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Glucocorticoid modulation of the innate antiviral immune response in bronchial epithelial cells

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"If we knew what it was we were doing, it would not be called research, would it?"

Albert Einstein

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Chapter 1: Introduction

1.1 Asthma and COPD

Asthma and chronic obstructive pulmonary disease (COPD) are among the most common chronic respiratory diseases (CRDs). Though they are not curable, the available treatments improve shortness of breath or reduce inflammation in the airways to control symptoms and increase the quality of life for people with these diseases [1]. Asthma and COPD are among the main causes of mortality and morbidity in the world and the number of patients is continuously increasing across the decades. Moreover, healthcare costs for chronic respiratory diseases have a strong impact on the global economic burdens [2].

1.1.1 Asthma

Asthma is defined as a chronic inflammatory disease of the airways, according to the Global Initiative for Asthma (GINA), presenting typical respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough, associated with episodes of airflow obstruction [3]. Generally, these symptoms resolve spontaneously or following an appropriate treatment. Chronic airways inflammation induces airways bronchial hyperresponsiveness (AHR) to a variety of allergic, infectious or irritant stimuli [4]. The consequent bronchoconstriction is a relevant clinical issue in asthmatic subjects.

Asthma is a heterogeneous disease of the lung and its severity and frequency are variable from person to person. A clinical classification of disease severity has been proposed in the past to include: intermittent, mild persistent, moderate persistent and severe persistent asthma [5].

An important feature of asthma pathology is the remodelling of the airways over time that may lead to an irreversible airflow obstruction.

1.1.1.1 Epidemiology

The World Health Organization reports that about 235 million people worldwide suffer from asthma [1]. However, this figure may be an underestimation due to the overlapping of asthma symptoms with other age-related disorders [3]. In addition, some people living in high-income countries and most of the people living in low or middle-income ones also have limited access to healthcare [6]. Moreover, different asthma definitions and different methodologies to collect and report data in epidemiological studies may be used. There is a need for a more standardized operational definition [7].

A study on the global burden of disease (GBD) program estimated that asthma affects 358 million people worldwide and reveals that in 2015 asthma was the most prevalent

chronic respiratory disease worldwide, ranked 23rd among the 315 GBD causes and that 0.4 million people died from asthma. Although the number of cases is continuously increasing, it is registered a decrease of all-age deaths of about 27% in 2015 compared with 1990 [8].

Asthma is also the most common chronic disease among children [9].

1.1.1.2 Aetiology

A complex combination of genetic and environmental factors determines the onset of asthma and its phenotypes. Rather than a single disease, asthma is a disorder defined by different phenotypes. The level of severity, the frequency of exacerbation, the presence of chronic airflow obstruction, the age of asthma onset, are among criteria to define clinical asthma phenotypes. Again, the presence or absence of specific inflammatory cell types such as eosinophils or neutrophils in sputum discriminates between immuno-pathological categorisation of asthma phenotypes.

A large proportion of asthmatic patients are also associated with atopic asthma and show allergic reactions to environmental triggers [10]. Atopy is defined a genetic tendency to develop allergic diseases (American Academy of Allergy, Asthma and Immunology; AAAAI) [11]. Exposure to environmental common inhaled allergens such as pollen, mould, pollutants, pet dander, house dust mite (HDM), or food allergens is typically associated with heightened adaptive immune response in atopic individuals. Soluble protein allergens activate dendritic cells (DCs) which through the lymphatic system reach lymph nodes, where antigen presentation to naïve T $CD4^{+}$ cells induce their differentiation into T helper (Th2) lymphocytes. These cells migrate toward the site of inflammation and produce type-2 cytokines IL-4, IL-5, IL-9 and IL-13, widely expressed in bronchial submucosa of asthmatic patients, inducing an excess of mucus secretion, airways smooth muscle constriction and the activation of eosinophils, B-lymphocytes, producing allergen-specific immunoglobulin E (IgE) and mast cells, producing histamine and leukotrienes [12, 13]. IL-5 is a major cytokine for eosinophils activation [14]. IgE has a central role in allergic asthma inflammation. In sensitised patients exposed to an allergen, this activates the induced Th2 lymphocytes to produce IL-4 and IL-13 triggering B-cells to release allergen-specific IgE, which subsequently bind to the high affinity IgE receptors (FcεRI), expressed on the surface of mast cells and basophils. A second exposure to the allergen leads to the degranulation of these cells and to the release of mediators including histamine, leukotrienes and proteases, inducing the typical and immediate allergic response, characterised by edema and bronchoconstriction. The late allergic response is mediated by other cytokines and chemokines-driven recruitment of eosinophils, basophils,

neutrophils, T-cells and macrophages to the site of inflammation. It takes hours to develop and it leads to mucus hypersecretion, airway inflammation and hyperresponsiveness [15, 16]. Furthermore, lung injured epithelial cells produce innate immune regulatory cytokines, including thymic stromal limphopoietin (TSLP), IL-25 and IL-33, which stimulate immune responses [17, 18]. The group-2 innate lymphoid cells (ILCs) represent other recently described tissue-resident lymphocytes, defined by their capacity to produce IL-4, IL-5 and IL-13 [19]. However, how they contribute to the type-2 immunity pathway is under investigation. One of the severe asthma phenotype is characterised by a corticosteroid-resistant scenario and may reflect a non Th2-driven mechanism associated with low number of eosinophils [20] and high number of neutrophils [21] in sputum. Type-1 immunity, mediated by IFN-γ and TNF-α producing Th1 cells and IL-17 producing Th17 cells may have a role in neutrophilic asthma phenotype [13].

Overall, a more accurate definition of asthma phenotypes is necessary for a further comprehension of the driving factors associated with the pathogenic mechanisms underlying asthma heterogeneity and the development of specific biomarkers would bring to define personalised therapies.

1.1.1.3 Diagnosis

Following an analysis of the patient history of respiratory symptoms and a physical examination, a spirometry is a reliable test for lung function. Asthma is characterised by variable expiratory airway obstruction that can be assessed by spirometric testing [3].

1.1.1.4 Treatments

Currently available therapies for asthma include corticosteroids and β2-agonists as main treatments to contrast the two most characterizing and invalidating aspects of the disease, bronchoconstriction and inflammation, respectively. Inhaled corticosteroids (ICS) can be prescribed in association with long acting β2-agonists (ICS/LABA). As needed, short acting β2-agonists (SABA) are a choice for an immediate relief. ICS/LABA therapy is effective in a large proportion of patients, but around 10% of asthmatic people with a clinical picture of severe asthma do not respond to these treatments even at high doses or with the use of oral corticosteroids [22]. These patients are defined as severe asthma patients.

Other options for asthma treatment include cysteinyl leukotriene receptor antagonists (CYS-LTRA) such as montelukast and zafirlukast, for initial controller treatment when patients are unable or unwilling to use ICS and the long acting muscarinic antagonist, tiotropium, when asthma is not well-controlled with ICS/LABA [3].

New humanised monoclonal antibodies are a biologic therapy in the management of severe asthma [23]. Omalizumab was the first approved anti-IgE antibody for severe allergic asthma [13]. Omalizumab is a humanised anti-IgE antibody developed for allergic asthma. The reduction of circulating free IgE supresses eosinophilic and T cell inflammation in asthmatic airways and may allow a reduction of inhaled corticosteroid dosage [24]. Omalizumab also downregulates the receptor FcεRI on the surface of mast cells and basophils, which become less sensitive to allergen stimulation [16]. Anti-IL-5 such as mepolizumab, reslizumab and benralizumab are other indicated antibodies in case of severe eosinophilic asthma [25].

1.1.2 Chronic Obstructive Pulmonary Disease (COPD)

Chronic Obstructive Pulmonary Disease is a major respiratory disorder in adults and is characterised by persistent common symptoms such as cough, excessive sputum production and shortness of breath. Lung dysfunction due to obstructive bronchiolitis or parenchymal destruction, known as emphysema, may also be associated with airways inflammation and chronic airflow limitation. Airflow limitation is irreversible and usually progressive in this condition. COPD is a heterogeneous disease and all these typical conditions do not necessarily occur together over time [26].

1.1.2.1 Epidemiology

The WHO estimates that Chronic Obstructive Pulmonary Disease will become the third leading cause of death worldwide by 2030. More than 3 million people dye for COPD each year, 6% of all deaths, 90% of which occur in low and middle-income countries. Globally, COPD affects about 65 million people [1]. Notably, during the last two decades, the number of deaths for COPD has almost doubled. Prevalence of COPD varies within and between countries, depending on geographical position, tobacco smoking, industrialization, pollution and access to healthcare [27].

1.1.2.2 Aetiology

In western countries, the main cause of chronic obstructive pulmonary disease is tobacco smoke. One other relevant risk factor is the indoor air pollution, provoked by organic fuels used for cooking and heating, especially in low-income countries. Outdoor air pollutants such as noxious particles or gases and occupational inhalants such as vapours, irritants and fumes are among other risk factors for COPD [28]. Further, the genetic predisposition to the disease is an adding factor.

Inhalation of cigarette smoke or other irritants increase oxidative stress and induces an amplified airway inflammation in COPD patients, with accumulation of different types of

immune cells, including macrophages, neutrophils, dendritic cells and $CD8^+$ T lymphocytes in the lungs. The release of a number of cytokines and chemokines such as IL-6, IL-8, IL-1β and TNF-α, in addition to proteases and also the recruitment of other inflammatory cells leads to a chronic state of airway inflammation and to a dysregulation of lung functions, associated with loss of elasticity and small airways collapse during exhalation [29]. At the molecular level, active smoking reduces the expression of histone deacetylase in alveolar macrophages. This effect could partially motivate the reduced efficacy ICS in suppressing airway inflammation in COPD, as one mechanism of corticosteroid gene repression involves the recruitment of histone deacetylase [30].

The association of COPD with co-morbidities such as cardiovascular disorders, obesity, lung cancer and pneumonia may also link to a systemic inflammation condition.

Different types of patients with distinct clinical characteristics, prognosis and response to treatments are described as patients with different 'clinical phenotypes'. In relation to airway inflammation, most patients with COPD present neutrophilic inflammation, but they may also have an enhanced eosinophilic component. This is an important feature in order to predict the treatment response. Eosinophilic inflammation, in fact, is associated with a more positive response to corticosteroids, while neutrophilic inflammation has a negative correlation with ICS and is associated with severe airflow obstruction [31].

Ad hoc therapies based on different phenotypes may improve their efficacy.

1.1.2.3 Diagnosis

Spirometry represents the most reproducible test for lung function. A post-bronchodilator $FEV_1/FVC < 0.7$ confirms a suspected case of COPD and the presence of persistent airflow limitation, which can occur many years after the onset of chronic cough and sputum production [26].

As both asthma and COPD are heterogeneous conditions, there is increasing evidence of overlapping clinical expressions. This asthma-COPD overlap (ACO) is due to common clinical symptoms such as cough, mucus hypersecretion and wheeze, rather than development of a new disease [32]. For instance, around 30% of people with asthma are also smokers and may develop chronic airflow limitation, which is a typical feature of COPD. Airflow obstruction in asthma is a reversible condition and symptoms are usually responsive to corticosteroids. Differently, airflow obstruction in COPD is persistent and generally not responsible to steroidal anti-inflammatory treatments [29].

Thus, differentiating the diagnosis of COPD from asthma may sometimes be very difficult. ACO is associated with higher acute exacerbation rate [33].

1.1.2.4 Treatments

COPD management include long-acting inhaled muscarinic antagonists (LAMA), also in combination with long acting β2-agonists (LABA) [34]. Inhaled combination of corticosteroids (ICS) with LABA are also widely used for exacerbation prevention, however the question about the efficacy of ICS to control inflammation in COPD is controversial [35] as they do not appear to reduce the progressive decline in lung function, added to numerous side effects associated with their use. Clinical trials have shown that prolonged therapy with ICS is effective in reducing airway sputum inflammatory cells in stable COPD, in contrast to shorter duration of the therapy, which not demonstrates a beneficial effect [30].

PDE-4 inhibitors (PDE4I) such as roflumilast, act as modulators of lung inflammation and bronchodilators through inhibition of 3',5'-monophosphate (cAMP) degradation. They are new approved drugs for COPD management, in alternative to theophylline, a non-selective phosphodiesterase inhibitor with a narrow therapeutic index and not infrequent gastrointestinal adverse effect [36, 37].

1.2 Asthma and COPD exacerbations

The term exacerbation indicates an acute worsening of respiratory symptoms that result in increased airway inflammation and reduced lung function. Hospitalisation is a frequent consequence of severe episodes and together with the need of additional therapy accounts for more than 50% of asthma-related costs [38]. Again, severe COPD exacerbations account for less than 10% of total exacerbations, but an estimation of the socioeconomic burden related to hospitalization reveals that they are associated with a high mortality rate and account for approximately 60-70% of healthcare costs [39].

