obtain using buffers provided in NE-PER® kit (Pierce Biotechnology, Rockford, IL) and following manufacturer's instructions. Fifteen µg of total protein or

nuclear fraction was boiled for 5 min in SDS loading buffer before being electrophoresed on 7.5% SDS-polyacrylamide gels and then transferred to PVDF membrane (Biorad, Hercules, CA). Membranes were blocked overnight at 4°C with 10% milk/PBS–Tween and probed with anti-GR, anti-IPO13 anti-GAPDH (total loading control), and anti-TBP (nuclear loading control; Santa Cruz Biotech., Santa Cruz, CA) for 1 h. After several washes in PBS-Tween buffer, membranes were incubated with HP-conjugated secondary antibodies. The ECL Plus Western Blotting Detection System was used for protein detection (GE Healthcare, Buckinghamshire, UK).

***Cytospins and Immunofluorescence***

An aliquot of cell suspension was taken at each time point and cells were pelleted down. Cells were then resuspended in cold saline, cytospin slides (Cytospin 4; Shandon, Pittsburgh, PA) prepared, air dried and fixed. For immunofluorescence experiments, slides were coded to mask sample identity. They were rehydrated though a series of decreasing ethanol washes, rinsed with PBS-0.03% Triton and incubated in warm 10mM sodium citrate for antigen retrieval. Slides were then incubated in $H_2O_2$ and methanol for 20 min to block endogenous peroxidase activity. To block non-specific binding, slides were incubated in PBS-0.03% Triton containing 5% normal goat serum and 1% BSA. GR primary antibody (Santa Cruz Biotech.) was incubated overnight at 4°C and the following day in corresponding fluorescent conjugated secondary antibody for 30 min at room temperature. Slides were washed with PBS-0.03% Triton and mounted with pro-long anti-fade media containing DAPI (Invitrogen).

***Statistical Analysis***

All results are presented as mean ± SEM. Statistical significance was determined by Student t-test or two-way ANOVA followed by pair-wise group comparisons using

Student-Neuman-Keuls test. Correlations were analysed by Pearson Product-Moment Correlation. All statistical analyses were conducted using SigmaPlot-Systat Software. Significance was defined as $p<0.05$.

## 3.4 Results

### *PSR lymphoblasts express abnormal levels of GR mRNA and protein*

To determine if the differences in GC response among asthmatic children was due to reduced expression of the receptor we first assessed the levels of GR mRNA by qRT-PCR. Lymphoblasts derived from children classified as PSR had significantly higher levels of GR mRNA at baseline ($p<0.05$, Figure 3.1A). Among PSR asthmatics the higher the levels of GR mRNA at baseline the smaller the decrease in lung function, as measured as change in $FEV_1$ after 12 months of steroid treatment ($p=0.0171$, $r=0.983$, Figure 3.1B).

We next assessed GR protein levels by Western blots. Lymphoblasts derived from GSR asthmatics had significantly higher levels of GR protein when compared to PSR cells ($p<0.05$, Figure 3.2A), suggesting that the amount of GR protein could be a predictor of GC response. To verify this association, a Pearson correlation analysis was conducted between GR protein levels and change in lung function over time. GR protein levels in GSR and PSR lymphoblasts positively correlated with change in lung function after 2 ($p=0.03$, $r=0.672$, Figure 3.2B) and 12 ($p=0.0319$, $r=0.676$, Figure 3.2C) months of inhaled steroid treatment.

### *PSR lymphoblasts display altered GC-induced nuclear translocation of GR*

Having identified decreased GR protein expression in PSR cells, we next wished to examine if these reduced receptor levels affected GR nuclear translocation. To determine if lymphoblast derived from PSR asthmatics displayed abnormal nuclear translocation of GR in response to GC treatment, cells were grown in culture and treated

with steroids for 15, 30 min, and 2 h. Nuclear protein fraction and cytospin slides were prepared and Western blots and immunofluorescence assays were performed. Steroid treatment caused the accumulation of GR protein in the nucleus of GSR and PSR lymphoblasts. However, there was a marked difference in temporal regulation of nuclear import. In lymphoblasts derived from GSR children, GC addition to culture media caused a rapid (15 min) nuclear translocation of GR. High nuclear GR protein levels were maintained by 30 min and continued to increase until at least 2 h following GC treatment ($p<0.05$, Figure 3.3A, 3.3C). In PSR lymphoblasts, nuclear GR also increased 15 min after GC addition to the media ($p<0.001$, Figure 3.3B, 3.3D). However, the large influx of GR was short-lived and by 30 min the levels of GR protein decreased to less than half of those observed at 15 min ($p<0.001$, Figure 3.3B, 3.3D). These results were confirmed by immunofluorescence experiments (Figure 3.3E, 3.3F).

***Effects of GC exposure on GSR and PSR lymphoblasts in the presence/absence of VitD***

Having established that GSR and PSR lymphoblasts display differences in GR mRNA, protein and hormone-induced nuclear translocation, we next wished to evaluate the response of these cells to sustained GC exposure. Given that VitD has been suggested to enhance steroid sensitivity we also exposed these cells to VitD alone or in combination with GC.

To determine the long-term effects of GC and/or VitD on GR expression, lymphoblasts were treated with GC and/or VitD for 30 min, 2 and 24 h. We first assessed GR mRNA expression by qRT-PCR. In GSR lymphoblasts, none of the treatments had any effect on the levels of GR mRNA in the early time points (30 min and 2 h). However, after 24 h of GC addition to the media, GR mRNA levels decreased ($p<0.001$, Figure

3.4A). VitD by itself had no effect on GR mRNA expression and when in combination with GC it did not prevent the downregulation of GR. On the other hand, in PSR lymphoblasts 2 h of GC treatment led to decreased levels of GR mRNA, an effect that was maintained after 24 h ($p<0.001$, Figure 3.4B). In these cells VitD prevented the GC-induced downregulation of GR mRNA observed at 2 h ($p<0.05$, Figure 3.4B).

To determine the effects of GC and/or VitD treatments on cellular GR protein levels, Western blots were conducted using total protein extracts. In GSR lymphoblasts, GR protein levels decreased after 2 h of steroid treatment and were even lower after 24 h ($p<0.05$, Figure 3.5A, 3.5C). VitD alone had no effect on the levels of cellular GR protein and when in combination with GC it did not prevent the GC-driven downregulation of GR. In contrast, PSR lymphoblasts displayed reduced levels of GR protein after 30 min of GC addition to the media ($p<0.001$, Figure 3.5B, 3.5C) and reached its lowest point after 24 h of treatment ($p<0.001$). In these cells treatment for 30 min with VitD alone caused a significant increase in the levels of GR protein and when in combination with GC, it prevented the steroid-induced reduction in GR protein levels.

***GC effects on GR nuclear influx in the presence/absence of VitD***

Given that PSR lymphoblasts displayed a faster downregulation of GR in response to GC than GSR lymphoblasts and that VitD seemed to modify this response, we next wished to determine the effects of these agents on GR nuclear influx. Nuclear extracts were obtained from GSR and PSR lymphoblasts after treatment with GC and/or VitD for 30 min, 2 and 24h. Consistent with our previous experiment, steroid treatment caused a large influx of GR into the nucleus of GSR lymphoblasts that peaked at 2 h after the addition of steroids to the media ($p<0.001$, Figure 3.6A, 3.6C). In PSR lymphoblasts GC also caused an increase in GR nuclear protein, but the levels never exceeded those

observed at 30 min ($p<0.001$, Figure 3.6B, 3.6C). VitD by itself had no effect on GR nuclear levels in lymphoblasts derived from neither GSR nor PSR children. However, in PSR lymphoblasts only, treatment for 30 min with GC and VitD led to higher levels of nuclear GR than those observed in cells treated with GC alone ($p<0.001$, Figure 3.6B, 3.6C), suggesting that VitD could prolong the initial influx of GR observed in these cells.

To determine if VitD enhanced GR translocation in PSR lymphoblasts by increasing the expression of the nuclear transport receptor IPO13, we assessed the levels of this importin by qRT-PCR and Western blot after GC and/or VitD treatment. No significant differences in the levels of IPO13 mRNA or protein were found neither between GSR and PSR lymphoblasts at baseline or after treatment with GC and/or VitD (data not shown).

***GC fails to enhance VDR mRNA expression in PSR lymphoblasts***

Given our intriguing findings that VitD appeared to modulate GR levels in the presence of steroids in PSR lymphoblasts, we next assessed the impact of these two agents on the VitD receptor (VDR). We conducted qRT-PCR for VDR on cells treated with GC and/or VitD. Treatment with GC for 2 h led to an increase in VDR mRNA levels in GSR lymphoblasts ($p<0.005$, Figure 3.7A). After 24 h treatment with steroids the levels of VDR were lower than in the 2 h treated group but still higher than in control cells ($p<0.05$). On the other hand in PSR cells GCs had no effect on VDR mRNA levels at any time point (Figure 3.7B). VitD treatment had a similar effect on GSR and PSR lymphoblasts and caused an increase in VDR levels only after 24 h ($p<0.001$, Figure 3.7A, 3.7B). VDR mRNA levels in GSR cells treated for 2 h with GC and VitD were higher when compared to controls ($p<0.05$) but were not any different from cells treated with GC alone. However, 24 h combination treated GSR cells had higher VDR mRNA

levels when compared to GC treated cells ($p<0.05$), suggesting that VitD prolongs the GC-induced upregulation of VDR.

Even though in PSR lymphoblasts neither GC nor VitD alone had any effect after 2 h of treatment, when in combination they significantly increased the levels of VDR mRNA ($p<0.05$, Figure 3.7B). Additionally, after 24 h VDR mRNA levels in combination-treated cells were higher than in cells treated with VitD alone ($p<0.05$). Among cells derived from PSR asthmatics a negative correlation was found between the effect of the combination treatment on VDR expression and age of the patient ($p=0.00550$, $r=-0.972$, Figure 3.8).

### 3.5 Discussion

It has been proposed that diminished receptor levels and defective transport into the nucleus could be associated with reduced steroid responsiveness in asthma [57, 70, 71]. We show that even though lymphoblasts derived from PSR asthmatic children had lower GR protein expression than GSR lymphoblasts, they had significantly higher expression of GR mRNA. Peripheral blood mononuclear cells (PBMCs) from severe asthmatics, which tend to be less GC responsive, express higher levels of GRα mRNA at baseline than PBMCs of mild stable asthmatics and healthy individuals [88, 240]. The reduced levels of GR protein without concomitant levels of mRNA, suggest that lymphoblasts derived from PSR children have defective translational and/or post-translational regulation of GR, leading to low amounts of protein. It should be noted that even though we used an anti-GRα/β antibody in all experiments, only one single band corresponding to GRα was detected in Western blots. This is consistent with previous reports that failed to detect the GRβ isoform in PBMCs of healthy and asthmatic individuals [86, 88].

Due to its involvement in vital cellular functions, the GC pathway is tightly controlled to maintain cell homeostasis. It is reasonable to speculate that in face of low amounts of GR, PSR cells might turn on a compensatory mechanism by which transcription of the receptor is stimulated and/or the half-life of the messenger increased, explaining the discrepancy observed between protein and mRNA expression. Furthermore, it is possible that GR mRNA degradation and ongoing protein translation are linked, in a way that when translation is inhibited the mRNA is stabilized [249]. Interestingly, among PSR asthmatics increasing the expression of GR mRNA seemed beneficial since higher mRNA levels correlated to a smaller decline in pulmonary function over time.