1.2.1 Asthma exacerbations

Increased symptoms such as shortness of breath, cough, wheezing, chest tightness and sputum production identify an episode of asthma exacerbation, which can occur in people with pre-diagnosed asthma or occasionally as initial event. Among others risk factors, including exercise, air pollution and exposure to inhaled environmental allergens or irritants, respiratory viral infections represent the most common precipitating cause of asthma exacerbations, with a rate of about 50% in adults [40, 41] and more than 80% in children [42]. Rhinovirus (RV) is the most frequently detected virus in nasal aspirate during exacerbations [43-45] and recently identified RV-C, followed by RV-A, is the most common virus associated with more severe asthma manifestation in children [46, 47].

Other detected viruses include respiratory syncytial virus (RSV), enterovirus, coronavirus and influenza virus [48-50].

1.2.1.1 Viral infection and interferons (IFNs)

Experimental investigations have shown cellular and molecular effects of RV infection. Johnston et al. reported the first evidences of RV implications with asthma exacerbations [43]. Additional studies revealed that in primary bronchial epithelial cells (BECs) from asthmatics, RV replication was significantly higher, compared to healthy controls, whilst the production of interferon beta (IFN-β) decreased, suggesting an impairment of the innate immune response [51]. Rhinovirus infections are related with deficient type-III interferon production in asthmatic patients [52]. Further, *ex vivo* experiments identified a significant lower expression of IFN-α, IFN-β, IFN-λ and interferon stimulated genes (ISGs) in asthmatics compared to healthy subjects [53, 54]. *In vivo* experiments also confirm the association between the deficient expression of IFN-α and IFN-β in the bronchial epithelium of asthmatic patients and the deficient number of sub-epithelial IFN-α/β expressing monocytes/macrophages during rhinovirus infection [55]. A recent study also proposes the identification of a pro-inflammatory state at baseline in circulating peripheral blood mononuclear cells (PBMCs) from asthmatics, characterized by elevated STAT1, ISGs and IL-15 expression profile, as a signature of increased frequency of asthma exacerbations triggered by respiratory infections [56]. In bronchial epithelial cells from both atopic and non-atopic children, the induction of IFN-λ and IFN-β following RV infection is reduced compared to normal controls. Epithelial cells from atopic asthmatic children show similar results, suggesting a correlation between the condition of atopy and the impairment of interferon production [57]. Synergism between allergy and viral infection represents a risk factor for asthma exacerbation and a major cause of hospitalization [58, 59]. However, some studies failed to detect defective RV-induced IFN-β protein production in asthmatic bronchial epithelial cells (HBECs) compared to healthy cells, suggesting that the response to RV infection may vary depending on the severity of the disease and the consequent lower degree of inflammation associated with mild and well-controlled asthma phenotype [60]. Other investigators reported that bronchial airway epithelial cells from asthmatic patients are more susceptible to RV infection and show an impaired IFN-β production compared with asthmatic nasal epithelial cells. Thus, different conclusions may come from different cell types, also considering the integrity of the epithelium [61].

1.2.1.2 Allergic asthma

Accumulating evidence led to the conclusion that a sensitization to allergens in early life increases the risk to develop asthma and predispose to a greater susceptibility to RV infections [48, 62, 63]. Wheezing is a symptom occurring frequently in infants and early childhood in association with rhinovirus or other virus infections and for nearly 90% of children by age of 3 was a predictor of subsequent development of asthma at age of 6 in the Childhood Origins of Asthma (COAST) high-risk birth cohort study [64]. Further, a correlation between high levels of Immunoglobulin-E (IgE) and a reduced response to viral infections is associated with their high affinity for plasmacytoid dendritic cells, which results in the reduced ability of these cells to produce IFNs [65].

Atopic asthmatic patients, with an allergic component of the disease, are considered more susceptible to episodes of exacerbation, especially following RV infections. RV-induced IL-25 and IL-33 drive type-2 immunity and allergic pulmonary inflammation in asthma exacerbations [66, 67]. A positive correlation between total serum IgE levels and eosinophilia may also exist during asthma exacerbation in the presence of rhinovirus [68]. Neutrophils infiltration in the airways is also increased during RV-induced exacerbations. As anti-microbial strategy, neutrophils can form neutrophil extracellular traps (NETs), which contain histones, neutrophil elastase (NE) and myeloperoxidase (MPO). Toussaint and colleagues described a molecular mechanism through which RV-induced release of host double stranded DNA (dsDNA) by neutrophils boosts type 2 allergic inflammation, promoting severe asthma exacerbations [69].

1.2.1.3 Eosinophilia

As mentioned before, eosinophilic airway inflammation, revealed by percentages of eosinophils in sputum higher than 3% of the total cells, is indicative of an enhanced risk of severe asthma exacerbation and corticosteroid responsiveness [20, 70]. A large cohort study in UK recently reported that asthmatic patients with more than 400 cells per µL blood eosinophils experience more severe episodes of asthma worsening in a year [71]. As IL-5 triggers the activation of eosinophils, anti-IL-5 have been recently approved drugs to control eosinophilic inflammation in severe asthma [13, 72]. In non-Th2 phenotypes asthma exacerbations may be associated with greater sputum neutrophil counts [73].

1.2.1.4 Bacterial co-morbidity

It is not clear if the co-morbidity with other infections have a role in the pathogenesis of asthma exacerbations. However, infections with bacteria such as *Haemophilus influenzae*, *Streptococcus pneumonia* and *Mycoplasma pneumonia* may be critical factors [74].

1.2.1.5 Pharmacological approach

Inhaled corticosteroids (ICS) and long acting β2-agonists (LABA) represent the main treatments for the control of inflammation and bronchoconstriction in asthma, often in combination ICS/LABA such as fluticasone, budesonide or beclometason with formoterol or salmeterol. Tiotropium is a long acting muscarinic antagonist (LAMA) used only in people aged more than 12 years for airways smooth muscle relaxation in severe asthma [75]. Although the classical treatment ICS/LABA reduces the number of episodes [76, 77], almost half of asthma patients in clinical trial with ICS alone continue to experience exacerbations [78]. Furthermore, experimental evidences associate ICS fluticasone propionate (FP) with the impairment of the innate and acquired antiviral immune response and the consequent reduction of host ability to clear the virus [79]. As the production of IFN-β is impaired in asthmatics, the administration of an inhaled formulation of exogenous IFN-β is a therapeutic option under consideration. However, no clinical evidence demonstrates an improvement of asthma symptoms caused by viral infection following IFN-β treatment, but the ability to enhance morning peak expiratory flow (PEF) and the innate immune response suggest the potentiality of this therapy [80].

For these reasons, new therapeutic approaches are highly needed in severe asthma. Clinical studies using omalizumab, a humanized anti-IgE antibody and mepolizumab or reslizumab, antibodies against IL-5, reported a reduced rate of exacerbations by 58% and 50%, respectively [78].

Further, many pharmaceutical companies afforded considerable efforts in the last decades with the purpose to identify and develop selective synthetic corticosteroids, which may have an unvaried anti-inflammatory effect but may spare the impairing effect on the anti-viral immunity. Nevertheless, no selective therapies have been approved until now.

1.2.2 COPD exacerbations

Acute worsening of symptoms such as cough, dyspnea and increased sputum production, identifies episodes of exacerbations, which frequently affects people with COPD. Bacterial and viral infections together with other non-infectious causative factors such as pollution and variations in air temperature represent the main triggering factors [26]. *Picornaviruses* are detected in 36% of cases [81]. Among them, it has become clear that rhinovirus (RV) infections are the primary cause of COPD exacerbations [78]. During naturally occurring COPD exacerbations, RV is the most prevalent detected pathogen and the viral load is significantly higher than in stable asthma. Furthermore, RV infection detected at exacerbation increases the frequency of further similar episodes [82]. COPD exacerbations are also associated with increased airway and systemic inflammation, with

higher production of cytokines and chemokines, such as IL-6, TNF-α, IL-8 and CCL5/regulated on activation, normal T cell expressed and secreted (RANTES), other than increased number of neutrophils and eosinophils in the lung, which aggravate the airway inflammation. Degranulation of activated neutrophils causes the release of neutrophil elastase and consequent epithelial damage and mucous hypersecretion [83, 84].

1.2.2.1 Viral infection and interferons (IFNs)

As discussed, RV infections trigger the innate immune response in airway epithelial cells and immune cells. IFN production is impaired in COPD, leading to increased susceptibility to infection and severity of exacerbations. It has been reported an impaired IFN production associated with higher viral load in *ex vivo* bronchoalveolar lavage (BAL) cells, mostly macrophages, infected with RV, comparing COPD subjects with control subjects [85]. As smoking is a leading condition to develop COPD and RV infections represent the main cause of COPD exacerbations, *in vitro* experiments have been performed in order to correlate these two elements. Cigarette smoke extract (CSE) reduces IFN-β production, in addition to CXCL10 (IP-10), RANTES and other host defence genes, thereby altering the response of airway epithelial cells to RV infection [86, 87].

1.2.2.2 Neutrophilic inflammation and role of eosinophils

Airway inflammation in chronic obstructive pulmonary disease is characterised by neutrophilic infiltration and prevailing resistance to ICS treatment. Nevertheless, ICS reduce COPD exacerbations by approximately 20%-30%. Increased numbers of macrophages and CD8+ T lymphocytes have also been detected in the airways of COPD patients especially in the later stages of the disease and the evidence that ICS significantly reduce lymphocytic inflammation may correlate with the positive outcome in the use of ICS in COPD [88].

Papi and colleagues demonstrated that COPD exacerbations are associated with an increased number of sputum neutrophils related to the severity of the disease, while sputum eosinophils count increase during virus-associated exacerbations, suggesting a correlation between the presence of eosinophils in sputum and the viral aetiology of an exacerbation [89]. In COPD, the number of eosinophils in the airways may increase during exacerbation and may partly explain the clinical improvement associated with the use of corticosteroids [31]. Subjects with higher percentage of eosinophils in sputum, more than 3%, have more chance of experiencing an exacerbation after withdrawal of ICS [90]. Furthermore, blood eosinophil count is a promising biomarker of response to ICS in patients with COPD [91]; levels of 300 cells per μL or more are indicative of higher rate of

moderate or severe exacerbation associated with ICS withdrawal in patients still treated with LAMA and LABA [92].

1.2.2.3 Bacterial infections

The presence of bacteria during COPD exacerbations [89] is generally associated with an increased production of purulent sputum and although the role of bacteria in COPD exacerbation is controversial, a secondary bacterial infection is common after RV infection and this may motivate exacerbation recurrence in COPD patients [82]. The degradation of antimicrobial peptides as consequence of RV infection [93] may be related to the increased risk of pneumonia frequently reported in COPD exacerbation and use of immunosuppressive ICS [94].

1.2.2.4 Pharmacological approach and use of corticosteroids (CS)

Even if widely prescribed in severe COPD or as an exacerbation preventive treatment in moderate COPD, the use of inhaled corticosteroids (ICS) is controversial. However, the association ICS/LABA is more effective than LABA alone in eosinophilic exacerbations [91, 95]. During acute exacerbations of COPD, the use of oral corticosteroids (OC) can be an option [96].

Singanayagam et al have recently demonstrated that Fluticasone propionate (FP), an inhaled corticosteroid therapy, suppresses the innate and acquired antiviral immunity in RV-induced COPD exacerbations. In addition, they reported an increase in pulmonary bacterial load and mucus production [97]. Similarly, Contoli et al produce evidence that long-term use of FP in association with the long-acting β2-agonists, in stable moderate COPD, is associated with a significant increase in sputum bacterial load, compared with salmeterol alone. Interestingly, this effect was observed in patients with lower baseline sputum or blood eosinophils, but not in patients with higher baseline eosinophils [98], showing a better responsivity to ICS in subjects with eosinophilic inflammation.

Other recommended treatment for COPD exacerbations, according to the Global Initiative for Chronic Obstructive and Lung Disease (GOLD), include PDE4 inhibitors in severe COPD patients with chronic bronchitis phenotype [26].

1.3 Human Rhinovirus

Human Rhinovirus (RV) is the virus of the common cold and is the main responsible for respiratory tract infections. Its transmission occurs through the saliva by contact from person to person or by aerosol. Although RV infections are usually self-limited, they

represent the major cause of asthma and COPD exacerbations. Although it was discovered in 1950s, no anti-rhinovirus treatment nor vaccine is currently available. The existence of more than 100 serotypes gives an idea of its high mutability and explains the reason of recurrent RV infections and the currently lack of an approved vaccine for the prevention [99]. The development of anti-viral drugs remains a big challenge.

1.3.1 Classification

Rhinovirus (RV) is a single-stranded RNA (ssRNA) virus, member of the genus *Enterovirus,* belonging to the *Picornaviridae* family. Based on their genome, rhinovirus genotypes are divided in three different species: RV-A, RV-B and RV-C. One other classification divides rhinoviruses in groups. The 'major group' includes approximately 90% of all RV serotypes, the entire RV-B group and most of RV-A group, whilst a subset of RV-A types are included in the 'minor group'. The intracellular adhesion molecule-1 (ICAM-1) and the low-density lipoprotein receptor (LDLR) are the two surface receptors that RVs of the major and minor group, respectively, use to enter the cell [100]. The cadherin-related family member 3 (CDHR3) is a receptor for the more recently discovered RV-C [99, 101].

1.3.2 Viral infection and entry pathways

The first step of infection is the RV uptake via receptor-mediated clathrin-dependent or independent endocytosis.

The clathrin-mediated endocytosis (CME) is the most well characterised mechanism and represents the way that minor-group RVs use to reach the cellular uptake. Virus internalisation occurs following the LDLR binding on the cell membrane [102]. The adapter complex AP-2 recognises a signature amino acid (AA) sequence in the N-terminal cytoplasmic tail of the receptor and orchestrates the polymerisation of soluble clathrin monomers on the inner side of the membrane to form a coat around an invagination created in correspondence with the bound receptors. The protein dynamin severs the plasma membrane to release mature clathrin-coated vesicles inside the cytosol, which are uncoated under ATP hydrolysis. The vesicles fuse together to form endosomes that move on to fuse with lysosomes. The disassembly of the virus occurs during this process, while the pH drops from neutral to about pH 5.6, depending on the cell type, reaching pH 5 into the lysosomes. The virus transfers its undamaged RNA into the cytosol through pores formed in the endosomal membrane, whilst the capsid proteins reach the lysosomes for degradation [103].

Differently, RVs of the major group enter the cell through a clathrin-independent pathway. The binding with the receptor ICAM-1 triggers the virus internalisation, which results in a

sort of untypical form of macropinocytosis, which involves actin fibres to direct the transport of extracellular liquid and membrane-bound ligands [103]. However, the mechanism of viral uptake for the major group has not fully elucidated. The release of viral RNA and proteins into the cytosol occurs via lysis of the endosomal membrane, without lysosomal degradation [104]. Recent findings suggests that a major group ICAM-1 associated serotype may involve clathrin to entry the cell and the so named endocytic recycling compartment (ERC) for uncoating [105, 106].

The mechanism involved in RV-C infection, a recently discovered and highly pathogenic specie is still unknown. The comprehension of the features underlying RV entry and uncoating is essential in order to identify new targets and to develop new potential antiviral drugs.

1.3.3 Virion structure and genomic organisation

Rhinovirus, a positive-sense single-stranded RNA (ssRNA) virus of approximately 7.2 kb, is a small particle of 25-30 nm diameter with a non-envelope icosahedral capsid, made of 60 copies each of the four structural protein, VP1-VP4. VP1, VP2 and VP3 are external proteins responsible for the antigenic diversity, while VP4 anchors the ssRNA to the capsid. A canyon on VP1 represents the site of attachment of the virus to the membrane-receptor on the cell surface [99, 107]**.** The RV icosahedral shell protect the viral RNA. Rhinovirus genome has a short viral priming protein typical of Picornaviruses, 3B/VPg, covalently linked to the 5’ untranslated region (5’UTR), which 5’ terminal portion is modelled into a cloverleaf, quite similar between species, followed by a spacer, highly variable between serotypes but also variable within serotype. Adjacent to the spacer, moving 3’ direction, are the internal ribosomal entry subunit (IRES) and the intervening sequence, preceding the open reading frame (ORF), in turn divided in three translated regions, P1-P3 [108]. P1 encodes for the capsid proteins, while P2 and P3 encode for the non-structural proteins (NSPs). A short 3’ untranslated region (3’UTR) and a poly-A tail complete the genomic structure [99] (Fig.1.1).

Inside the cytoplasm, the entire viral genome is translated in a large polyprotein by binding of the host 40S ribosomal subunit to a 5’ triplet, e.g. AUG, in the IRES and subsequent processing into P1, P2 and P3 by virally encoded proteases [108]. Subsequently, P1 is cleaved to form VP1, VP3 and the precursor-protein VP0, which is further cut to form VP4 and VP2 [109]. VP1-VP4 represent the structural proteins to build the viral capsid. P2 is cleaved to form $2A^{pro}$ and 2BC, which is further cut to form 2B and 2C, while P3 is cleaved to form the precursor 3AB and 3CD, further cut to form 3A and 3B/VPg the former and $3C^{pro}$ and $3D^{pol}$ the latter. The non-structural proteins generated from P2 and P3 are

involved in viral genome replication and assembly. Finally, the host transcriptional machinery produces eleven viral proteins, in total [110].

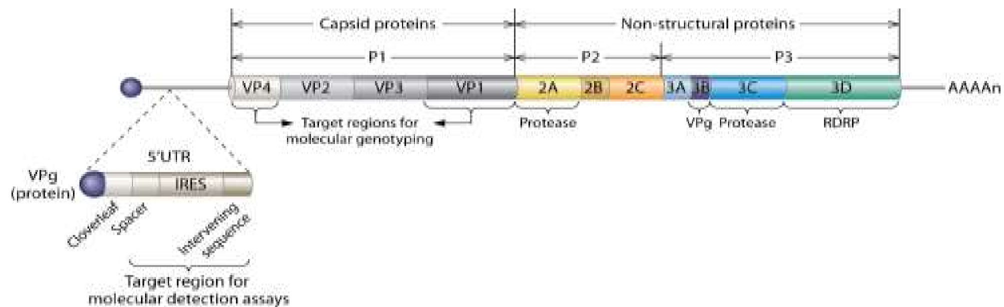


Figure 1.1 RV genomic structure

Human rhinovirus is an enterovirus, which 7.2 Kb positive-sense single-stranded RNA encodes for the synthesis of a large polyprotein. The translation starts with the ribosomal binding to the internal ribosomal entry subunit (IRES) on the 5' untranslated region (5'UTR), which is linked to the genome virion protein VPg, typical of Picornaviruses. The polyprotein is subsequently processed and further cleaved in four capsid proteins (VP1-VP4) and seven non-structural proteins, involved in viral replication, including proteases and the RNA-dependent RNA polymerase (RDRP), by the viral proteases 2A and 3C.

1.3.4 Viral life cycle

Rhinovirus infection in airway epithelial cells occurs via receptor-mediated endocytosis or macropinocytosis. After internalisation and viral capsid break down inside the endosomes, the viral genome is released into the cytosol through a pore formed by viral proteins or following membrane lysis and is translated into a large polyprotein, which is eventually processed by the viral proteases 2A and 3C to produce four structural and seven non-structural proteins (Fig.1.2-(1) and 1.2-(2)). After translation of the P1 region, encoding for the structural proteins, the protease 2A of the P2 region is the first translated protein. It subsequently cleaves itself and the rest of the P2 region from the P1 region, before the completion of the full polyprotein translation. Once even the second protease 3C is translated, it further cleaves the polyprotein including the precursors generating the rest of the viral proteins, except for the VP0 precursor, which cleavage mechanism is unknown [111]. Following these processes, viral replication takes place. The viral polymerase 3D copies the positive-sense single-stranded RNA in its complementary negative-strand RNA, which in turn acts as a template for synthesising a large number of copies of the viral genome (Fig.1.2-(4)). Multiple cycles of translation in viral proteins and viral genome replication lead to the virus amplification. Capsid proteins are assembled incorporating the

viral genome to produce mature virions, which are released into the extracellular environment via cell lysis or in extracellular vesicles, spreading a new progeny of infective virus particles [99, 107] (Fig.1.2-(5)).

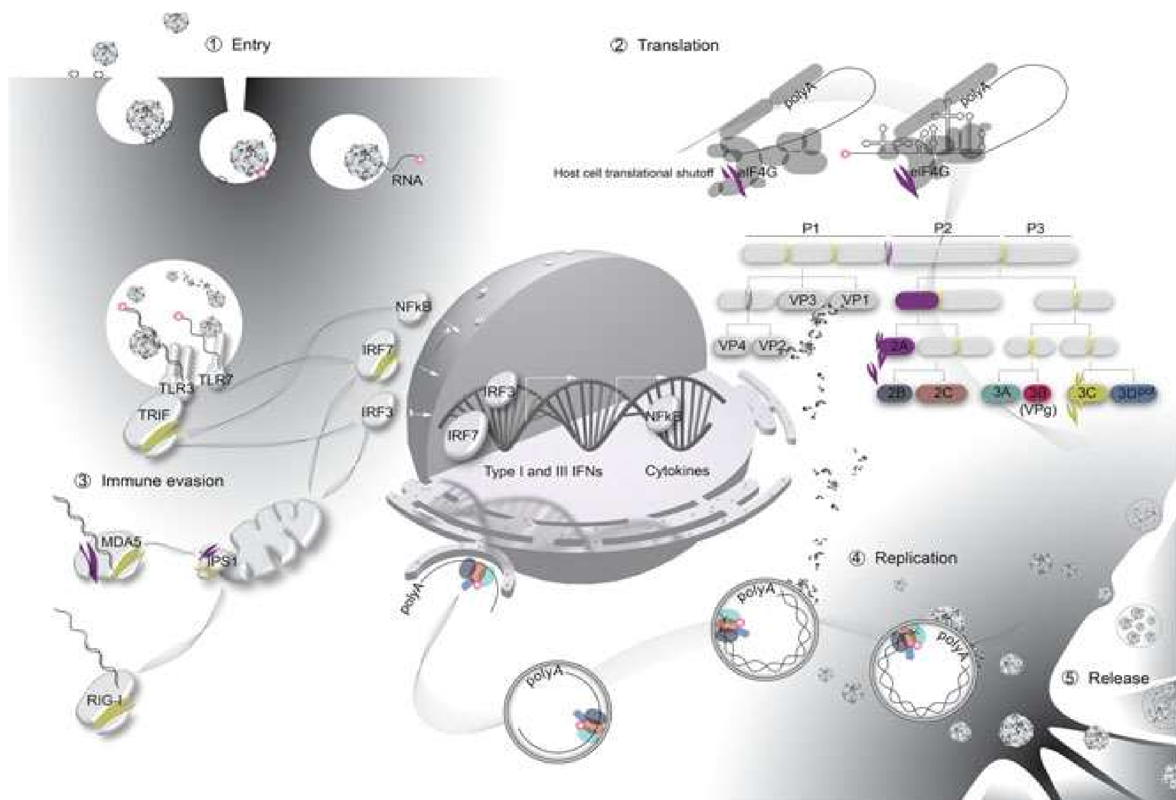


Figure 1.2 Enterovirus life cycle

(1) Entry. After surface receptor attachment and virus internalisation, viral capsid is disassembled and viral RNA released into the cytoplasm. (2) Translation. Viral RNA is translated in a large polyprotein, which is processed by the proteases 2A and 3C to produce four structural and seven non-structural proteins. (3) Immune evasion. The virus blunt the host immune response, by proteolysis of the intracellular receptors MDA5 and RIG-1 and of the innate signalling inducer IPS-1, blocking the production of interferons and cytokines. (4) Replication. Viral proteins interact with cellular lipids and proteins to promote viral RNA replication in specialized membranous vesicles (ROs). (5) Release. The virions are assembled incorporating the viral positive-sense RNA genome into the viral capsid and are released via cell lysis or in extracellular vesicles.

1.3.5 Viral proteins function and replication complex

Functional studies in Picornaviruses, but not necessarily in Rhinoviruses, elucidated the main role of single viral proteins. The proteases 2A and 3C not only cleave viral polypeptides, but also inhibit several host cellular mechanisms (Fig.1.2-(3)). 3C inhibits

host transcription by cleavage of various transcription-associated factors and regulators, while 2A shuts off host proteins synthesis by cleavage of the eukaryotic initiation factor 4 gamma-1 (eIF4GI) and gamma-2 (eIF4GII). Additionally, 2A and 3C induce cell apoptosis [110]. Positive-strand RNA viruses, such as Rhinoviruses, remodel endoplasmic reticulum (ER) and Golgi apparatus membranes in host infected cells, to build specialized membranous vesicles with unique lipid composition. These vesicles have been associated with the viral replication complex and thus named replication organelles (ROs), which, additionally, protect viral RNA from cellular RNAses degradation and immune antiviral responses triggered by cytosolic RNA sensors. The ROs evolve from single-membrane tubule into double-membrane vesicle as replication progresses (Fig.1.2-(4) [112, 113]. Viral protein 2B and its precursor 2BC contain two hydrophobic regions, important for their integration with host membranes, and are responsible for changes in membrane permeability and disassembly of ER and Golgi complex, also increasing the efflux of Ca^{2+}. The reduction of the Ca^{2+} level blocks the protein transport from ER to Golgi. Protein 2C also participates in vesicle formation. Protein 3A plays a key role in the formation of the replication complex. 3A is a membrane binding protein, inhibiting cellular protein trafficking by redistribution of ADP-ribosylation factors (Arfs), important components of the membrane secretion pathway. 3A, in fact, recruits to the RO membrane the protein Arf, which also participate in viral RNA replication, by involvement of its nucleotide exchange factor (NEF) Golgi-specific brefeldin-A (BFA) resistance factor 1 (GBF1) [110] , inducing the recruitment of phosphatidylinositol 4-kinase IIIβ (PI4KB), which promotes the production of phosphatidylinositol 4-phosphate (PI4P) from phosphatidylinositol (PI) [114, 115]. This lipid-rich microenvironment, in turn, enhance the recruitment of the RNA-dependent RNA polymerase 3D [116]. In many Picornavirus, 3A can also interact with acyl-coenzyme A binding domain-containing 3 (ACBD3) to recruit PI4KB [117]. The recruitment of all these elements to the ROs creates the conditions to promote viral replication. Membrane lipids are fundamental components for determining membrane properties such as curvature, fluidity and charge, and are involved in protein recruitment to the membranes. However, lipids play a crucial role in RO. The lipid PI4P recruits oxysterol-binding protein (OSBP) from ER membrane. Then, OSBP mediates the accumulation of the structural lipid cholesterol from ER to the RO membrane through an exchange-flux with PI4P from RO to ER. The redistribution of membrane lipids creates the optimal conditions for generation and functioning of replication organelles [112, 118]. 3AB acts as a membrane-associated protein and may serve for viral RNA-dependent RNA polymerase 3D recruitment on the replication complex. Protein 3B/VPg, linked to the 5'UTR, serve as a primer in both positive and negative-strand RNA synthesis. Protein 3CD, stimulated by the protein 3AB, also exhibits protease activity [110].