GR-induced transcriptional responses are proportional to the number of receptor molecules present in the cell [250]. These results at the cellular level seem to have a physiological effect and are consistent with our findings that increased amounts of GR protein are present in GSR lymphoblasts and correlate with better therapeutic outcome. Although the phenotypic differences between GSR and PSR children could be explained by the levels of GR protein per se, it is unlikely that steroid responsiveness is a result of solely variances in receptor expression.

We also investigated the hormone-induced nuclear translocation of GR, a key step in the normal function of the GC pathway. Interestingly, there was a clear temporal difference in the nuclear localization of GR between GSR and PSR lymphoblasts which was not dependent on the expression of the nuclear transport receptor IPO13. After GC addition to the culture media, GR nuclear levels peaked at 2 h in GSR lymphoblasts and at 15 min in PSR cells. It is possible that the temporal differences in nuclear GR are a reflection of the initial levels of cellular GR protein. While in GSR lymphoblasts there is

sufficient number of receptors available for activation, keeping a constant nuclear influx over time, in PSR cells the reservoir of GR in the cytoplasm gets saturated only after 15 min of treatment. Even though this explanation is supported by the cellular expression levels of GR, we cannot rule out the possibility that the observed differences in nuclear translocation are a result of dysregulation of import receptors other than IPO13. Several other nuclear transport receptors, including importin 7 and importin α/β, have been shown to be able to transport GR into the nucleus [14].

GSR and PSR lymphoblasts not only show a difference in baseline GR expression but more importantly they display variances in the homologous downregulation of the receptor mRNA and protein. Consistent with previous reports, steroid treatment led to decreased expression of GR, which occurred earlier and was more pronounced at the protein than at the mRNA level [79]. This is not surprising, homologous downregulation of the receptor involves direct destabilization of the existing protein while decreased levels of GR mRNA are believed to be mainly a result of reduced transcription [79, 251]. We show that GR expression decreased more rapidly in cells derived from PSR asthmatics at both the protein and mRNA levels, suggesting that the negative feedback mechanism in PSR children has a lower GC threshold for activation. Even though the existence of this mechanism is necessary to limit the effects of long-term exposure to the hormone, if it gets activated too soon it could be maladaptive, making cells unresponsive to further steroid treatment [79]. PSR lymphoblasts have low expression of GR protein and when treated with steroids these levels decrease at higher rates than in GSR cells, affecting the bioavailability of active GR in the nucleus. These results further support the hypothesis that the differences in GR nuclear influx between GSR and PSR lymphoblasts

is a result mainly of receptor levels in the cytoplasm and not a consequence of defects on the actual nuclear translocation mechanism.

Steroid treatment leads to a twofold decrease in GR protein half-life reducing cellular responsiveness to ligand [79, 252]. Residues in the PEST degradation motifs of GR get ubiquitinated upon GC binding, targeting the receptor for rapid degradation by the proteasome [51, 53]. Other studies have provided further evidence for the involvement of the proteasomal system in limiting GC responsiveness; for example, treatment with a proteasome inhibitor increases GR transactivation and overcomes steroid resistance in certain cancer cells [51, 253, 254]. Given the epidemiological evidence that VitD enhances steroid responsiveness we were interested in exploring the molecular mechanism by which VitD might improve GC sensitivity. Despite our small sample size, we show that in this group of PSR lymphoblasts VitD prevents the early ligand-induced reduction in GR expression, increasing the levels of the receptor in the nucleus of PSR lymphoblasts. Interestingly, it has been suggested that VitD represses the ubiquitin-proteasome system [255]. In our study, treatment with VitD alone for 30 min led to a significant increase in GR protein only in cells derived from PSR children, suggesting that there is ongoing GR degradation at the resting state and that VitD might increase receptor protein levels by preventing degradation.

Extensive research on the co-regulation of the VitD and GC pathways has been done in other systems, where steroids stimulate VDR expression, enhancing VitD effects [256]. Herein we report that GC induces VDR expression in lymphoblasts derived from GSR children, but fails to do so in PSR lymphoblasts even after 24 h treatment. In some cell lines, GCs regulate VDR mRNA levels by increasing VDR *de novo* transcription in a GR-dependent manner [256]. Our findings suggest a similar mode of regulation in

lymphoblasts. Interestingly, in PSR cells GC induction of VDR is regained when VitD is present in the media, supporting the idea that lower levels of GR protein are in part responsible for the steroid insensitivity of PSR children and that VitD modulates this response. Recently Goleva and coworkers reported that serum VitD levels were associated to steroid requirement and GC responsiveness *in vitro* only in pediatric and not adult asthma, and that these associations were stronger in younger children [257]. Consistent with this report, despite our limited numbers, we found a strong negative correlation between age of PSR asthmatics and the levels of VDR expression after combination treatment. These results suggest that VitD supplementation might have a beneficial effect on steroid responsiveness in pediatric asthma by increasing GR stability and enhancing the positive GR/VDR co-regulation, particularly in younger children.

The differences between GSR and PSR lymphoblasts in all parameters assessed were mainly evident during the early time points after treatments. The GC pathway is tightly regulated and it is likely that small variations on GR expression/translocation early on have a crucial effect on normal steroid response. Despite our limited sample size, our results support the idea that steroid insensitivity in asthmatic children could partially be a result of increased degradation of GR, a defect that becomes more apparent after steroids induce the downregulation of the receptor. VitD in this scenario seems to have a positive effect on PSR cells by stabilizing the levels of GR protein. This is of extreme interest, since it provides molecular evidence of the possible therapeutic properties of VitD on steroid insensitive pediatric asthma.

**Figure 3.1 (A)** Relative GR mRNA expression by qRT-PCR in lymphoblast cell lines of good (GSR) and poor steroid responders (PSR). Means (n=5) are presented ± SEMs, *$p<0.05$. **(B)** Correlation between the change in $FEV_1$ following 12 month of inhaled steroid treatment and baseline GR mRNA levels in lymphoblasts from PSR children, $p=0.0171$, $r=0.983$, $n=5$.

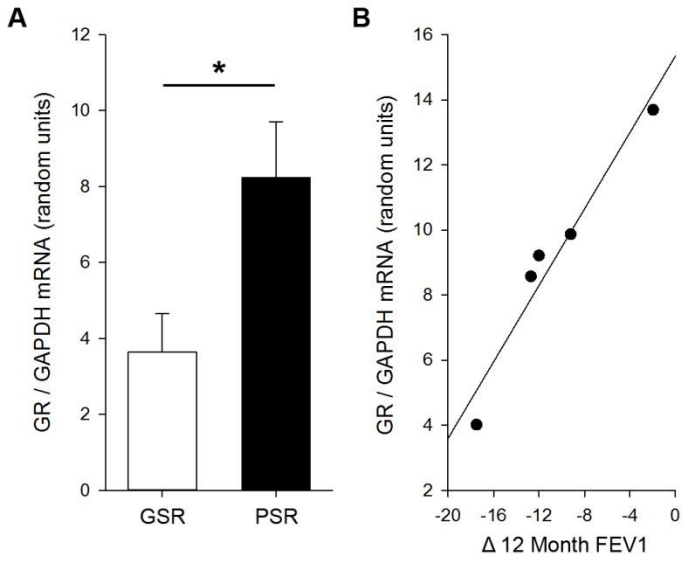

A
B
GR / GAPDH mRNA (random units)
*
GSR
PSR
Δ 12 Month FEV1

**Figure 3.2 (A)** Relative GR protein expression by Western blot in lymphoblast cell lines of good (GSR) and poor steroid responders (PSR). Means (n=5) are presented ± SEMs, *p<0.05. Representative Western blot showing baseline GR protein levels in GSR and PSR lymphoblasts. GAPDH was used as a loading control. Correlation between the change in $FEV_1$ following **(B)** 2 or **(C)** 12 months of inhaled steroid treatment and baseline GR protein levels in GSR ( ▲ ) or PSR ( ● ) lymphoblasts. p=0.03, r=0.672 and p=0.0319, r=0.676 respectively, n=10.

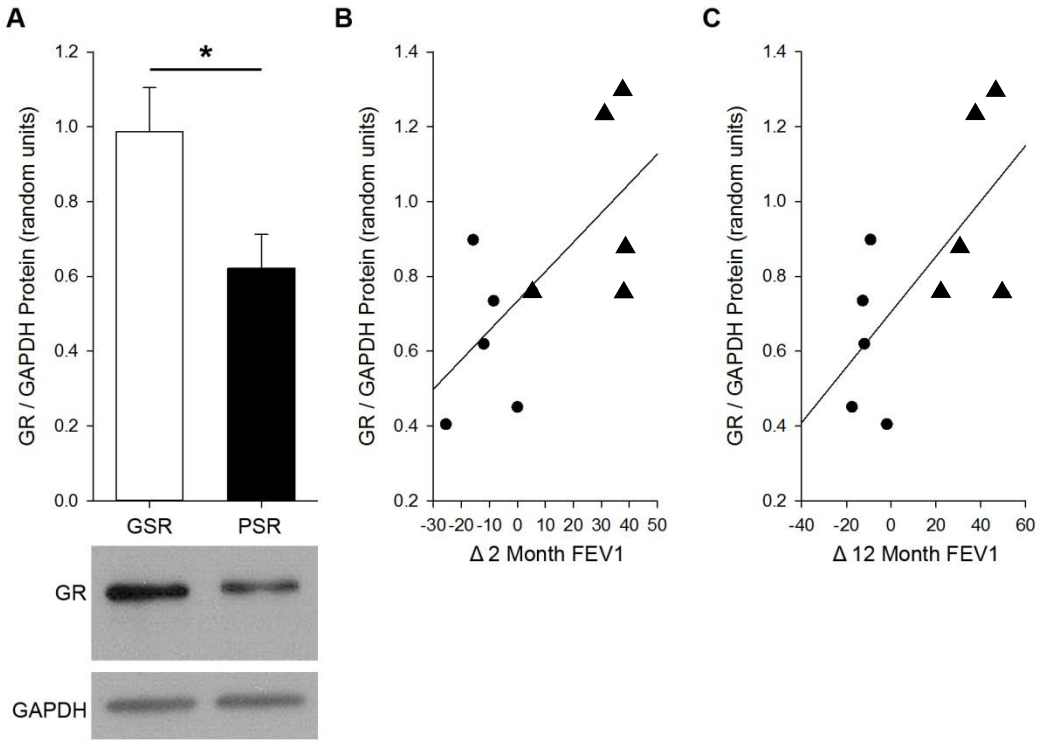

A
B
C
*
GR / GAPDH Protein (random units)
GSR
PSR
GR
GAPDH
Δ 2 Month FEV1
Δ 12 Month FEV1

**Figure 3.3** Nuclear GR protein levels over time by Western blot in **(A)** GSR and **(B)** PSR lymphoblast cell lines following glucocorticoid (GC) treatment (solid bars) and respective controls (open bars). Means (n=5) are presented ± SEMs, *$p<0.05$, **$p<0.001$ as compared to respective control or as indicated. Representative Western blot for nuclear GR protein in **(C)** GSR and **(D)** PSR lymphoblasts following GC treatment. TBP was used as a nuclear protein loading control. Representative immunofluorescence image showing GR (red color) translocating into the nucleus (blue color) of **(E)** GSR and **(F)** PSR lymphoblasts after 0, 0.25, and 2 h after the addition of GC to the media.

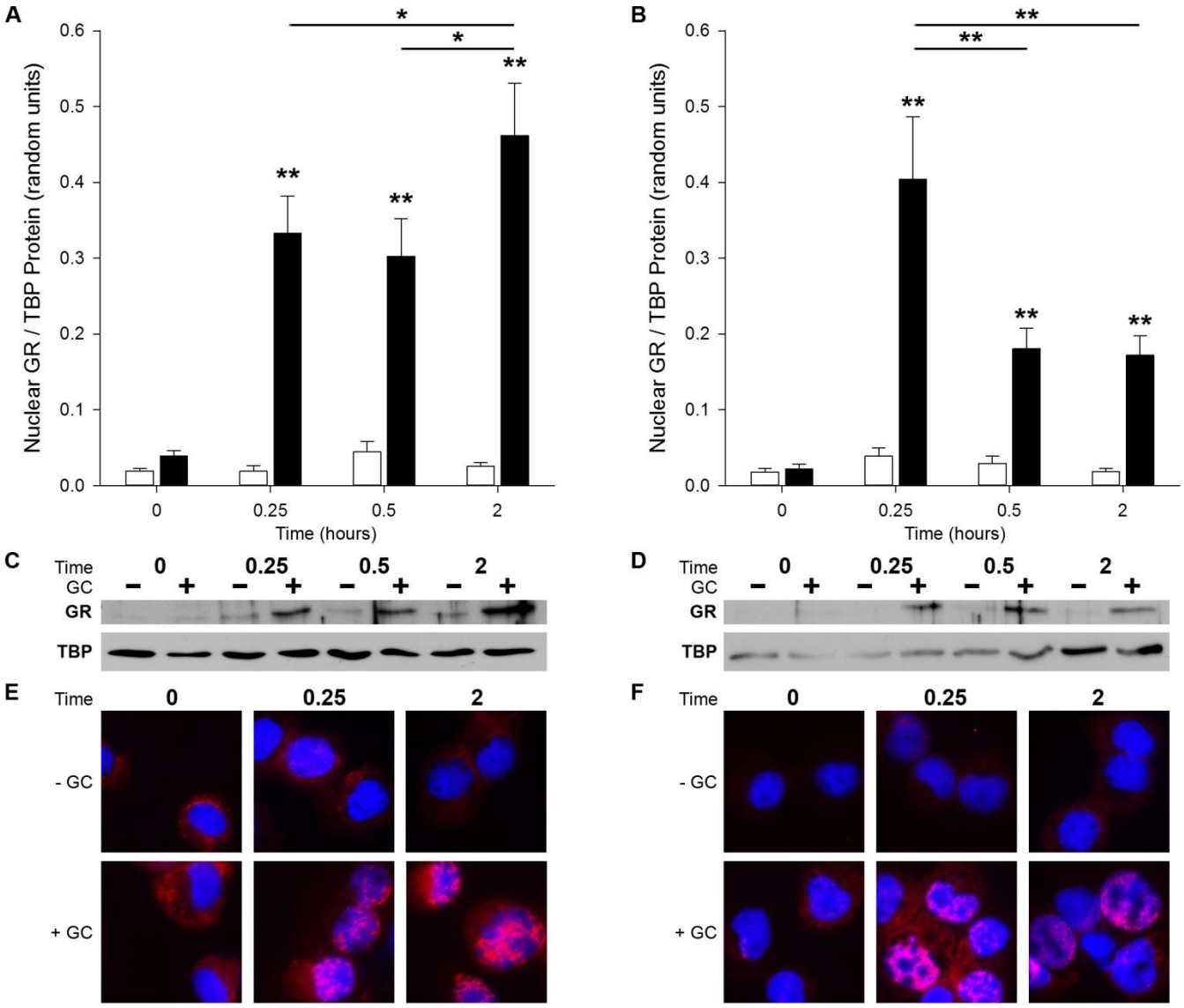

A
B
Nuclear GR / TBP Protein (random units)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
0.25
0.5
2
Time (hours)
*
**
C
D
Time
GC
GR
TBP
E
F
- GC
+ GC

**Figure 3.4** Fold change of GR mRNA from time 0 by qRT-PCR in **(A)** GSR and **(B)** PSR lymphoblast cell lines following treatment with glucocorticoid (GC), vitamin D (VitD) or both for 0.5, 2 and 24 h. Means (n=5) are presented ± SEMs, *$p<0.001$ as compared to respective control (CTL), ‡$p<0.05$ as compared to GC treated cells.

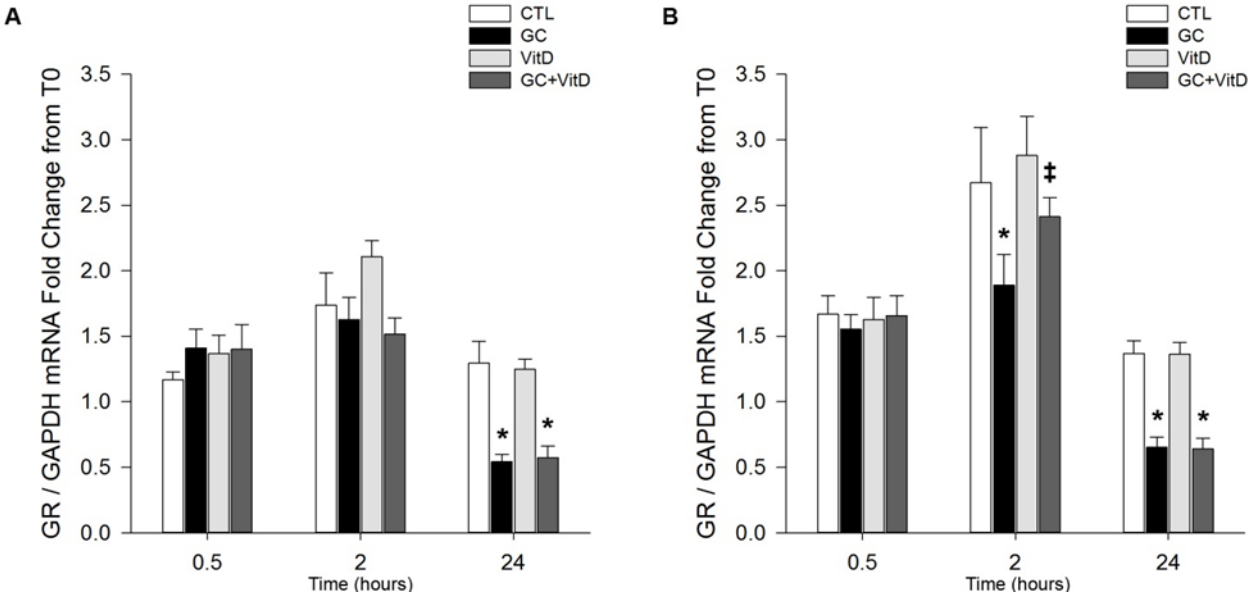

A
CTL
GC
VitD
GC+VitD
GR / GAPDH mRNA Fold Change from T0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.5
2
24
Time (hours)
*
*
B
CTL
GC
VitD
GC+VitD
GR / GAPDH mRNA Fold Change from T0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.5
2
24
Time (hours)
*
‡
*
*

**Figure 3.5** Fold change in total cellular GR protein expression from time 0 by Western blot in **(A)** GSR and **(B)** PSR lymphoblast cell lines following 0.5, 2, and 24 h treatment with glucocorticoid (GC), vitamin D (VitD) or both. Means (n=5) are presented ± SEMs, *$p<0.05$, ‡$p<0.001$ as compared to respective control (CTL). **(C)** Representative Western blot for total GR in cellular extracts of GSR and PSR lymphoblasts after 0, 0.5, 2, and 24 h treatment with GC, VitD or both. GAPDH was used as a loading control.

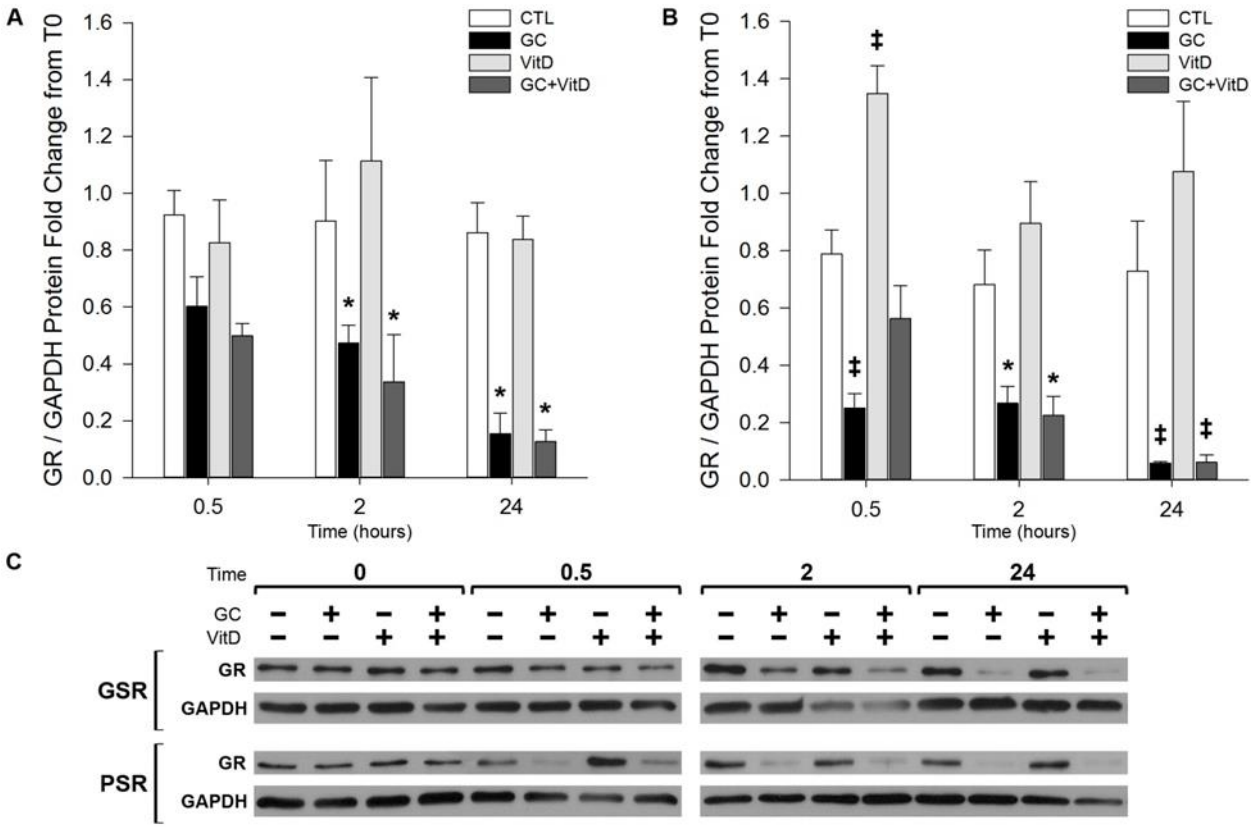

A
B
C
CTL
GC
VitD
GC+VitD
GR / GAPDH Protein Fold Change from T0
Time (hours)
0.5
2
24
Time
0
0.5
2
24
GSR
PSR
GR
GAPDH

**Figure 3.6** GR protein levels over time by Western blot in nuclear extracts from **(A)** GSR and **(B)** PSR lymphoblast cell lines following treatment with glucocorticoid (GC), vitamin D (VitD) or both. Means (n=5) are presented ± SEMs, *p<0.05, **p<0.005, ***p<0.001 as compared to respective control (CTL) or ‡ p<0.001 as compared to GC treated cells. **(C)** Representative Western blot for GR in nuclear extracts of GSR and PSR lymphoblasts after 0, 0.5, 2, and 24 h treatment with GC, VitD or both. TBP was used as a nuclear protein loading control.