The comprehension of the mechanisms underlying enterovirus replication complex is important for the development of new anti-viral targets.

1.3.6 Antiviral drugs discovery challenge, a brief overview

The discovery of an effective therapy against enteroviruses has always been a big challenge. The main issue is represented by the high mutability that characterises these viruses, resulting in the rapid emergence of drug-resistance. The consequence is that there are currently no approved anti-viral drugs for the treatment of enterovirus infections, included RV. Furthermore, the identification of around 170 RV serotypes gives a reason of why there are also no vaccines available for preventing RV infections [99].

Several antivirals have been developed targeting various viral components. Pleconaril, for instance, is a small capsid-binding molecule that integrates in the hydrophobic pocket of VP1 inhibiting virus attachment to its cognate surface cellular receptor, rather than the following uncoating that is the release of viral RNA in the cell cytoplasm. However, the emergence of resistance to pleconaril emerged during clinical trials of RV infections [119]. Non-structural proteins can also be a target for antiviral drugs. Rupintrivir has been selected as a potent peptidomimetic inhibitor of the viral protease 3C in vitro. However, despite its efficacy in reducing RV load in a human experimental challenge trial, its clinical development was halted because it was not significantly effective against naturally occurring cold [120,121]. Viral proteins 3D and 2C are other largely investigated targets in the anti-viral drugs discovery challenge [122-124].

An alternative approach for the identification of a therapeutic strategy against enteroviruses is targeting host cellular proteins. Some examples include: brefeldin A (BFA), a fungal metabolite that inhibits GBF1 [125]; inhibitors of PI4KB [126] and OSBP inhibitors, such as itraconazole [127]. All these molecular targets are involved in viral replication of RNA viruses, such as RV, in specialized membranous vesicles within airway epithelial cells, as explained above. Other molecules can also potentiate the antiviral properties of airway-derived nitric oxide synthase 2 (NOS2) or reduce ICAM-1 transcription, thereby inhibiting RV replication and inflammation in airway epithelial cells [128]. Guedán and colleagues have also identified protein kinase D (PKD), which is involved in the control of vesicular and lipid transport at Golgi membranes, as a novel antiviral target for drug discovery [129]. Most interesting, Mosnier and colleagues have recently proposed IMP-1088 as a potent inhibitor of the N-myristoyltransferases 1 (NMT1) and NMT2, eukaryotic enzymes responsible for transferring myristate from myristoyl coenzyme A (Myr-CoA) to VP0 in the N-terminus of RV polyprotein, a mechanism involved in viral infectivity. The effect of IMP-1088 is the blockage of RV capsid assembly,

resulting in rapid and complete prevention of viral replication. Thus targeting host myristoylation is a promising strategy in developing new effective and safe drugs for RV infections and thereby treating the common cold [130].

1.4 Host antiviral responses

Rhinovirus infection of the airways occurs primarily in nasal and bronchial epithelial cells, which trigger both anti-viral and pro-inflammatory responses in order to control virus infectivity and eliminate the pathogen [61, 131]. The effect of RV infection in epithelial cells is the recruitment of other cells *in situ*, which contribute to airway inflammation and respiratory symptoms. Activated macrophages secrete interferons and pro-inflammatory cytokines and chemokines, such as TNF-α, MIP-1α, IL-1 and IL-8. During acute infections, increased neutrophils infiltration leads to the production of elastase, cathepsin G and neutrophil proteinase 3, inducing pathogen eradication and airways remodelling. Eosinophilic inflammation has been associated with an increased risk of virus-induced exacerbations [132]. Cell production of several mediators orchestrates an effective link between innate and adaptive immune responses against the virus [133-135].

1.4.1 Innate immunity

Several pattern recognition receptors (PRRs) recognise pathogen associated molecular patterns (PAMPs) and activate intracellular signalling pathways. Following surface RV uptake and receptor-associated endosomal internalisation, toll-like receptors (TLRs), a well-known group of transmembrane PRR, mediate the innate host defence against the virus and play a crucial role in recognising various RV constituents. TLR2 interacts with the viral capsid on the cell surface, inducing the activation of nuclear factor-kB (NF-kB) through recruitment of the adaptor protein myeloid differentiation primary-response gene 88 (MyD88), which results in the production of pro-inflammatory cytokines, such as IL-6 and IL-8, and anti-viral interferons (IFNs) (Fig.1.3). Furthermore, TLR2 can activate the mitogen-activated protein (MAP) kinase p38, leading to the activation of one other mediator of the inflammatory cascade, the activator protein 1 (AP-1) [136]. Upon endosome formation, TLR7 and TLR8 mediate the recognition of the viral single-stranded RNA (ssRNA), while TLR3 senses the double-stranded RNA (dsRNA), once the viral replication has started [137]. TLR7/TLR8 recruits MyD88 and activates IkB kinase alpha (IKKα) and IKKβ through TNF receptor-associated factor 6 (TRAF6). This protein association leads to IkB phosphorylation and subsequent degradation, which in a non-phosphorylated state is coupled to NF-kB. The dissociation of NF-kB from its inhibitor enable the translocation into the nucleus and the activation of pro-inflammatory gene

expression. Also the interferon-regulatory factor 7 (IRF7), highly constitutively expressed in plasmacytoid dendritic cells (pDC) as a unique feature of this cell type, is activated as a dimer, following TLR7/TLR8 stimulation, and is mainly associated with IFNα induction. Moreover, upon dsRNA interaction, activated TLR3 dimerises and recruits the TIR domain-containing adaptor protein inducing IFN-β (TRIF) to activate IKKε and TRAF family member-associated NF-kB activator (TANK)-binding kinase 1 (TBK1). TBK1/IKKε subsequently induces IRF3 dimerisation, leading to type-I interferon expression and to IRF7 upregulation in responding cells, through a positive feedback loop. Similar to IRF3, when upregulated, IRF7 is phosphorylated by TBK1/IKKε, stimulating further type I IFN release. However, IRF3 is essential for viral induction of IFN-β. TLR3/TRIF also activates NK-kB through IKKα/IKKβ [138, 139]. Within the intracellular compartment, TLR3 triggers the upregulation of RIG-like receptors (RLRs) including retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated protein 5 (MDA5), a second group of cytosolic PRRs, which also sense newly synthesized single stranded and double-stranded viral nucleic acids, respectively. The engagement of RNA helicases RIG-1 and MDA5 induces the production of type I-III IFNs including IFN-β, IFN-γ and IFN-λ, as well as of pro-inflammatory cytokines, such as IL-6, T-cell chemokines IP-10 and RANTES, and neutrophil chemokines IL-8 and ENA78 [99, 140, 141]. The molecule IFN-β promoter stimulator 1 (IPS-1) has been identified as a signalling mediator for RIG-I and MDA5, which downstream events lead to IRF3, IRF7 and NF-kB activation through similar mechanisms described above. Noteworthy, IRF7, RIG-I, MDA5 and TLR3 are themselves ISGs [142, 143].

All together, the transcription factors IRF3, IRF7, NF-kB and AP-1 regulate both anti-viral and pro-inflammatory immune responses.

Overall, the combined recognition of different RV constituents by different TLRs and RLRs in host epithelial cells results in the activation of different signal transduction pathways to fight the virus, also responsible for clinical symptoms of the common cold.

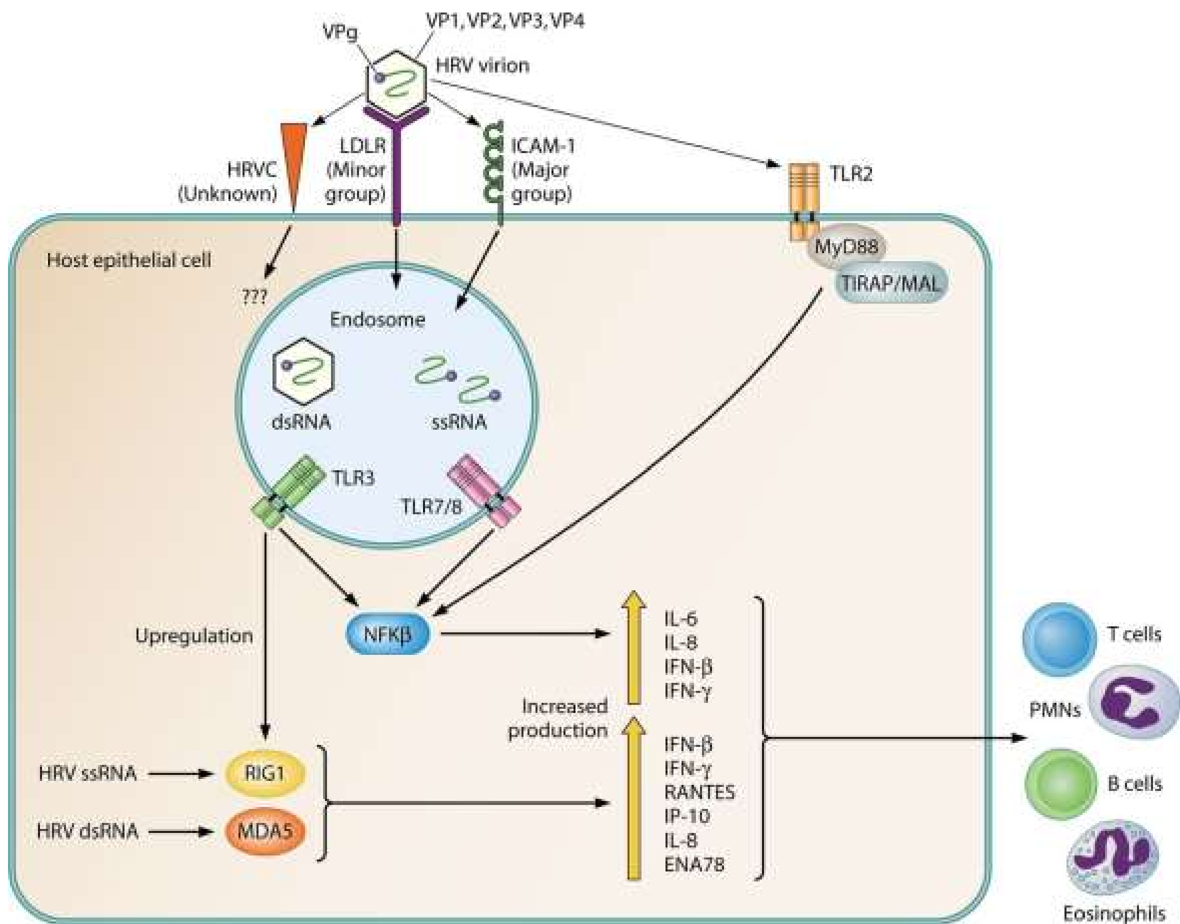


Figure 1.3 RV activation of innate immune response

Airways epithelial cells represent the first site of RV infection and interferons modulation is one of the first lines of antiviral response. Virus uptake via surface receptor binding and subsequent endosomal internalisation trigger the host innate immune response. Different pathogen associated molecular patterns (PAMPs) are recognised by several pattern recognition receptors (PRRs). Among them, toll-like receptors (TLRs) are membrane-located while retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated protein 5 (MDA5) are solubilized in the cytosol. Nuclear factor kB (NF-kB)-mediated pro-inflammatory and antiviral signalling is activated by myeloid differentiation primary-response gene 88 (MyD88)-dependent TLR-2 pathway, following recognition of viral capsid, and by TLR-7/TLR-8 and TLR-3, activated by single-stranded (ss) and double-stranded (ds) RNA, respectively. TLR-3 also triggers RIG-1 and MDA5 to recognise newly synthesized ssRNA and dsRNA. Overall, RV induces the production of antiviral type I-III interferons including IFN-β, IFN-γ and IFN-λ, as well as of pro-inflammatory cytokines and chemokines, such as IL-6, IL-8, IP-10 and RANTES.