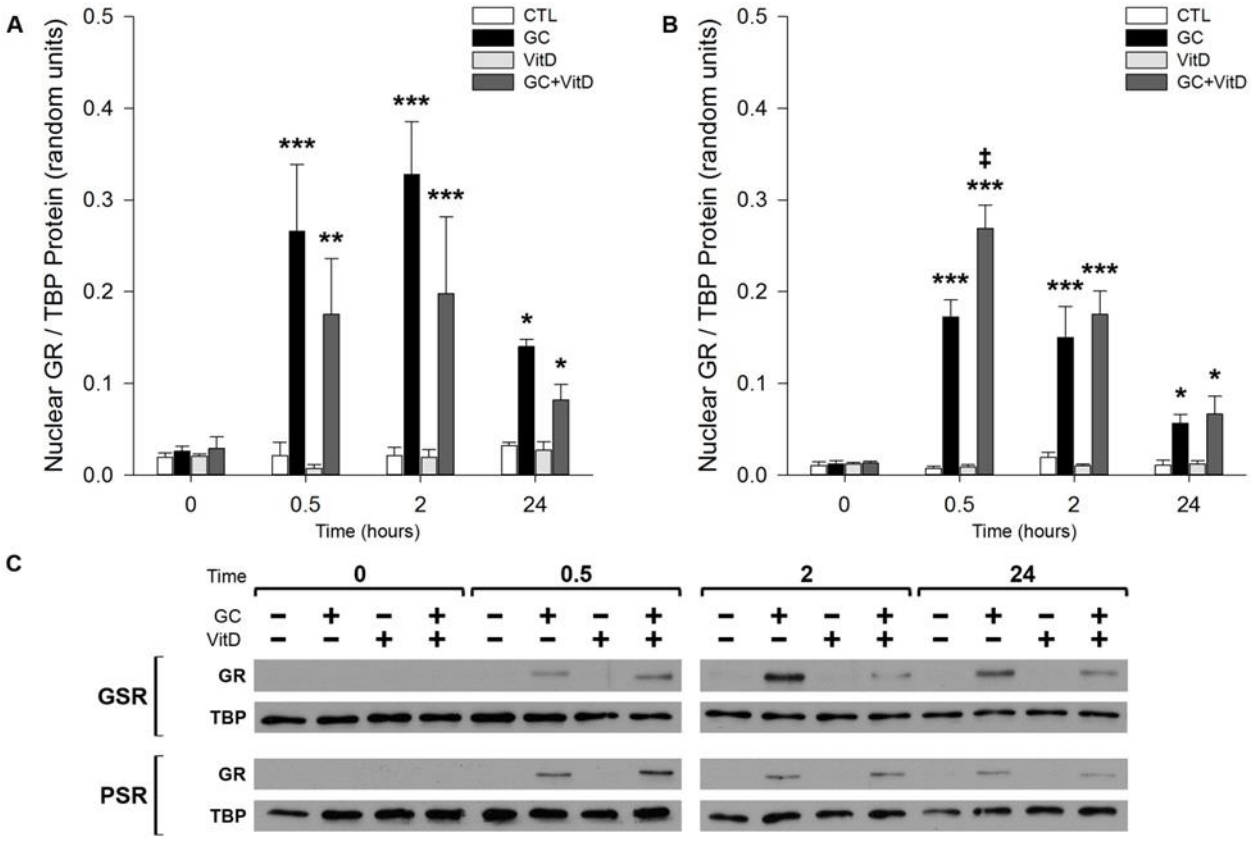

A
B
C
CTL
GC
VitD
GC+VitD
Nuclear GR / TBP Protein (random units)
Time (hours)
0
0.5
2
24
Time
GC
VitD
GSR
PSR
GR
TBP

**Figure 3.7** Fold change of VDR mRNA from time 0 by qRT-PCR in **(A)** GSR and **(B)** PSR lymphoblast cell lines following glucocorticoid (GC), vitamin D (VitD) or combination treatments. Means (n=5) are presented ± SEMs, *p<0.05, **p<0.005, ***p<0.001, as compared to respective control (CTL), ‡p<0.05, §p<0.001 as compared to GC treated cells.

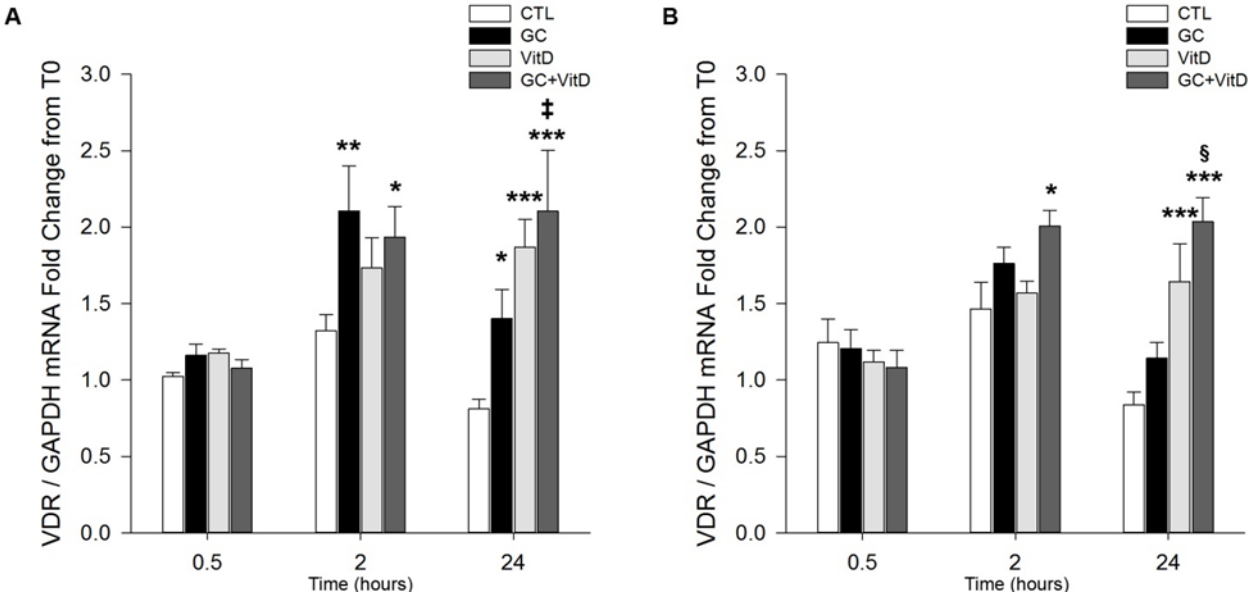
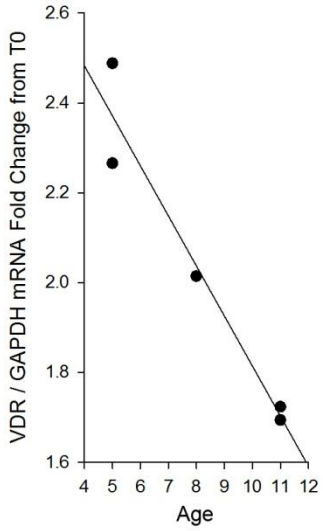

A
B
CTL
GC
VitD
GC+VitD
VDR / GAPDH mRNA Fold Change from T0
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.5
2
24
Time (hours)
**
*
*
***
***
‡
*
***
§

**Figure 3.8** Correlation between the fold change in VDR mRNA from time 0 and the age of PSR patients. p=0.00550, r=-0.972, n=5.


VDR / GAPDH mRNA Fold Change from T0
2.6
2.4
2.2
2.0
1.8
1.6
4
5
6
7
8
9
10
11
12
Age

*In Chapters II and III possible mechanisms of steroid insensitivity in an animal model of asthma and in lymphoblasts from asthmatic children were explored. As stated in the introductory chapter, GC plays an important regulatory role in late phase lung organogenesis and variations in steroid function during this time period might have long lasting effects in respiratory risk. The final objective of this thesis was to study steroid responsiveness during a critical time in lung development. Additionally, since the results presented in Chapter III suggest that VitD modulates GC responsiveness in pediatric asthma, I investigated the molecular interaction between these two agents using primary lung epithelial cell cultures derived from rat strains with different respiratory profiles. These results are presented in the following chapter.*

## CHAPTER IV:

# Variant Steroid Response in Developmental Airway Epithelium of Normoresponsive and Atopic Rats

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## 4.1 Abstract

Perturbation of gene expression during critical developmental windows may alter lung development, in turn affecting long-term lung function and risk for pulmonary disease. Glucocorticoids (GCs) regulate gene expression in late lung development. Vitamin D (VitD) modulates the effects of GCs in the developing lung. While much is known about the effects of variable steroid responsiveness in the treatment of pulmonary disease – including asthma and postnatal bronchopulmonary dysplasia – little is known about how variable GC responsiveness affects the developing lung. In the current study we investigated the molecular interactions between GC and VitD in primary lung cell cultures isolated from postnatal alveolarizing lungs of atopic and normoresponsive rat pups.

Primary airway epithelial cells were isolated from postnatal day 14 Lewis (control) and Brown Norway (BN, atopic) rats and exposed to hydrocortisone ($10^{-6}$M) and/or $1\alpha,25(OH)_2D_3$ ($10^{-8}$M) over time. Effects of these treatments on mRNA levels were assessed by qRT-PCR for the GC receptor (GR), importin (IPO)13, VitD receptor (VDR), and Ly6B (a cell surface antigen linked with the BN atopic phenotype). Cellular and nuclear GR levels were assessed by Western blot.

BN epithelial cells showed lower GR levels than Lewis cells. Both GC and VitD stimulated GR and IPO13 expression in BN but not in Lewis cells. However, when added to GC, VitD reduced the GC-induced GR and IPO13 expression. Ly6B levels increased in response to GC only in BN cells and VitD attenuated this effect. VitD also suppressed GC-induced GR nuclear translocation in cells of both strains.

GC responsiveness and VitD modulation of this response in epithelial cells of the developing lung is rat strain-specific. The developmental epithelium of the atopic rat

seems more GC sensitive and VitD attenuates steroid response in this model. Our results suggest that variable GC responsiveness associated with altered GR nuclear levels are likely to impact lung development, lung function and long-term pulmonary risk.

**4.2 Introduction**

Early effects on lung development are believed to influence pulmonary risk. Existing evidence shows that lungs of children who will become asthmatics have pre-existing structural and functional abnormalities [1]. Important morphological changes during late phase lung development are regulated by a wide range of molecules, including glucocorticoids (GCs). GCs, via the GC receptor (GR), stimulate alveolar wall thinning, epithelial cell differentiation and surfactant synthesis [258]. Even though progress has been made in understanding the mechanisms responsible for variable GC responsiveness in the context of inflammation and immunity, very little is known about how normal lung development proceeds in the face of steroid insensitivity. Among the proposed molecular mechanisms for variable GC response in asthma are: irregular GR numbers and altered nuclear translocation of GR, possibly involving a dysregulation of nuclear transport receptors such as importin 13 (IPO13) (Reviewed in [55]).

Vitamin D (VitD), an important modulator of the immune system, has also been implicated in late phase lung development [150, 259]. The role of VitD however, is less well understood. VitD is believed to regulate epithelial cell maturation during lung growth and increase surfactant production [143]. Further evidence for a role for VitD in lung organogenesis comes from work of Zosky and colleagues, who demonstrated that VitD deficiency during development in mice leads to altered lung structure and deficits in lung function [142]. Interestingly, epidemiological studies suggest that VitD enhances steroid responsiveness [213, 214, 257, 260]. In a more recent study we showed that VitD

modifies the GC response in lymphoblasts derived from patients in a pediatric asthma cohort [261].