1.4.2 Interferon signalling

Interferons are a class of soluble glycoproteins produced in a variety of cells in response to viruses or bacteria infection. IFNs are released in the extracellular space, where they

can be detected by specific receptors on the cell membrane surface in an autocrine or paracrine loop, inducing an antimicrobial state in infected and neighbouring uninfected cells. IFNs also show antitumor and immunomodulatory activity. Activated IFN signalling events induce interferon stimulated gene (ISGs) expression and modulation of innate and adaptive immune response. IFNs are classified into three types, based on their receptor structure, however all IFNs signal through the Janus Kinase (JAK)-signal transducer and activator of transcription (STAT) pathway [144, 145].

Type I IFNs include IFN-α, with 13 subtypes encoded by 14 genes and IFN-β, ε, k and ω, encoded by single genes; IFN-γ is the only representative of type II IFNs, while type III IFNs include IFN-λ1, IFN-λ2, IFN-λ3, also known as IL-29, IL-28A and IL-28B, respectively, and the recently described IFN-λ4 [146, 147].

1.4.2.1 Type I IFNs

Different cells produce type I IFNs during virus infections. Non-immune cells, such as epithelial cells and fibroblasts predominantly produce IFN-β, inducing ISGs expression in infected and neighbouring cells. ISGs activate a series of intracellular events to limit pathogen infection. Innate immune cells, including macrophages and dendritic cells (DC), produce IFN-α and IFN-β after sensing pathogen component via PRR and respond in turn to interferons, which promote antigen presentation and the production of immune response mediators. In particular, plasmacytoid DCs produce large quantities of IFN-α. Interferons also stimulate natural killer cell function. In addition, adaptive immune cells modulation by interferons induce antibody production in B cells and increase effector function of T cells [148]. Type I IFN modulation of innate and adaptive immunity represents the first line of host defence against viruses.

1.4.2.1.1 The canonical pathway

IFN-α and IFN-β, the most well defined type I IFNs, bind the transmembrane interferon-alpha receptor (IFNAR) on the surface of nearby cells. IFNAR is expressed by nearly all cell types and is composed of two subunits, IFNAR1 and IFNAR2, respectively associated with the receptor proteins tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1), which are both Janus family kinases (JAKs). These two kinases, which are in an inactive state in absence of stimulus, undergo a rapid auto-phosphorylation and activation upon IFN binding. In turn, TYK2 and JAK1 phosphorylate the signal transducer and activator of transcription (STAT) proteins, activating type I IFN-induced signalling pathways [149, 150], through specific interactions between STAT Src-homology 2 (SH2) domains and receptor phospho-tyrosine residues (Fig 1.4) [151].

In the canonical pathway, IFNα/β receptor-binding triggers the rearrangement and dimerisation of IFNAR subunits, promoting the recruitment of the latent cytoplasmic transcription factors STAT1 and STAT2 and their subsequent phosphorylations on tyrosine residues Y701 and Y690, respectively. STAT1 Y701 (pSTAT1 Y701) and STAT2 Y690 (pSTAT2 Y690) phosphorylations are crucial to activate STAT1-STAT2 dimerisation through their SH2 domains, and further association with interferon regulatory factor 9 (IRF9), forming a complex called interferon stimulated gene factor 3 (ISGF3) [152-154]. This complex translocates into the nucleus and promotes the expression of a distinct subset of interferon-stimulated genes (ISGs), binding to DNA sequences, known as IFN-stimulated response elements (ISREs), on target promoters. IRF9 directly bind to the DNA and STAT1 provides additional DNA contacts, stabilising the complex. The role of STAT2 is the recruitment, through its transcriptional activation domain (TAD), of important transcriptional co-activators, such as the p300/CREB binding protein (CBP), which shows a histone acetyltransferase (HAT) activity. STAT2, in fact, is not able to directly bind the DNA [155, 156]. However, for full transcriptional activity and biological function, STAT1 must also be phosphorylated on serine S727 residue within the COOH-terminal amino acidic sequence [157-159]. STAT1 Y701 phosphorylation, its nuclear translocation and DNA binding have been demonstrated to be necessary for IFN-induced S727 additional phosphorylation [157].

Although STAT1-STAT2 heterodimers are major involved in type I IFN response, STAT1 can secondarily form STAT1-STAT1 homodimers. This complex do not recruit IRF9 to induce the transcription of pro-inflammatory genes, following interaction with gamma-activated sequences (GAS) in the promoter region [160].

Moreover, other STAT dimers are activated in response to type I interferons; the STAT3-STAT3 complex, for instance, interact with the promoter GAS element, activating the expression of as not well-known inflammatory pathway repressors, counterbalancing STAT1 activation and ISGF3 function. The SIN3 transcription regulator homologue A (SIN3A) complex is described for its ability to bind STAT3, acting as a co-repressor of STAT3-mediated gene induction [148, 150].

Regarding the kinetics of activation, type I IFN antiviral immune response occurs rapidly in case of acute infections. STAT1 phosphorylation on tyrosine residue Y701 occurs within 5 minutes or less, whereas STAT1 phosphorylation on serine S727 takes more than 10 minutes [149].The kinetics of IFN-β induced S727 phosphorylation is delayed by around 15 minutes after Y701 phosphorylation and nuclear accumulation [157]. Additionally, small amounts of IFN-β maintain high basal levels of STAT1 and IRF9 in homeostatic condition [161]. Furthermore, type I interferons include several subtypes with different receptor

affinity, likely contributing to modulate the temporal IFN production and the strength of the innate immune response [162].

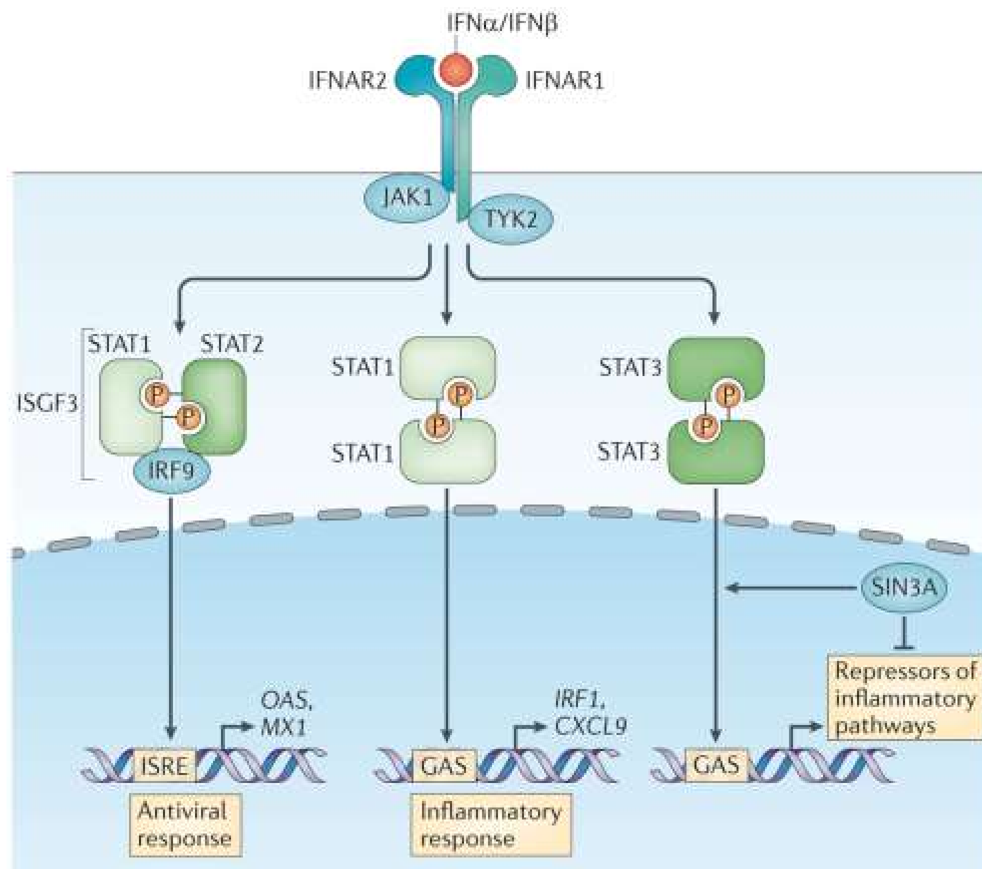


Figure 1.4 The canonical type I interferon signalling pathway

Type I interferons (IFN-α/IFN-β) specifically bind to interferon-alpha receptor (IFNAR) which is composed of IFNAR1 and IFNAR2 subunits, respectively associated with the protein kinases TYK2 and JAK1 in their cytoplasmic domains. Upon IFN binding, these two kinases undergo reciprocal phosphorylation, which in turn further phosphorylate IFNAR, predisposing it to function as docking site for signal transducer and activator of transcriptions (STAT) proteins. The recruitment of STAT1 and STAT2 to the receptor induce STAT1 Y701 and STAT2 Y690 phosphorylations on their tyrosine residues, leading to STAT1-STAT2 dimerisation and association with IRF9, forming a complex called interferon stimulated growth factor 3 (ISGF3). Once activated, the complex translocates to the nucleus and triggers interferon stimulated gene (ISG) expression, by recognition of interferon-stimulated response elements (ISRE) within their promoters, activating the antiviral response. To a smaller extent, STAT1-STAT1 homodimers directly induce pro-inflammatory gene expression, whilst STAT3-STAT3 homodimers indirectly repress the inflammatory response, both interacting with GAS elements on DNA sequences.

1.4.2.1.2 Signalling regulation

STAT1 Y701 and STAT2 Y690 tyrosine phosphorylations are crucial cytoplasmic events for dimerisation and nuclear localisation sequences (NLSs) activation. Once activated, ISGF3 complex (STAT1/STAT2/IRF9) and STAT1 homodimers are accumulated in the nucleus through an importin α5-mediated mechanism [163, 164]. Conversely, serine S287 phosphorylation of STAT2 negatively regulates type I IFN induced cellular response [165]. The post-translational conjugation of STAT1 lysine K703 with a small ubiquitin-like modifier (SUMO) protein, a process similar to ubiquitination known as SUMOylation, was also reported as inhibitory signal of transduction, which event also inhibits STAT1 phosphorylation at Y701 [166]. pSTAT1 Y701 chromatin association enables the transcriptional machinery to initiate the expression of IFN-stimulated genes. By contrast, STAT1 Y701 de-phosphorylation inactivates the protein in both STAT1 homodimers and ISGF3 complexes, causing STAT1 loss of DNA-binding ability and its relocation to the cytoplasm. It has been demonstrated that promoter occupancy of phosphorylated STAT1 Y701 gradually decreases during progress of the transcriptional process. The promoter dissociation represents the regulatory step for transcriptional cessation, followed by phosphatases-driven STAT1 and STAT2 de-phosphorylation [167]. The nuclear T cell protein tyrosine phosphatase (TC-PTP) identified as TC45 is the major phosphatase directed to Y701 [168]. SH2 domain-containing protein-tyrosine phosphatase 2 (SHP2) is another phosphatase involved in STAT1 dephosphorylation at both tyrosine 701 and serine 727 residues in nuclei [169]. However, as explained above, for full transcriptional activity nuclear STAT1 S727 phosphorylation, driven by kinases such as cyclin-dependent kinase 8 (CDK8) [170] or by protein kinase C-delta (PKC-δ), is strictly required. PKC-δ further shows a dual effect, being involved as an upstream regulator of the p38 mitogen-activated protein kinase (MAPK) pathway, which participates in IFN-α dependent transcriptional regulation, besides mediating the inflammatory response [171]. The p38 MAPK signalling is activated by IFNAR and cooperates with the STAT pathway, as evidenced by the abrogation of IFN-dependent ISG expression via ISRE caused by the inhibition of p38 activation, without inhibiting STAT function [172, 173]. However, although p38 MAPK signalling is not required for S727 and Y701 phosphorylations, it contributes to type I IFN-dependent transcriptional regulation of ISGs in a non-canonical way [174]. Moreover, stress signals such as UV, lipopolysaccharide (LPS) or TNF-α can induce phosphorylation of STAT1 S727, but not Y701 via the p38 MAPK pathway, in an IFN-independent manner. An increased ISG transcriptional activation is also due to p38 pathway-dependent enhancement of IRF1 expression, independently of S727 phosphorylation [175]. Collectively, different molecular mechanisms can balance the antiviral immune response acting on STAT1/2 activity.

Considering the IFN-α receptor (IFNAR), acetylation by p300/CBP plays a crucial role for its function as docking site for STAT1, STAT2 and IRF9, which in turn undergo acetylation by CBP, activating the downstream signalling [176]. By contrast, pro-inflammatory cytokines such as IL-1 promote IFNAR internalisation via p38 kinase and casein kinase II (CK2)-mediated receptor phosphorylation, limiting cellular IFN responsiveness. Strong activation of immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors, which seems be able to sense the extracellular environment and tune cellular responses, can also recruit PKC-β or PKC-δ, which phosphorylates the SHP2 phosphatase, leading to dephosphorylation of signalling intermediates, likely including JAK protein. Ubiquitin carboxy-terminal hydrolase 18 (USP18) is an additional cytokine-induced protein that displaces JAK1 from IFNAR2 subunit [148, 177].