Our laboratory has focused on the impact of gene-environment interactions in the developing lung in asthma susceptibility. We showed that distinct developmental respiratory phenotypes and highly diverse transcriptome signatures distinguish rat models of atopy from normoresponsive rats [219]. In the present study we use primary airway epithelial cells derived from postnatal day (PN)14 Lewis (normoresponsive) and Brown Norway (BN, atopic) pups to evaluate steroid responsiveness during late phase lung development. Additionally, we explored the modulatory role of VitD on steroid function during this developmental period. We hypothesized that the demonstrated genetic differences between rat strains would influence "normal" GC responsiveness and that VitD would have a modulatory role in steroid action.

We show that GC responsiveness in lung epithelial cells isolated from these rat strains is highly variable and that VitD modulation of this response is strain dependent. We postulate that the complex interaction between GC and VitD during late phase lung development will impact epithelial cell differentiation, which in turn may also influence the asthmatic and GC-responsive phenotypes later in life.

## 4.3 Materials and Methodology

### *Rat Lung Primary Cell Culture*

All procedures involving animals were conducted according to criteria of the Animal Care Committees of the Canadian Council and the Montreal Children's Hospital Research Institute, McGill University Health Centre (Montreal, QC, Canada). PN14 Brown Norway and Lewis rats were purchased from Charles-River Laboratories. Isolation and culture of primary rat lung epithelial cells were performed as we previously

described [262]. Each culture represented pooled lungs from at least four animals. Lung cell cultures were routinely tested for purity, as assessed by positive staining for cytokeratin (mouse monoclonal antibody; Santa Cruz Biotech., Santa Cruz, CA), and negative staining for vimentin (monoclonal antibody; Sigma-Aldrich, Oakville, ON, Canada).

***Cell Treatment Protocols***

Epithelial cells were grown in 100mm plates and allowed to reach 75-80% confluence in MEM-EBSS (Invitrogen, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT), penicillin (100 units/mL) and streptomycin (100 µg/mL) (Invitrogen). Cells were then serum-starved for 24 h in MEM-EBSS media containing 10% charcoal-stripped FBS (HyClone), penicillin (100 units/mL) and streptomycin (100 µg/mL) before being treated with hydrocortisone ($10^{-6}$M), 1α,25-Dihydroxyvitamin $D_3$ ($10^{-8}$M) or both for 0.5, 2 and 24 h (Sigma-Aldrich). Control plates contained equal volumes of ethanol in 10% charcoal stripped FBS-media ($n \geq 4$).

***RNA Isolation and Quantitative Real-Time RT-PCR***

Cells were lysed using 1ml Trizol reagent (Invitrogen) and total RNA was isolated following manufacturer's protocol. RNA was resuspended in RNASecure reagent (Ambion, Austin, TX, USA) and traces of DNA were removed using the Turbo DNase-free kit (Ambion). Quantitative real-time RT-PCR (qRT-PCR) was performed on the Mx4000 QPCR system (Stratagene, La Jolla, CA) with the QuantiTect SYBR green RT-PCR kit (Qiagen, Mississauga, ON, Canada) as previously described [219]. Gene-specific primers for SYBR green detection of GR, IPO13, vitamin $D_3$ receptor (VDR), lymphocyte antigen 6-B (Ly6B), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed. Synthesis of cDNA was prepared from an initial 200 ng/ml of

RNA. RNA was incubated at 65°C for 5 min with 1 mg/ml of random primers and 10 mM dNTPs. To this, 5X first-strand buffer, 1 mM DTT, 1 ml of RNase OUT and 1 ml of Superscript II (Invitrogen) was added and incubated at 42°C for 1 h and at 70°C for 15 min. RT-PCR was performed in 25 ml reactions for 40 cycles with 1 µl of cDNA. Results were analyzed using the delta-delta cycle threshold method ($n \geq 4$ for all assays).

***Protein Isolation and Western Blots***

Total protein was isolated using KPO4 buffer and nuclear and cytoplasmic fractions were obtain using buffers provided in NE-PER® kit (Pierce Biotechnology, Rockford, IL) and following manufacturer's instructions. Fifteen µg of total protein or nuclear fraction was boiled for 5 min in SDS loading buffer before being electrophoresed on 7.5% SDS-polyacrylamide gels and then transferred to PVDF membrane (Biorad, Hercules, CA). Membranes were blocked overnight at 4°C with 10% milk/PBS–Tween and probed with anti-GR, anti-GAPDH (total loading control), and anti-TBP (nuclear loading control; Santa Cruz Biotech.) for 1 h. After several washes in PBS-Tween buffer, membranes were incubated with HRP-conjugated secondary antibodies. The ECL Plus Western Blotting Detection System was used for protein detection (GE Healthcare, Buckinghamshire, UK).

***Promoter Analysis***

DNA sequence of the IPO13 (1606bp) and Ly6B (1102) promoters were obtained (University of California at Santa Cruz genome tools, Nov. 2004 (Baylor 3.4/rn4) assembly) and a TRANSFAC® matrix match analysis was conducted (Biobase Corp., Beverley, MA) to identify putative transcription factor binding sites using the Biobase "vertebrate, non-redundant, minimize false positives" matrices and cut-offs.

### *Statistical Analysis*

All data are presented as mean ± SEM. Statistical significance was determined by either Student's t-test or two-way Analysis of Variance (ANOVA) as appropriate. After ANOVA, pair-wise group comparisons were assessed using Student-Neuman-Keuls test. Significance was defined as $p<0.05$.

## 4.4 Results

### *GR expression/regulation in developmental airway epithelial cells of Lewis and BN rats*

In rat development, "bulk alveolarization" is completed by PN14 and is followed by a surge in circulating GCs [263]. To evaluate the effects of steroids and VitD on developmental epithelial cells, primary cultures were derived from PN14 Lewis and BN rats and treated with GC, VitD or both for 0.5, 2 and 24 h. We first assessed the effects of these treatments on GR expression by qRT-PCR. Baseline GR mRNA levels were lower in BN compared to Lewis epithelial cells ($p<0.05$, Figure 4.1A). Treatments for 0.5 h with GC and/or VitD did not alter the levels of GR mRNA in either of the rat strains studied (Figure 4.2).

PN14 Lewis lung epithelial cells incubated for 2 h with GC, VitD or both showed decreased expression of GR mRNA when compared to controls ($p<0.05$, Figure 4.2A). Cells treated for 24 h with GC or VitD recovered from the initial suppression of GR and showed mRNA levels similar to that of Lewis controls. Interestingly, GR mRNA levels in cells exposed to the combination treatment remained significantly lower ($p<0.05$). Thus at 24 h, VitD appeared to have an effect in prolonging the GC-inhibition of GR observed at 2 h.

Incubation of BN cells with GC and/or VitD for 2 h had no effect on GR mRNA expression. Following treatment for 24 h with GC ($p<0.001$, Figure 4.2B) and/or VitD ($p<0.05$) GR mRNA expression increased. However, the stimulation observed in the VitD and combination treated cells was significantly lower than that of GC alone ($p<0.05$), suggesting that VitD attenuates the GC-induced GR expression.

Western blots for total cellular GR were conducted at all the different time points. Consistent with baseline mRNA measurements, untreated Lewis cells showed higher GR protein levels when compared to untreated BN controls ($p<0.001$, Figure 4.1B). Neither GC and/or VitD led to a significant difference in GR protein levels at the early time points (0.5 and 2 h) in any of the strains studied (data not shown). However, all treatments decreased GR protein levels in the Lewis cells after 24 h ($p<0.001$, Figure 4.3), paralleling the GR mRNA expression at 2 h. The reduction in GR protein levels was more profound in GC or combination treated cells than in cells exposed to VitD alone ($p<0.05$). In BN epithelial cells VitD treatment had no effect on GR protein levels. However, GC and the combination treatments led to a decrease in GR protein ($p<0.05$), pointing out a disparity between levels of mRNA and protein, and suggesting increased protein degradation in the presence of GC.

***VitD modifies GC regulation of IPO13 mRNA in developmental airway epithelium***

Given that GC and VitD differentially regulate the expression of GR in Lewis and BN developmental epithelial cells, we wanted to assess the effects of these agents on IPO13 expression. No significant changes in IPO13 mRNA expression were observed at 0.5 and 2 h in response to GC and/or VitD in cells of either strain (Figure 4.4). After 24 h treatment, the IPO13 expression profiles in response to GC and/or VitD were similar to

those observed for GR expression. Lewis epithelial cells treated with the combination of GC and VitD had reduced levels of IPO13 mRNA when compared to controls ($p<0.001$, Figure 4.4A). In BN cells, all treatments led to an increase in IPO13 mRNA expression. However, the effect on cells treated with GC ($p<0.001$) was greater than in VitD or combination treated cells ($p<0.05$, Figure 4.4B). Similar trends of IPO13 protein expression were detected by Western blots (data not shown)

***VitD attenuates the GC-driven nuclear translocation of GR***

Having determined that GC and VitD alter the expression of IPO13, we decided to further investigate if these expression changes led to variations in the GC-induced nuclear translocation of GR. The effects of the different treatments on the nuclear localization of GR were similar in epithelial cells of the two rat strains studied. Treatment for 0.5 h with GC alone or in combination with VitD led to a large nuclear influx of GR, which decreased in magnitude by 2 h (data not shown). There was no difference in the levels of nuclear GR between GC and combination treated cells of the two rat strains. However, after 24 h of treatment, the level of nuclear GR was significantly higher in GC treated cells compared to combination treated cell, suggesting that VitD attenuates the GC-driven nuclear translocation of GR (Figure 4.5).

***VitD enhances the GC-induced expression of VDR***

Having shown that GC and VitD have different effects on GR expression in the cells of the two rat strains studied, we were interested in investigating if these agents also led to differential regulation of the VitD receptor (VDR) expression. Treatment for 24 h with GC and/or VitD led to increased expression of VDR mRNA in both Lewis and BN epithelial cells (Figure 4.6). In Lewis cells the increase was modest and of similar magnitude in the three treatments ($p<0.05$, Figure 4.6A). In BN epithelial cells, VDR

stimulation was higher in the GC-treated compared to the VitD-treated cells ($p<0.05$, Figure 4.6B). Furthermore, the combination treatment had an additive effect leading to a 4 fold increase in the expression of VDR compared to controls ($p<0.001$).

***VitD modulates the GC-induced expression of Ly6B in atopic rat epithelial cells***

We have previously shown that the rat models used in this study have unique respiratory gene expression profiles in the perinatal lung [219]. To further characterize the role of GC and VitD in lung development, we wanted to study their transcriptional effect on a gene previously associated with the atopic rat model. The Ly6 genetic locus controls the expression of a superfamily of antigenic proteins on multiple cell types, including bronchial epithelial cells [264]. Consistent with our previous report, lung epithelial cells of BN express higher levels of Ly6B at baseline when compared to Lewis ($p<0.001$, Figure 4.7). None of the treatments had any effect on the mRNA expression of Ly6B in Lewis epithelial cells (Figure 4.7A). In BN epithelial cells, GC treatment for 24 h led to an upregulation of Ly6B mRNA ($p<0.001$, Figure 4.7B). Even though VitD by itself had no effect, when in combination with GC, it significantly attenuated the GC-induced upregulation of Ly6B ($p<0.05$).