Type I IFN signalling is negatively regulated in cells to prevent excessive pathway activation, and return to a homeostatic state. A loss of regulation can induce tumorigenesis, chronic inflammation and autoimmune diseases [178, 179].

Various cytokines can stimulate the expression of the suppressor of cytokine signalling (SOCS) proteins family. These proteins share a conserved structure consisting of an N-terminal region of varying length and sequence, a central Src Homology 2 (SH2) domain and a C-terminal SOCS box motif, which promotes the inhibition of the IFN signalling pathway through binding and ubiquitination of proteins directed to proteasome degradation [180]. SOCS1 and SOCS3 can both inhibit JAK enzymatic activity, directly or via SH2 domain pre-association with IFN-receptor, thus inhibiting ISGs production [181]. A competition between SOCS proteins and STATs for IFNAR binding is also a reported inhibitory mechanism. However, several details about SOCS regulatory activity of IFN signalling still need to be clarified [182]. Gielen and colleagues reported an induction of SOCS1 mRNA and protein by IL-4 and IL-13, Th2 cytokines strongly implicated in asthma pathogenesis, by TNF-α and IL-1β, pro-inflammatory cytokines, and by RV-1B (minor group), RV-16 (major group) and poly I:C (a synthetic analog of viral dsRNA) in *in vitro* experiments in primary bronchial epithelial cells (BECs) from asthmatic patients. They also proposed a novel nuclear role of SOCS1 and correlated increased SOCS1 protein level in bronchial epithelium with stable severe asthma *in vivo* [183, 184], which is in line with studies reporting impaired IFN induction during RV-induced asthma exacerbations [54, 55, 185, 186].

Relative to the transcriptional regulation of ISGs, the organisation of the chromatin in higher-ordered nucleosomes prohibits transcription factors binding and consequent gene expression. Thus, IFN-induced ISG expression requires chromatin remodelling in a more relaxed structure. Histone acetylation and deacetylation are essential to maintain

chromatin in an open or closed conformation. Enzymes with histone acetyltransferase (HAT) or deacetylase (HDAC) activity respectively catalyse these reactions. HDAC are also reported to be required for RNA polymerase II recruitment to the promoter of ISGs, but the mechanism remain unclear. As mentioned before, p300/CBP, a HAT family member, is an important co-activator of ISG expression. By contrast, other negative regulators, such as protein inhibitors of activated STAT protein (PIAS) family, act as co-repressor. Further, the forkhead box O3 (FOXO3) protein, together with HDAC3 and the nuclear corepressor 2 (NCOR2), forms a complex that close the chromatin structure, regulating ISG expression under basal levels. Type I IFNs stimulate ISG expression through activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which in turn leads to FOXO3 degradation [174]. The balance between HAT and HDAC regulates gene expression, including inflammatory and immune genes. For example, HDAC2 expression and activity are reduced in macrophages and blood cells from severe asthmatics and smoking asthmatics as well as COPD patients, contributing to enhance lung inflammation and to reduce steroid responsiveness observed in these patients [187]. Glucocorticoids (GCs), widely used to reduce inflammation in asthma and COPD, also show a suppressive effect on ISG by binding and displacing glucocorticoid receptor-interacting protein 1 (GRIP1) from its interaction with IRF9 within ISGF3 complex, thus impairing ISGF3 function to promote ISG expression [188].

Furthermore, ISG transcription can be regulated through different non-canonical or IFN-independent mechanisms. Cytokines such as tumour necrosis factor alpha (TNF-α) can directly drive ISG expression by NF-kB protein complex activation [189]. The inhibitor of NF-kB kinase-ε (IKKε) mediates STAT1 phosphorylation, but favouring ISGF3 activation, rather than STAT1 homodimerisation [148]. Interestingly, IKKε also phosphorylates serine S708 of STAT1, an additional phosphorylation point for an effective antiviral response [190]. Moreover TNF can also indirectly promote a delayed JAK/STAT signalling activation through IFN-regulatory factor 1 (IRF1) promoted STAT1 phosphorylation [191, 192]. Interestingly, the DNA sequence for IRF1 recognition overlaps with ISRE. Conversely, the same sequence can be recognised by IRF2 to repress IRF1-induced transcription [174]. The transcription factors IRF3 and IRF7 can also induce a subset of ISGs in an IFN-independent manner [193, 194].

Overall, type I IFN signalling pathway activation induce an antiviral immune response in most cells, counterbalanced by a complex network of suppressive signals that regulates the magnitude of the immune response, limiting toxicity for the host.

1.4.2.2 Type II IFNs

Immune cells such as NK, Th1 and CD8+ cells, produce IFN-γ, the only representative of type II IFN class [195], which shows proper structural features, differentiating it from other IFNs. Despite this marked difference, it is included in the IFN family because of its ability to 'interfere' with viral infections. IFN-gamma receptor (IFNGR), localised on the surface of most cells, is composed of IFNGR1 and IFNGR2 subunits, respectively associated with JAK1 and JAK2. After IFN-γ receptor binding, activated JAK proteins phosphorylate each other, promoting IFNGR docking site function for STAT1 protein. Further phosphorylation of STAT1 results in the formation of STAT1-STAT1 homodimers, which move to the nucleus, where they recognise GAS elements to initiate the transcription of IFN-γ stimulated genes, such as IRF1 and interferon gamma-inducible protein (IP10) [150, 196-198]. As explained above, STAT1 requires Y701 phosphorylation for nuclear translocation and subsequent S727 phosphorylation for full transcriptional activity [199]. IFN-γ activates protein kinases PKC-ε and PKC-δ in a PI3K-dependent way to induce STAT1 S727 phosphorylation [200, 201]. In addition, IFN-γ mobilizes a Ca^{2+} flux in cells, activating the Ca^{2+}/calmodulin-dependent kinase II (CAMKII), which directly phosphorylates STAT1 in position S727, contributing to maximal activation of type II IFN induced transcriptional pathway [202]. Therefore, in contrast to type I IFNs, which canonical activation of ISGF3 complex induce ISRE-containing gene expression, the IFN-γ induced pathway follows a different transcriptional route, leading to pro-inflammatory and anti-viral gene expression [203].

1.4.2.3 Type III IFNs

Recently described IFN-λ1, -λ2 and -λ3 (alias IL-29, IL-28A and IL-28B) are identified as a new class of interferons or IFN-like molecules [204] produced by most cell types, but thought to be active predominantly at anatomic surface barriers, included the lung mucosal barrier [205]. IFN-λs have been reported to likely be the principal IFNs produced by the innate immune response during respiratory viruses infections in bronchial epithelial cells [206]. The fact that only a small subset of cells, including some immune cells, are responsive to type III IFNs limits possible side effects usually related to persistent type I IFN production at systemic level [207, 208]. Exploring new therapeutic strategies, in order to prevent exacerbations caused by impairment of IFN production in cells isolated from asthmatic and COPD patients upon RV infections, exogenous administration of IFNs have been studying. It has been reported that the treatment of airway epithelial cells with IFN-β (type I) and IFN-λ1 (type III) *in vitro* had a protective effect against the virus, inducing up-regulation of ISGs. However, while IFN-β induced expression of ISGs decreased over time, IFN-λ1 induced a sustained or even increased ISG expression over time, which

together with its more localised action, could make IFN-λ1 a more promising candidate for prophylactic treatment than IFNβ [209].

These type III IFNs share with type I IFNs the same STAT1-STAT2-IRF9 (ISGF3) complex-mediated pathway to transduce the signal from the cell-surface to the nucleus, leading to ISGs expression. However, it has been shown that type III IFNs activate the pathway after type I IFNs peaks and subsides, showing different kinetics [210]. Moreover, IFN-λs bind a proper heterodimeric receptor, composed of IFN-lambda receptor chain 1 (IFNLR1) and IL-10 receptor chain 2 (IL-10Rβ), associated with the tyrosine-kinase proteins JAK1 and TYK2, respectively. Regarding IFN-λ4, the last discovered member of this IFN family, its role has not yet been understood [211].

1.4.3 Interferon stimulated genes (ISGs)

IFNs can induce the expression of hundreds of ISGs. The eradication of pathogen infections is the role of ISG-encoded proteins. Many ISGs exert antiviral action by several mechanisms targeting different steps of the virus life cycle, including the inhibition of viral transcription, translation and replication, the degradation of viral nucleic acids and the alteration of cellular lipid metabolism [148]. However, the ISGs effector system appear to be redundant and as new ISGs have been identified and characterised in recent years, the function of the majority of them and/or their contribution to the immune response *in vivo* remain unknown or to be further elucidated [212].

Classical ISGs include double-stranded RNA-activated protein kinase (PKR), which inhibits cellular and viral translation through phosphorylation of initiation factor EIF2α; myxovirus resistance (Mx) proteins, which appear to target viral nucleocapsid during exocytosis thus impeding virus replication; tripartite motif (TRIM) protein, which induces inhibition of viral transcription and sequestration of viral proteins. TRIM have been identified to also conjugate ISG15, another interferon stimulated gene, to cellular proteins, modifying their function through a mechanism known as ISGylation, similar to ubiquitination [212, 213].

The key orchestrators of the innate immunity, such as STAT1, IRF proteins, RIG-1 and MDA5, are constitutively expressed in most cells. Interestingly, they are themselves ISGs, which enhanced expression contribute to amplify and prolong the activated antiviral response. As described above, IFNs stimulate ISG expression through the JAK/STAT pathway, but ISGs can also be directly induced by IRF1, IRF3 and IRF7 in an IFN-independent pathway [193, 214].

Among ISGs genes, some have only ISRE or GAS elements in their promoter, other have both elements. Thus, for the optimal transcriptional activation of a particular gene, the

combination of different STAT-containing complexes may be required. However, the mechanisms underlying the differential gene regulation by STATs are not understood [150].

Other important antiviral ISGs of the immune system are the virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible (viperin) and 2’-5’ oligoadenylate synthetase (OAS).

1.4.3.1 Viperin

Viperin protein is encoded by the radical S-adenosyl methionine domain-containing 2 (RSAD2) gene that shows an inhibitory effect on several virus-type replication. Its expression is induced by IFNs in response to viral infections and therefore is classified as ISG [215]. Viperin associates with ER and ER-derived lipid droplets, which are important for cellular protein and lipid trafficking and that are thought to be a site of viral replication, through an amphipathic N-terminal domain, required for their localization [216, 217]; it also associates with mitochondria [218]. Viperin is involved in lipid biosynthetic or fatty acid metabolic pathways [212] and it further interacts with cell membrane integrity [219]. In addition, viperin promoted TLR signalling, stimulating IFN production in plasmacytoid dendritic cells [220]. Regarding the airways, nasal epithelial cells, from naturally acquired human rhinovirus (HRV) infections *in vivo*, were harvested and cultured *in vitro*, demonstrating an increased expression of viperin during infections. Increased RV replication were also observed in infected epithelial cells following viperin knockdown with short interfering RNA (siRNA) [221].

1.4.3.2 2’-5’ oligoadenylate synthetase (OAS)

IFN-α and IFN-β selectively induce OAS expression [150]. OAS family consists of three genes encoding for active enzymes OAS1, OAS2, OAS3 and a gene encoding for inactive protein OAS-like (OASL) [222]. OAS 1-3 are activated by double-stranded RNA and catalyse the formation of 2’-5’-oligoadenylates (2-5A) from ATP, which activate the latent ribonuclease L (RNaseL) to induce viral and host RNA degradation, thereby blocking viral replication and protein synthesis. OAS1 is the most well characterised protein. It produces shorter 2-5A relative to OAS2 and OAS3 and is thought to exhibit less ability to activate RNaseL. However, it has been demonstrated that enzymatically inactive OAS1 is still able to inhibit viral replication *in vitro*, suggesting that OAS immune functions have yet to be investigated [223]. OASL protein lacks the 2-5A synthetase activity, however it binds dsRNA showing an antiviral function [224]. Rhinovirus infections have been shown to up-regulate OAS gene expression in nasal epithelial cells [221].

1.5 Glucocorticoids (GCs)

Natural glucocorticoids are steroid hormones derived from cholesterol and secreted by the kidney-associated adrenal glands, in particular by the zona fasciculata of the cortex. Dynamic circadian and ultradian rhythm and environmental and physiological stress regulate the hypothalamic-pituitary-adrenal (HPA) axis to produce GCs and maintain metabolic and homeostatic functions in the human body. An imbalanced regulation can result in pathological conditions such as Cushing's disease and Addison's disease as the consequence of chronic hyperactive and hypoactive HPA axis, respectively. The hypothalamus secretes the corticotropin-releasing hormone (CRH) which stimulates the release of the adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. In turn, ACTH induces the synthesis and secretion of glucocorticoids, such as cortisol, from the cortex of the adrenal glands into the bloodstream, where the hormone is mostly bound to corticosteroid-binding globulins. The biological active form is the unbound cortisol, which can be locally converted in cortisone, its inactive form, by type 2 11β-hydroxysteroid dehydrogenase (11β-HSD2). Conversely, 11β-HSD1 converts the inactive cortisone to the active cortisol. A negative-feedback loop suppresses CRH and ACTH levels to maintain GC homeostatic conditions [225, 226]. According to the well-known immunosuppressive effects of GCs, an association between basal salivary cortisol secretion and susceptibility to clinically relevant upper respiratory infections in the short-term period has been established. In healthy adults, the endogenous production of cortisol shows inter-individual variability. Higher levels increases the risk of rhinovirus (RV) infections, which in chronic inflammatory respiratory diseases, such as asthma and COPD, have been linked to increased exacerbations rate. However, cortisol levels have not been related to severity of virally induced cold [227]. Moreover, basal salivary cortisol levels are lower in asthmatic children on moderate to high doses of inhaled corticosteroids (ICS) compared with those using no or low-dose ICS [228]. In addition, it has been reported that children with asthma have significantly lower long-term cortisol levels in their scalp hair than healthy children. Nevertheless, whether this is due to the chronic inflammatory disease *per se* or the effect of inhaled corticosteroids for asthma was not established [229]. Other studies drawn the conclusion that there is no evidence of suppression of cortisol production measured in scalp hair in asthmatic children using ICS compared to healthy controls. Further investigation is needed in the direction of potential HPA-axis suppression in asthmatics under ICS therapy with the additional purpose to evaluate whether the measurement of cortisol in scalp hair is a useful biomarker tool for the diagnosis of adrenal insufficiency. However, clinicians should consider this regardless of the dose of ICS prescribed [230]. Reducing steroid load and supplementing therapy with alternative treatments could reverse hypocortisolaemia and HPA-axis suppression in asthmatic children [231].

Synthetic glucocorticoids are drugs similar to natural glucocorticoids that differ in their potency and metabolic clearance. Synthetic GCs do not bind globulins and therefore their local availability is increased. Furthermore, dexamethasone does not undergo inactivation by 11β-HSD2. GC drugs are broad-spectrum anti-inflammatory molecules widely prescribed for the treatment of many chronic inflammatory conditions, including asthma, as well as for immunosuppressive disease [225, 232]. In addition, glucocorticoids have been used as co-treatment of patients with breast cancer (BC) for their ability to reduce chemotherapy-induced side effects such as nausea, lack of appetite, rather than inflammation. However, GCs may directly reduce tumour proliferation and angiogenesis, rather than apoptosis, induced by chemotherapy, radiation and cytokines [233].

1.5.1 Clinical use of GCs

Glucocorticoids (also called glucocorticosteroids, corticosteroids or steroids) are the most effective available therapy for asthma. However, a subgroup of asthmatic people show a corticosteroid-resistant phenotype, even at high doses of glucocorticoids. The same scenario of relatively inefficacy of GCs is observed in COPD patients, whether using inhaled or oral GCs [88, 90, 234].

A complex network of chemotactic mediators recruits and activates different inflammatory cell-types that chronically colonise the respiratory tract in both asthma and COPD. GCs suppress the production of these inflammatory mediators and adhesion molecules, inhibiting the recruitment and survival of eosinophils, T-lymphocytes, mast cells and dendritic cells, rather than inhibiting the production of inflammatory cytokines in alveolar macrophages. GCs also suppress many activated inflammatory genes in airway epithelial cells. Regular use of ICS can restore epithelial integrity. However, as described above, the nature of the inflammation defines the efficacy of the glucocorticoid therapy. GCs ability/failure to suppress inflammation in chronic inflammatory diseases have been widely investigated, leading to a better understanding of their underlying mechanisms of action. In a future perspective, the development of new selective classes of compounds may bring to improve the GC therapy and overcome the GC-resistance [235]. However, an additional partial explanation of GC resistance during RV-associated exacerbations may be the viral-induced enhanced activity of the transforming growth factor-β (TGF-β), which has been shown to impair GC action in human airway epithelial cells [236].

Inhaled corticosteroids (ICS) represent the first-line therapy in asthma. Their introduction in clinical practice reduced mortality and morbidity, restricting the use of oral glucocorticoids to severe episodes of asthma exacerbations [237, 238]. Commonly prescribed GCs include fluticasone propionate, beclomethasone monopropionate, which

is the active form of beclomethasone dipropionate, and budesonide. ICS suppress airway inflammation and therefore attenuate asthma symptoms, improve lung function, reduce bronchial hyperresponsiveness and the exacerbation rate. However, despite their efficacy, ICS safety in asthma treatment is a relevant debated issue for physicians and patients. Inhaled corticosteroids can produce local adverse effects such as dysphonia and thrush, whilst higher doses of ICS required for severe asthma more likely produce long-term side effects. In fact, an amount of GCs reaches the systemic level by absorption across lung and nasal mucosa or across the gastrointestinal tract absorption of swallowed drug. Adverse effects include suppression of HPA-axis, reduction in growth velocity, osteoporosis, diabetes, cataract, ocular hypertension and respiratory infections [239, 240]. Emerging evidence also suggests that GCs may have inhibitory effects on the innate immune response against respiratory virus infections, thus increasing viral replication [241, 242]. It is well-documented that rhinovirus (RV) infections are the primary cause of asthma and COPD exacerbations [78]. Overall, corticosteroid treatment is associated with several adverse effects, which can vary depending on several factors such as molecular differences among different GCs, coexistence of cellular GC receptor isoforms, intersubjective variability and environmental stimuli [243].

Current guidelines suggest the use of ICS in COPD in case of severe disease associated with frequent exacerbations, although high doses of corticosteroids showed little improvement in lung function or symptoms in around 20-25% of patients. Nevertheless, ICS are widely prescribed to control COPD symptoms and oral corticosteroids (OCS) can be a therapeutic option in case of acute disease exacerbations [235]. However, the overuse of ICS in COPD increases the risk for long-term side effects, including pneumonia [244]. Interestingly, Mitani and colleagues reported the potential of the polyphenol Quercetin to restore corticosteroid sensitivity in cells from patients with COPD [245]. Some patients with asthma-COPD overlap (ACO) are more responsive to corticosteroids than COPD patients, showing clinical features such as increased sputum/blood eosinophils and associated fractional concentration of exhaled nitric oxide (FENO), which are markers of inflammation, and greater bronchoconstriction reversibility, all characteristics that are generally associated with asthma [235, 246].

Overall, the clinical use of GCs in chronic inflammatory pulmonary diseases, especially in asthma, is due to their strong ability to suppress inflammation through activation of glucocorticoid receptors (GRs). The therapeutic effect of ICS is exerted through a dual mechanism of action: on one hand, GCs activate many anti-inflammatory genes and, on the other hand, repress many activated pro-inflammatory genes [243].

1.5.2 Glucocorticoid receptors (GRs)

The human NR3C1 gene, encoding for the ubiquitously expressed GR, is located on chromosome 5 and consists of nine exons. Exon 1 forms a 5'-untranslated region, while exons 2-9 form the protein-coding region (Fig 1.5-(A)) [225]. Alternative splicing of exon 9 generates the two highly homologous receptor isoforms GRα and GRβ, which are identical through amino acid 727. Then, GRα and GRβ diverge in their C-terminal region, having an additional 50 amino acids and 15 non-homologous amino acids, respectively, with final molecular weights of 97 and 94 KDa. Although their similarities, only GRα binds glucocorticoids in the cytoplasmic compartment and translocates to the nucleus, regulating the expression of GC-responsive genes. Therefore, GRα represents the classic glucocorticoid receptor, which functions as a transcription factor [247]. Differently, GRβ is thought to reside constitutively in the nucleus and to be transcriptional inactive, as it lacks the ability to bind GC agonists, acting as inhibitor of GCs action by interfering with GRα binding to glucocorticoid response elements (GREs) in the regulatory regions of glucocorticoid target genes. However, GRβ seems to have a proper independent transcriptional activity targeting genes not regulated by GRα and, additionally, to be able to bind the synthetic glucocorticoid antagonist RU486 (mifepristone) [248-250].

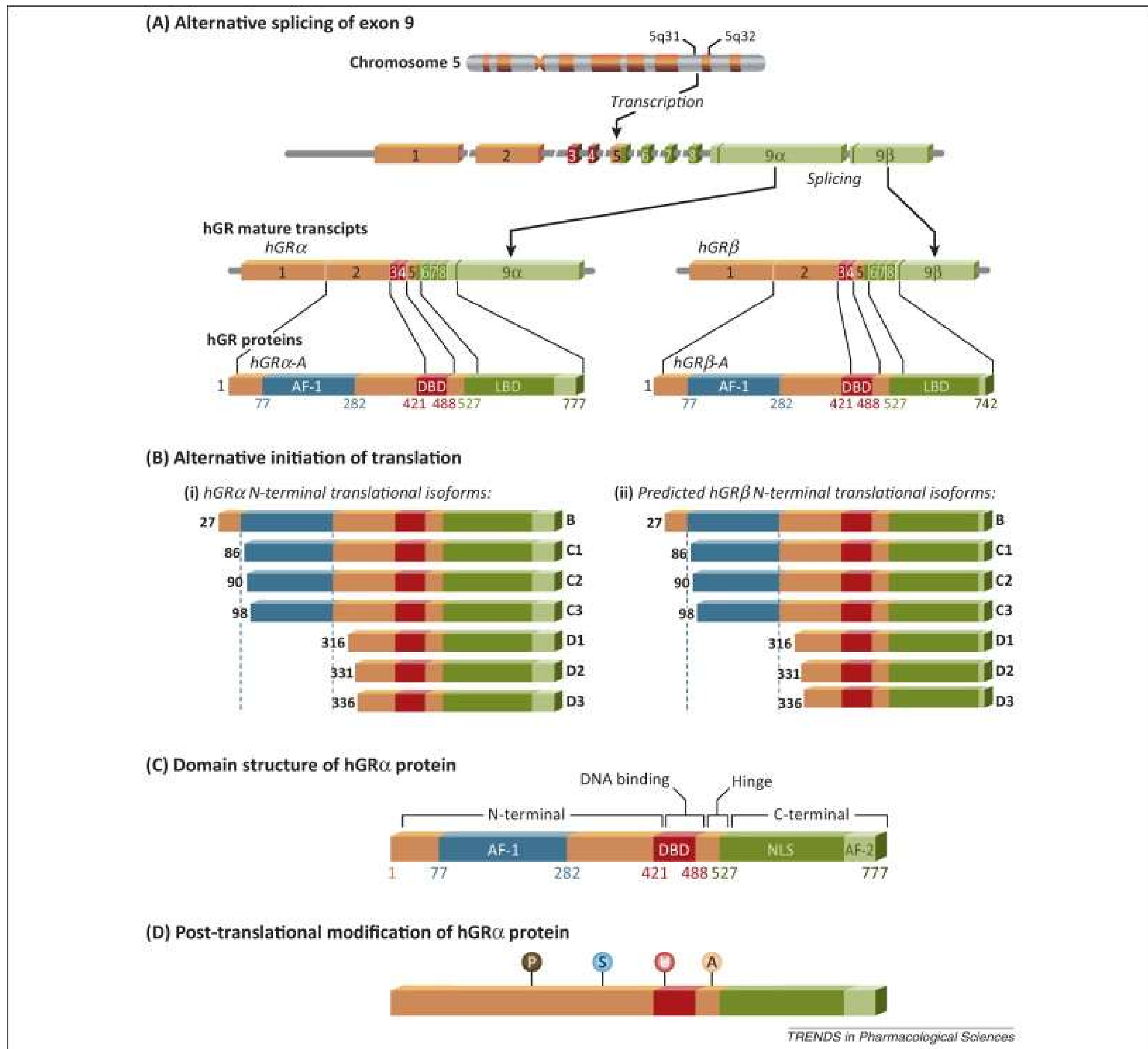


Figure 1.5 Human glucocorticoid receptor (GR) gene organisation and protein structure

(A) The human GR is encoded by the NR3C1 gene, located on chromosome 5 and composed of 9 exons. Alternative splicing of GR generates GRα and GRβ isoforms, which differ in their C-termini. **(B)** Additionally, different initiation sites in exon 2 generate eight GRα isoforms, seven of which have truncated N-termini (GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, GRα-D3). GRβ is predicted to similarly generate just as many β isoforms. **(C)** GR is a modular protein that comprise an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region (HR) and a ligand binding domain (LBD). The NTD contains a strong transcription activation function (AF-1), while the LBD contains a second activation function (AF-2). Nuclear localisation signal (NLS) identifies the protein portion that allow GR translocation to the nucleus, following GC activation. **(D)** GR undergoes post-translational modifications, including phosphorylation (P), SUMOylation (S), ubiquitination (U) and acetylation (A).