## 4.5 Discussion

Deviations from the normal developmental program could render lungs more susceptible to chronic diseases. Even though the role of steroids in lung growth and maturation is well established, little is known about how variations in GC responsiveness affect these processes. VitD has been implicated in late phase lung development and has been suggested to enhance steroid function. In the current study we used rat primary lung cell cultures to investigate the molecular interactions between GC and VitD in the developing lung.

GC mediates the downregulation of its own receptor in a number of different tissues, including airway epithelial cells [79, 225, 249]. In accordance with these reports, GC-treated Lewis epithelial cells show a temporal decrease in GR mRNA levels which are restored back to normal by 24 h. It is believed that this negative feedback mechanism is present and necessary in normal cells to limit hormone signal and maintain cell homeostasis [225]. In contrast, epithelial cells from the atopic BN rat showed decreased GR levels compared to Lewis and lacked the steroid-induced inhibitory phase on GR mRNA expression. Moreover, following 24 h of GC treatment GR mRNA levels were significantly elevated. A similar mode of GR expression in response to steroids is observed in inflamed tissues [85]. Interestingly, we have previously reported that the BN perinatal lung shows signs of inflammation [219]. In that same study, we demonstrated higher GR expression in whole lung tissue of BN rats compared to Lewis, while here we identify the opposite pattern of expression in PN14 epithelial cells. It is possible that in an intact lung, epithelial GR levels are influenced by factors of the surrounding mesenchyme. Also, total lung GR expression levels could be a reflection of mainly fibroblast expression. In fact, it has been previously reported that GR levels are much higher in the lung mesenchyme compared to the epithelium [265].

The observed steroid-induced upregulation of GR mRNA in epithelial cells of the atopic rat might seem positive in the inflammatory context of BN lungs. However, GC hypersensitivity during a critical window of development could interfere with proper lung organogenesis. For example, GCs have been given prophylactically to mothers expecting to deliver premature babies and to premature infants to treat bronchopulmonary dysplasia, reducing inflammation, promoting lung maturation and improving short-term lung mechanics [266]. Nevertheless, studies have shown that high levels of steroids disrupt

normal septation, causing a reduction in alveoli numbers and total lung growth [267]. Since steroids have such powerful effects on lung development, tight regulation of GC signalling is probably crucial. Our data suggests that the developing airway epithelium of the atopic rat is more GC sensitive, making it perhaps more prone to the deleterious effects of endogenous steroids. Interestingly, the perinatal lung of BN rats also shows structural changes when compared to the Lewis, including altered epithelial cell proliferation and collagen deposition [219]. In future studies it will be of interest to explore *in vivo* if and how GC affects the normal pulmonary structure of BN rats.

Several studies have suggested that VitD improves GC responsiveness in asthma [210, 213]. However, the mechanism by which VitD exerts its effect is not clear. To our knowledge, we are the first ones to report a modulatory role of VitD on GR expression in airway epithelium and this modulation is rat strain dependent. Interestingly, in cells of both strains VitD had similar effects to that of GC alone. However, after 24 h VitD attenuated the effects of GC on GR expression, suggesting that VitD makes them less steroid responsive.

Comparison of temporal levels of GR mRNA and protein suggests that in Lewis cells decreased protein levels in response to all treatments (24 h) partially reflect earlier changes in mRNA levels. However, GR protein levels are lower in GC-treated cells than in cells exposed to VitD alone. We speculate that while protein levels in the VitD treated cells could result from decreased GR mRNA expression, the levels in the steroid treated groups could probably reflect both decreased mRNA expression and increased protein degradation. In fact, GC-induced proteasomal degradation of GR has been previously proposed in other systems [53]. In contrast to the mRNA/protein expression observed in Lewis cells, BN epithelial cells exposed to GCs show reduced GR protein (24 h) without

changes (2 h) or even elevated mRNA levels (24 h), suggesting that these cells may have a defective translational and/or post-translational regulation of GR.

We also wished to determine if VitD modulates the expression of other genes involved in GC response during lung development. IPO13 is a GC-regulated nuclear transport receptor which facilitates GR nuclear import in developmental airway epithelial cells [15, 268]. Interestingly, IPO13 mRNA expression parallels that of GR after 24 h of treatment in both Lewis and BN cells. In order to clarify if these effects of GC and VitD on IPO13 expression where direct or indirect, we conducted a TRANSFAC® matrix match analysis of the IPO13 promoter. 2 GR and 3 VDR binding sites were detected in this region suggesting that GC and VitD could in theory regulate IPO13 expression by directly affecting its transcription. However, further studies are necessary to determine if these sites are active and it is impossible for us to conclude at this point if this is a direct effect on transcription or a modulation through other genes.

Epithelial cells of both strains exposed for 24 h to the combination treatment displayed lower levels of IPO13 than cells exposed to GC alone, suggesting that VitD could reduce GC responsiveness by attenuating IPO13 expression and affecting GR nuclear translocation. In fact, we observed less nuclear GR in cells treated with GC and VitD than in cells treated only with steroids. Since total cellular GR protein levels where not any different in the GC versus the combination treated cells in either strain, the detected differences in nuclear GR are most likely due to altered translocation in the presence of VitD.

Auto- and cross-regulation of the nuclear receptors are mechanisms to fine-tune hormonal signals in a temporal and tissue-specific manner [269]. GC-induced upregulation of VDR has been demonstrated in adipocytes, kidney and cancer cells [256].

VDR auto-regulation varies among tissues, for example, VitD increases the expression of VDR in kidney and skin but not intestine cells [269]. In the present study we wanted to clarify the interaction between GC, VitD and their receptors in airway epithelial cells during development. All treatments caused an upregulation of VDR mRNA. Interestingly, the combination treatment caused the maximal stimulation and this was most pronounced in cells of the BN rat. These findings suggest the presence of a positive feedback mechanism between GR and VDR in which VitD induces GR expression and this further increases VDR transcription. A crosstalk mechanism between the GC and VitD pathways which potentiates the effects of each other has been proposed in other systems [256].

Given the differential regulation of GR and IPO13 by GC and VitD in BN epithelial cells, we were interested in studying the impact of these agents on the expression of Ly6B, a gene highly expressed in the developing lung of atopic rats and which gets further stimulated when mothers are exposed to allergen (unpublished data) [219]. Very little is known about the exact function of Ly6B; however, increased expression of this gene has been observed during the acute phase of the inflammatory response which subsides as inflammation is resolved [270]. Ly6B was found significantly elevated in epithelial cells of the BN strain, confirming our previous report linking this gene with this asthma-associated phenotype [219]. Members of the Ly6B family including secreted mammalian Ly-6/urokinase-type plasminogen activator receptor-related protein (SLURP)-1 and SLURP-2, have been shown to be expressed in bronchial epithelial cells and to enhance cell survival [264, 271]. Interestingly, in BN epithelial cells GC treatment leads to a further increase in Ly6B expression, possibly altering even more the normal turnover of pulmonary cells and inducing morphological changes in the developing lung. No GR binding sites were detected in the Ly6B promoter, suggesting

that GC regulation of this gene is probably indirect. The attenuating effects of VitD on steroid response also seem reflected on Ly6B expression, since combination treated cells express significantly less Ly6B than cells treated with GC alone.

In conclusion, we show that GC responsiveness in lung epithelial cells of two rat strains with different pulmonary phenotypes is highly variable and this variability is likely due to unique respiratory gene expression profiles in the perinatal lung. Furthermore, we demonstrate the ability of VitD to modulate the GC response during late phase lung development in a strain dependent manner. Our results suggest that the epithelium of the atopic rat is more GC sensitive and VitD effects on gene expression lead to an attenuation of steroid response. We speculate that the observed variation in GC responsiveness during a critical window of development could potentially contribute to long term lung function and subsequent disease risk.

**Figure 4.1** GR Expression in primary developmental lung epithelial cells. **(A)** Baseline relative GR mRNA expression by qRT-PCR in cells from Lewis and Brown Norway (BN) rat strains. **(B)** Total cellular GR protein expression by Western blot. A representative blot for GR is shown. GAPDH was used as a protein loading control. *$p<0.05$, **$p<0.001$, $n\geq4$.

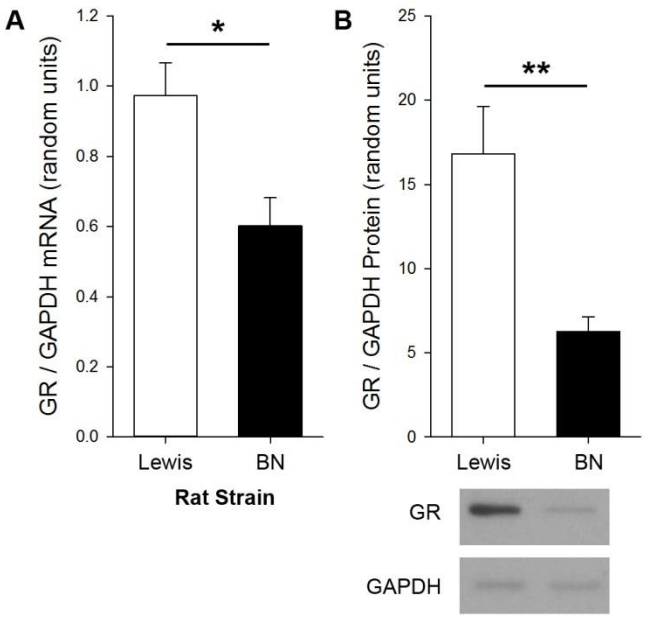

A
B
GR / GAPDH mRNA (random units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Lewis
BN
Rat Strain
GR / GAPDH Protein (random units)
25
20
15
10
5
0
**
Lewis
BN
GR
GAPDH

**Figure 4.2** Relative GR mRNA expression over time by qRT-PCR in primary lung epithelial cells of the **(A)** Lewis and **(B)** Brown Norway rat strains following glucocorticoid (GC), vitamin D (VitD) or combination treatments. **$p<0.001$, *$p<0.05$ as compared to respective control (CTL) or as indicated. $n \geq 4$.

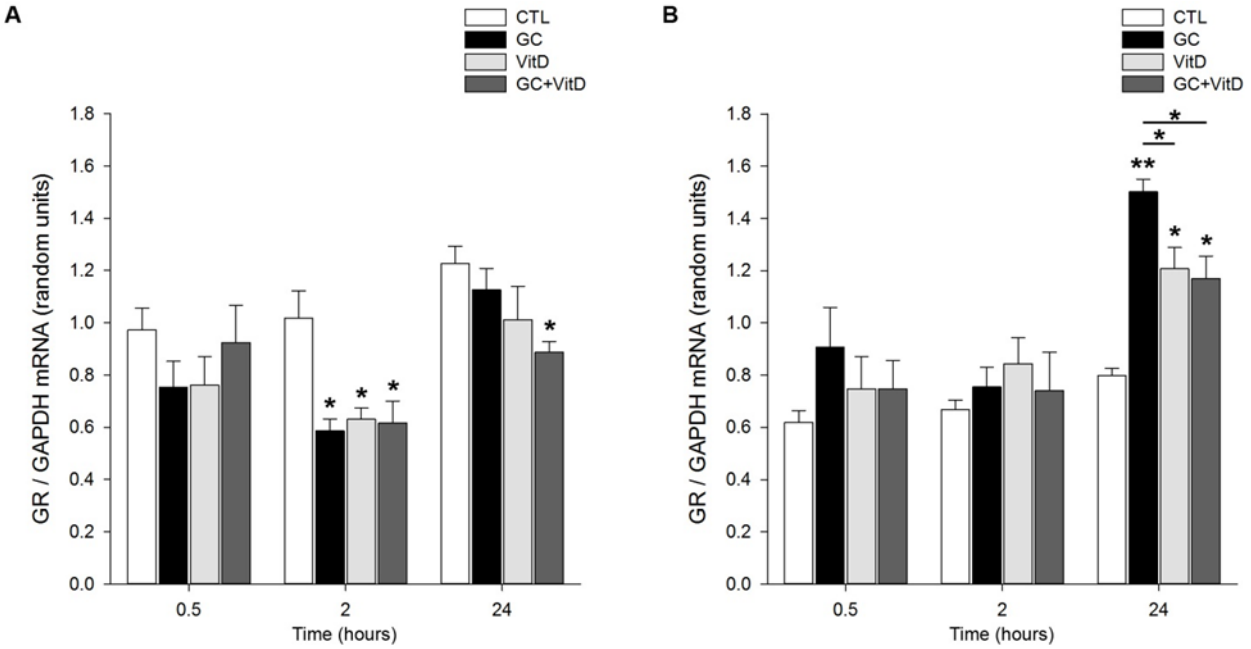

A
CTL
GC
VitD
GC+VitD
GR / GAPDH mRNA (random units)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.5
2
24
Time (hours)
*
*
*
*
B
CTL
GC
VitD
GC+VitD
GR / GAPDH mRNA (random units)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.5
2
24
Time (hours)
*
*
**
*
*

**Figure 4.3 (A)** Total cellular GR protein expression by Western blot in primary lung epithelial cells of the Lewis and Brown Norway (BN) rat strains following 24 h treatment with glucocorticoid (GC), vitamin D (VitD) or both. **$p<0.001$, *$p<0.05$ as compared to respective control (CTL), $n\geq4$**. (B)** A representative Western blot for GR in total protein extracts of primary airway epithelial cells after 24 h treatment with GC, VitD or both. GAPDH was used as a protein loading control.

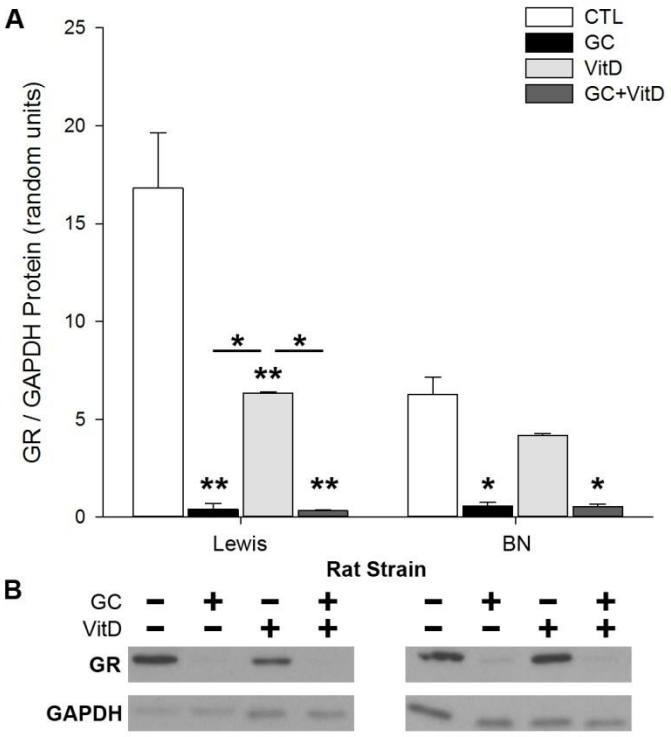

A
CTL
GC
VitD
GC+VitD
GR / GAPDH Protein (random units)
25
20
15
10
5
0
*
*
**
**
**
*
*
Lewis
BN
Rat Strain
B
GC
VitD
GR
GAPDH

**Figure 4.4** Relative IPO13 mRNA expression over time by qRT-PCR in primary lung epithelial cells of the **(A)** Lewis and **(B)** Brown Norway rat strains following glucocorticoid (GC), vitamin D (VitD) or combination treatments. **$p<0.001$, *$p<0.05$ as compared to respective control (CTL). $n\geq4$.

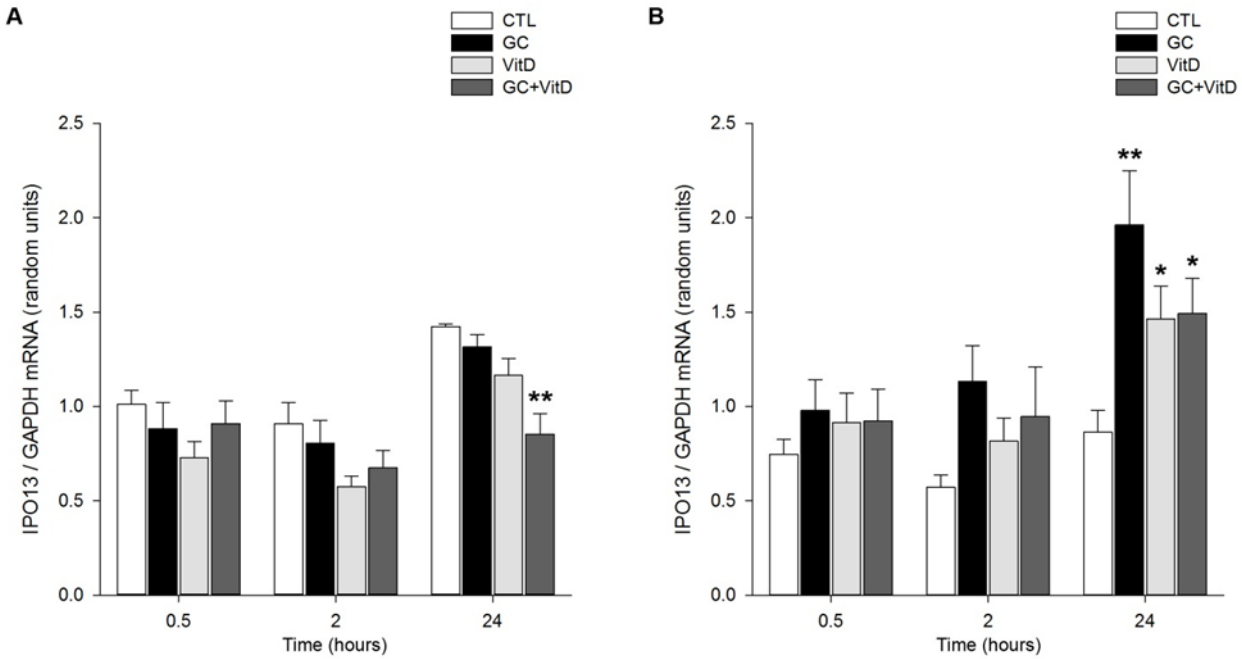

A
CTL
GC
VitD
GC+VitD
IPO13 / GAPDH mRNA (random units)
2.5
2.0
1.5
1.0
0.5
0.0
**
0.5
2
24
Time (hours)
B
CTL
GC
VitD
GC+VitD
IPO13 / GAPDH mRNA (random units)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
*
0.5
2
24
Time (hours)

**Figure 4.5 (A)** GR protein levels by Western blot in nuclear extracts from primary lung epithelial cells of the Lewis and Brown Norway (BN) rat strains following 24 h treatment with glucocorticoid (GC), vitamin D (VitD) or both. **$p<0.001$, *$p<0.05$ as compared to respective control (CTL) or as indicated. $n\geq4$. **(B)** A representative Western blot for GR in nuclear extracts of primary airway epithelial cells after 24 h treatment with GC, VitD or both. TBP was used as a nuclear protein loading control.

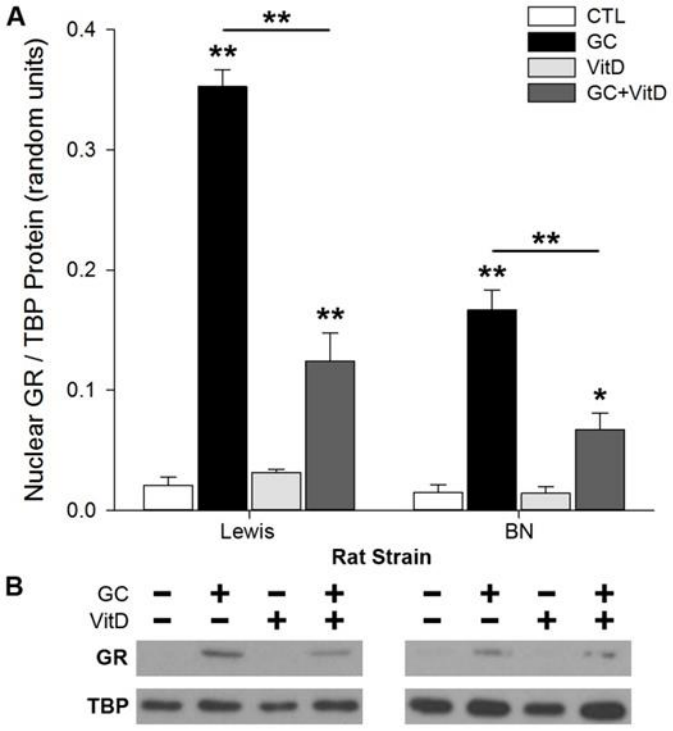

A
CTL
GC
VitD
GC+VitD
Nuclear GR / TBP Protein (random units)
0.4
0.3
0.2
0.1
0.0
**
**
**
**
*
Lewis
BN
Rat Strain
B
GC
VitD
GR
TBP

**Figure 4.6** Relative VDR mRNA expression by qRT-PCR in primary lung epithelial cells of the **(A)** Lewis and **(B)** Brown Norway rat strains following 24 h treatment with glucocorticoid (GC), vitamin D (VitD) or both. ***$p<0.001$, **$p<0.005$, *$p<0.05$ as compared to respective control (CTL) or as indicated. $n\geq4$.

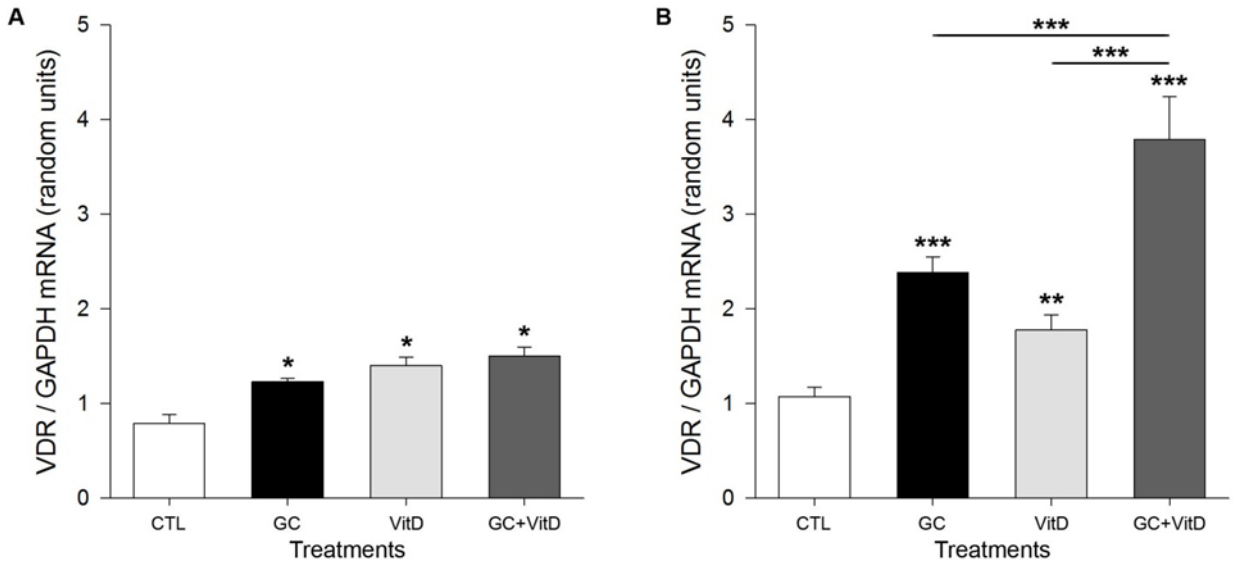
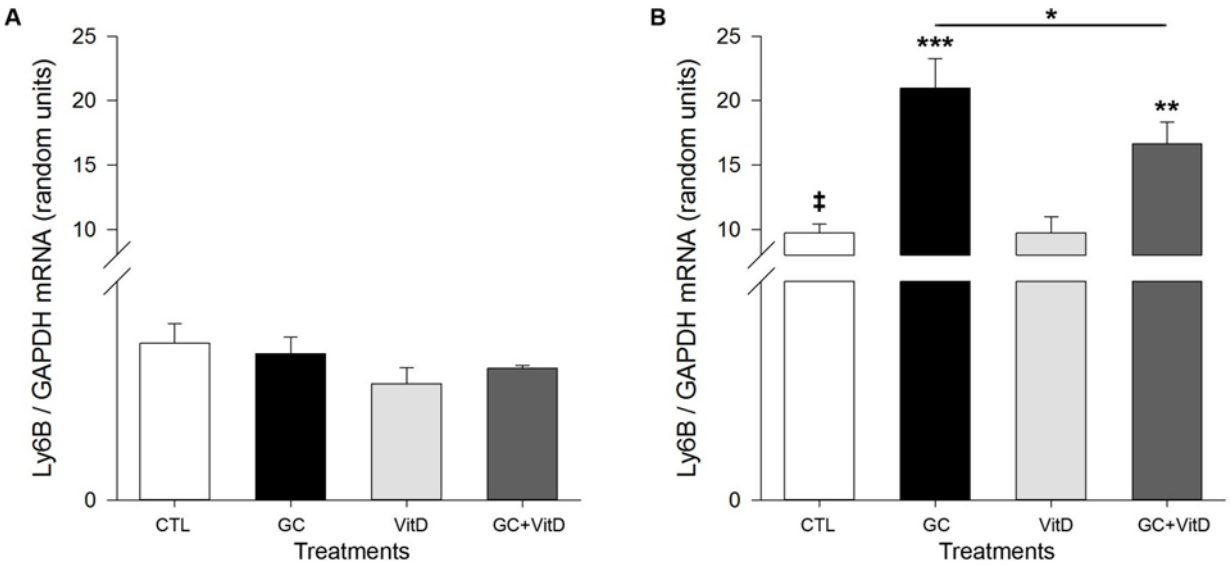

A
VDR / GAPDH mRNA (random units)
0
1
2
3
4
5
*
*
*
CTL
GC
VitD
GC+VitD
Treatments
B
VDR / GAPDH mRNA (random units)
0
1
2
3
4
5
***
***
***
***
**
CTL
GC
VitD
GC+VitD
Treatments

**Figure 4.7** Relative Ly6B mRNA expression by qRT-PCR in primary lung epithelial cells of the **(A)** Lewis and **(B)** Brown Norway rat strains following 24 h treatment with glucocorticoid (GC), vitamin D (VitD) or both. ***$p<0.001$, **$p<0.005$, *$p<0.05$ as compared to respective control (CTL) or as indicated. ‡$p<0.001$ as compared to Lewis CTL. $n\geq4$.


A
Ly6B / GAPDH mRNA (random units)
25
20
15
10
0
CTL
GC
VitD
GC+VitD
Treatments
B
Ly6B / GAPDH mRNA (random units)
*
***
**
‡
25
20
15
10
0
CTL
GC
VitD
GC+VitD
Treatments

# CHAPTER V

## General Conclusions

Steroids are hormones that play a crucial role in numerous biological processes, including lung development and the inflammatory/immune response. Due to their potent effects GC function is tightly regulated and insensitivity to the actions of GCs could have significant consequences. A better understanding of the mechanisms responsible for the observed variability in GC response is compulsory to tailor patient-specific therapies and to identify the long term effects of limited steroid response during development. The overall objective of this thesis was to study mechanisms of GC action in asthma and lung development and explore the modulatory role of VitD on GC responsiveness during these processes.

The use of murine models in asthma research has been an important tool in identifying molecular mechanisms responsible for the pathophysiology of the disease. Even though significant interstrain variability in the susceptibility to develop an asthma-like phenotype exists, little is known about interbred differences in steroid responsiveness. In the first section of this thesis I exploited the inbred mouse system to investigate possible mechanisms controlling variable GC responsiveness in asthma. I demonstrated that Balb/c mice, which tend to show more severe features of the disease, also display limited response to steroids. Interestingly, this correlation between disease severity and limited steroid response is also observed in asthmatics, which highlights the probable significance of the Balb/c mouse as a model of GC insensitivity [221].

Allergen sensitization/challenge led to increases in respiratory resistance, lung eosinophilia, inflammatory cytokines and circulating OVA-specific IgE levels, as well as reduced GR and IPO13 expression in both mouse strains studied. However, budesonide intervention corrected all the variables measured in C57BL/6 but not in Balb/c mice. Interestingly, only in Balb/c mice allergen challenge induced the phosphorylation of p38

MAPK, possibly modifying normal GR function. GR phosphorylation by phospho-p38 MAPK has been demonstrated to inhibit GR nuclear import and transcriptional activity [58, 63, 228]. In agreement with high levels of active p38 MAPK, a reduction in the proportion of nuclear GR in the lungs of Balb/c mice following allergen challenge was observed, and this was not improved by GC treatment. The overall results presented in Chapter II suggest that the steroid insensitive phenotype of Balb/c mice is associated with high levels of inflammatory cytokines following allergen challenge, leading to increased amounts of active p38 MAPK and subsequent inactivation of GR.

The development of a mouse model for steroid insensitive asthma is a significant outcome of the first part of my project. My results support previous studies that suggest an involvement of p38 MAPK and altered GR nuclear translocation in steroid insensitivity [58, 63, 64, 66]. However, the variability in GC responsiveness is believed to be multifactorial and the use of this model in experimental asthma research could provide further clues on other determinants of steroid sensitivity. Even though budesonide is a common inhaled GC used in the treatment of asthma, several others with different pharmacodynamic properties and resulting clinical effects exist [272]. In future studies, it would be of interest to analyze the effects of other GCs normally prescribed to asthmatic patients in the Balb/c model and determine if the insensitive phenotype of this mouse strain is exclusive to budesonide.

Asthma in adults and children does not display exactly the same characteristics. Interestingly, these phenotypic differences are more pronounced in patients with severe forms of the disease, which tend to be less steroid responsive [244]. The next objective of this thesis was to investigate mechanisms for variable GC responsiveness in pediatric asthma. Cell culture experiments using lymphoblast cell lines derived from asthmatic

children at two ends of the spectrum of steroid responsiveness were conducted. Furthermore, given the increasing body of evidence supporting a role of VitD in enhancing steroid sensitivity, experiments were performed to explore the possible modulatory role of VitD in GC function [210, 211, 213].

Lymphoblasts derived from asthmatic children with poor GC response exhibited decreased levels in GR protein when compared to those from children with a positive response to steroids. Furthermore, in cells from poor responders the hormone-induced nuclear translocation of GR was short-lived and more importantly, the homologous downregulation of GR was faster than in lymphoblasts derived from good GC responders. Interestingly, the presence of VitD in the media modified all the GC effects in cells derived from poor responders suggesting a probable positive effect on steroid sensitivity.

GC induced the expression of VDR only in cells derived from asthmatics classified as good GC responders. However, steroid induction of VDR was regained in lymphoblasts from poor GC responders when VitD was present in the media, supporting the hypothesis that lower levels of GR protein are in part responsible for the steroid insensitivity of these asthmatic children and that VitD improves this response.

The overall results presented in Chapter III suggest that in a subgroup of asthmatic children GC insensitivity could partially be a result of limited GR nuclear bioavailability as a consequence of decreased baseline expression and faster hormone-induced downregulation of GR. The VitD results presented herein are promising and support a positive role of this secosteroid on GC responsiveness by stabilizing the levels of GR protein. Despite the limited sample size, these results provide molecular evidence of the possible therapeutic properties of VitD on steroid insensitive pediatric asthma.

The group of lymphoblast cell lines used in this study represent limited phenotypes for GC responsiveness which could have skewed the results not allowing for more robust VitD effects. In future follow up studies, it would be important to expand the lymphoblast cell line selection criteria to include a wider range of steroid responsiveness, increasing the sample sizes and allowing us to draw stronger conclusions. Also, it would be extremely interesting to repeat the experiments presented in Chapter III using lymphoblasts derived from patients at two ends of the spectrum of circulating VitD levels in a way that VitD status could be directly correlated to steroid responsiveness *in vitro*.

The experiments carried out during this study allowed us to distinguish differences in steroid regulation of genes involved in the GC pathway but did not provide us with a direct measurement of functional steroid responsiveness at the molecular level. Therefore, this limitation could be addressed in the future by measuring the effects of steroids on the modulation of cytokines important in asthma and that are expressed in lymphoblasts. Finally, given the results presented in Chapters II and III, it would be interesting to explore if VitD modifies the steroid insensitive phenotype observed in Balb/c mice. Even though the effects of VitD on the "asthmatic" phenotype of murine models have been partially studied, VitD's modulation of GC responsiveness in these models has not been investigated.

In the first two data chapters of this thesis steroid responsiveness was studied in the context of asthma. However, since GC is a key player in lung organogenesis and little is known about how steroid insensitivity affects normal development, the final objective of this thesis was to explore molecular mechanisms for steroid responsiveness during a critical developmental window of the lung. Furthermore, given the results from Chapter III suggesting that VitD modifies GC function, molecular interactions between these two

agents were studied using primary lung epithelial cultures isolated from postnatal lungs of atopic and normoresponsive rats.

The results demonstrated that GC responsiveness, as measured by steroid regulation of GR and IPO13 expression, is strain dependent. Interestingly, GC stimulated the expression of GR and IPO13 only in epithelial cells from the atopic BN strain. Similarly, levels of Ly6B, a gene highly expressed in the developmental BN lung, significantly increased in response to steroids only in cells from this strain. Contrary to what was observed in lymphoblasts from asthmatics, in developmental epithelial cells VitD attenuated the GC-induced GR nuclear translocation and the GC effects on mRNA expression levels of all genes assessed.

These findings emphasize the role of temporal effects in the modulation of GC response. Based on gene expression following GC treatment, the developmental airway epithelium of the atopic rat appears to be more sensitive to steroids. This might seem positive in an inflammatory context but it could possibly be detrimental during development. There is already solid evidence that high levels of GCs terminate alveolarization [267]. However, little is known about how variation in the handling of endogenous steroids contributes to respiratory development and disease susceptibility. In future studies, it would be interesting to assess *in vivo* if and to what extent increased GC responsiveness in the atopic rat epithelium affects normal pulmonary development and if VitD attenuates these effects.

The collection of data presented in this thesis highlights the complexity of steroid function and the involvement of multiple mechanisms in the regulation of its response. With the use of a variety of experimental procedures including the use of animal models and cell culture work several molecular mechanisms responsible for variable steroid

responsiveness were identified, including altered expression, reduced activation, abnormal nuclear translocation and increased homologous downregulation of GR.

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