During the translational process of GRα transcript, different initiation sites in exon 2, which encodes for the N-terminal domain (NTD), bring to generate eight truncated GRα isoforms

(GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, GRα-D3) (Fig 1.5-(B)). However, despite substantial differences in the N-terminal region, all GRα isoforms interact similarly with GCs and GREs. Proceeding along the transcript in 3' direction, exon 3 and 4 encode for the DNA binding domain (DBD) and exons 5-9 for the hinge region (HR) and the C-terminal ligand-binding domain (LBD). GRβ transcript may also produce a similar set of N-terminal translational isoforms [225, 251]. Further, the NTD of GRα contains a transcriptional activating portion, termed activation function 1 (AF-1) (Fig 1.5-(C)), which is crucial for receptor interactions with transcriptional coactivators, RNA-polymerase II, chromatin modulators such as TATA-binding protein (TBP) and several TBP-associated proteins (TAFIIs). The DBD contains two zinc finger motifs, which allow GRα to bind specific DNA sequences, the GREs in the promoter region/s of target genes. The HR is involved in the formation of GRα dimers, conferring them a structural flexibility, thereby allowing the interaction with multiple GREs. The LBD contains the ligand-dependent portion AF-2 and plays a critical role in GRα activation. Other sequences within DBD and LBD have a role in receptor dimerization and translocation inside the nucleus. The LBD also interacts with coactivators and is important for binding to the heat shock proteins (hsp) [247].

In the cytosol, molecular chaperon heat-shock protein-90 (hsp90) and hsp70 associate each other to form a multiprotein hsp90/hsp70-based chaperone machinery, which play a key role for GR-acquiring of a high-affinity steroid-binding conformation, also interacting with other cofactors as well as immunophilins. Hsp90 has an intrinsic ATPase activity that induce a self-conformational change following ATP hydrolysis, which is required to induce a conformational change in the bound GR, resulting in the opening of a steroid-binding cleft, thus allowing GC access and binding. [252, 253]. It has been proposed that the impairment of hsp90 expression in pro-inflammatory lymphocytes may be associated with steroid resistance in COPD [254]. Immunophilins are regulatory proteins thought to be involved in GR signalling. Immunophilin FK506 binding protein of molecular weight 52 (FKBP52) is part of the heterocomplex GR/hsp90/hsp70/FKBP52, which recruits the tubulin-associated transport protein dynein to move through the cytoplasm toward the nucleus, where the dissociation of the complex release the GR to exert its molecular effect. FKB51 acts as a competitive inhibitor to FKB52. Upon GC binding to GR, the switching of FKB51 to FKB52 activate the complex and drives the intracellular trafficking of GR [255, 256]. The nuclear translocation of GR, which is essential for GC function, occurs via importin receptors, included importin-α [257]. It has been reported that oxidant hydrogen peroxide (H_2O_2) and oxidative stress in general, which are elevated in COPD and severe asthma, in particular in airway epithelial cells and macrophages, impair importin-7 and may in part explain the reduced GC responsiveness [258]. Common

importin-13 genetic variation has been associated with improved airways hyperresponsiveness in children [259]. Conversely, nuclear export of GR involves the Ca^{2+}-binding protein calreticulin (CRT), which directly interacts with the DBD to deliver GR from the nucleus to the cytoplasm [260].

Overall, human GR is a modular protein coexisting in multiple functionally distinct receptor isoforms, which relative availability in different cell or tissue-types, together with their interaction with variable GREs within DNA, determine the diversity in GR signalling. GR can also interact with GRE sequences that exert a negative regulation, as will be explained below. Thus, DNA is also involved in modulating GR function. In addition, GRE can allosterically modulate the structure and transcriptional activity of GR [225, 247].

In addition, post-translational modifications (PTMs) add a further level in the regulation of GR activity, mostly upon ligand binding (Fig 1.5-(D)). For example, GR phosphorylation on serine 226 has been associated with GC resistance in some patients with asthma and a reduced phosphorylation of the receptor at this site by administration of p38 MAPK inhibitors reverted GC responsiveness [261]. GR is acetylated in its DBD and GR deacetylation by HDAC2 is necessary for GR to inhibit NF-kB activation of inflammatory genes. In addition, GR is a target for ubiquitination, which is a marker for proteasome degradation, while a mechanism known as SUMOylation can regulate the transcriptional activity of GR. About methylation, little is known on how this modification can alter GR transcriptional activity. PTMs target not only GR but also key molecules involved in the regulation of GR activity, included Hsp90 and FKBP52, rather than GRIP1, which role will be discussed below. Phosphorylation of hsp90 affects its chaperone activity and phosphorylated FKBP52 does not interact with hsp90. Further, deacetylation of hsp90 is crucial for GR complex functionality. SUMOylation and ubiquitination can also modulate hsp90 function [262].

Moreover, GRγ, GR-A and GR-P are other functionally distinct less well-characterised GR isoforms deriving from alternative processing of the GR gene that have been associated with cancer-related GC resistance. GR-A and GR-P have a truncated LBD [263].

1.5.2.1 Genomic signalling

GCs activate the nuclear translocation of GR, regulating the transcription of several genes involved in different biological effects through DNA-binding dependent or independent mechanisms [264]. Relatively high concentrations of GCs are needed to activate the transactivation pathway, which is related to both therapeutic and adverse effects, while low concentrations can activate the transrepression pathway, which mainly accounts for the clinical efficacy in asthma [234, 235].

1.5.2.1.1 Transactivation pathway

Glucocorticoids diffuse across the cell membrane and bind to GRs in the cytoplasm. In the absence of binding to the ligand GR is part of a heterocomplex, which includes chaperones hsp90, hsp70 and immunophilins, as explained above. The ligand binding induce a conformational change in GR, activating multiple functional domains, including the nuclear-localisation sequences (NLS) NL1 and NL2, which are located adjacent to the DBD and in the LBD, respectively . The effect is the nuclear translocation of GR. Once in the nucleus GR dimerises and associates with simple GREs usually in the promoter of GC target genes to activate gene transcription. The mechanism of GC activation of anti-inflammatory gene expression is called 'transactivation' (Fig. 1.6). The DNA-bound GR interacts with coactivators such as p300 and the homologous cAMP-responsive element-binding protein (CREB)-binding protein (CBP), p300/CBP-activating factor (pCAF) and steroid receptor coactivator-2 (SRC-2; also known as GRIP1, NCOA2, or TIF2) which belong to the p160 family. These coactivators have an intrinsic histone acetyltransferase (HAT) activity that cause acetylation of core histones, particularly histone-4, on lysine residues. This is a crucial event in gene activation, as histone acetylation induce the recruitment of chromatin remodelling factors such as the switching/sucrose non-fermenting (SWI/SNF) complex and subsequent DNA association of RNA polymerase II. Several anti-inflammatory proteins are generated by GR-induced activation of anti-inflammatory genes [235, 247, 265]. Such proteins include secretory leukoprotease inhibitor (SLPI), inhibitor of NF-kB (IkB-α) and annexin-1/lipocortin-1, which is a phospholipase A_2 inhibitor [266]. Further, the glucocorticoid-induced leucine zipper protein (GILZ), which influences gene expression through interaction with transcription factors such as NF-Kb and AP-1 [267]. In addition, the mitogen-activated kinase phosphatase-1 (MKP-1) which inhibits the p38 MAP kinase pathway, also reducing the downstream activation of NF-kB and AP-1 [268]. A post-transcriptional effect of GC is also mediated by increased expression of tristetrapolin (TTP), a protein that destabilizes mRNAs of inflammatory proteins [269].

Interestingly, GCs activate the expression of cell surface β2-adrenergic receptors (β2ARs), validating the beneficial interaction of ICS/LABA in reducing inflammation and reversing bronchoconstriction in chronic inflammatory diseases, especially in asthma. Budesonide/formoterol combinatorial therapy through inhaler devices suppresses mucosal eosinophilic inflammation and alleviates airway hyperresponsiveness within few hours, likely rescuing from exacerbations. Furthermore, formoterol has additional inhibitory effects on neutrophils and mast cells. Thus, ICS/LABA combination effectively reduce airway inflammation in asthma and GCs also protect against the down-regulation of β2-

receptors after long-term administration [270]. In addition, as β2ARs are G protein-coupled receptor (GPCRs) and GCs modulate gene expression of GPCR inhibitors, β-arrestin-1 and β-arrestin-2, in a positive and negative manner, respectively, GCs can balance G-protein dependent response of β2-agonists in asthma treatment [271]. GCs can also reverse the uncoupling of β2-receptors that may occur in response to inflammatory mediators such as IL-1β through stimulation of a GPCR kinase [272]. On the other hand, Formoterol increases GC-activated GR translocation from cytoplasm to the nucleus [273]. Hence, ICS/LABA combination inhalers have a greater efficacy compared to increased doses of ICS.

However, several side effects are thought to be mediated via transactivation. For instance, the two most important enzymes of gluconeogenesis, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, are both induced by GCs, promoting the development of diabetes [274]. GCs also activate the transcription of glutamine synthetase (GS), a marker for muscle wasting [275].

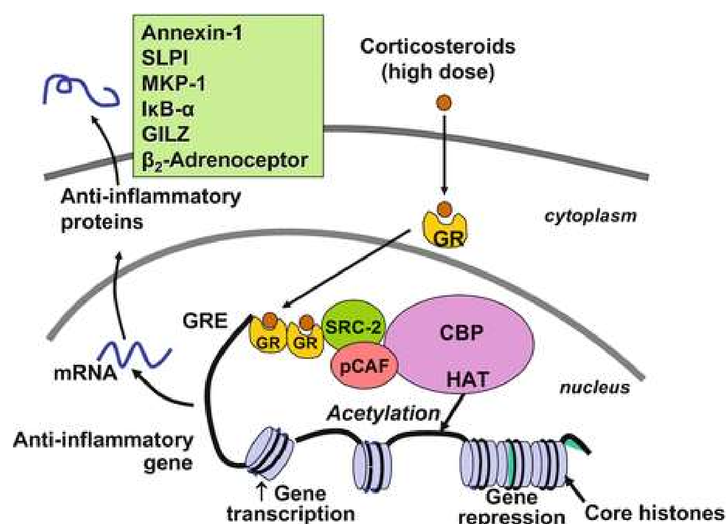


Figure 1.6 Glucocorticoid transactivation of anti-inflammatory gene expression

Glucocorticoids diffuse across the cell membrane and bind to glucocorticoid receptors (GRs) in the cytoplasm. The ligand binding induce a conformational change in GR, which translocates into the nucleus, where it forms a homodimers that bind to glucocorticoid response elements (GRE) in the promoter of GC target genes, thereby activating gene transcription. The DNA-bound GR interacts with coactivators such as steroid receptor coactivator-2 (SRC-2; also known as GRIP1, NCOA2, or TIF2), p300/CBP-activating factor (pCAF) and CREB-binding protein (CBP). These coactivators have an intrinsic histone acetyltransferase (HAT) activity that cause acetylation of core histones, particularly histone-4, thus recruiting chromatin remodelling factors such as the switching/sucrose non-fermenting (SWI/SNF) complex and subsequent DNA association of RNA polymerase II. This mechanism results in gene 'transactivation' and subsequent translation in several anti-inflammatory proteins and β2-adrenergic receptors (β2ARs).

1.5.2.1.2 Transrepression pathway

The therapeutic advantage of GCs in the management of chronic pulmonary diseases is their strong anti-inflammatory effect. Asthma and COPD are characterised by persistent activation of pro-inflammatory transcription factors, such as NF-kB and AP-1. Inflammatory stimuli, such as interleukin-1β and TNF-α, activate the inhibitor of IkB kinase 2 (IKK2) which phosphorylates and inactivates the inhibitor of NF-kB (IkB), thus activating the transcription factor. NF-kB proteins p50 and p65 form a dimer that translocates into the nucleus, where it binds specific kB recognition sites, also interacting with CBP and pCAF. As described above for the transactivation pathway, these coactivators have an intrinsic HAT activity and modulate the expression of multiple pro-inflammatory genes and consequent production of cytokines and chemokines, such as IL-6, IL-8 and TNF-α, adhesion molecules such as ICAM-1, receptors and enzymes involved in airways inflammation, included inducible nitric oxide synthase (iNOS) and inducible phospholipase A_2 ($cPLA_2$) [235].

The activated GC-bound GR translocates from the cytoplasm to the nucleus. Chromatin immunoprecipitation (ChIP) experiments, which reflect the true in vivo situation, demonstrate that GR does not decrease the recruitment of NF-kB to the promoter of IL-8 and ICAM-1, two different target genes repressed by GCs. Similarly, GR does not affect the recruitment of c-Fos and c-Jun, subunits of AP-1, to the promoter of target genes such as collagenase-1 and collagenase-3, supporting the idea that interference with DNA binding is not the mechanism responsible for GR transcriptional repression. Furtherly, this suggests a protein-protein interaction between GR and the repressed transcription factor at target genes, known as 'tethering' [276].

Further investigation revealed that GR directly interacts with coactivators of NF-kB inflammatory gene complex, inhibiting their HAT activity (Fig 1.7). In addition, GR recruitment of histone deacetylase-2 (HDAC2) induces histone deacetylation, thereby suppressing the activation of inflammatory genes. In fact, chromatin structure comprises DNA wound around nucleosomes, which are composed of two copies of histone H2A, H2B, H3 and H4 each for eight molecules in total. Histone acetylation by HAT enzyme leads to DNA unwinding, thus allowing gene transcription. Conversely, HDAC enzyme removes the acetyl groups, returning histones to their basal state. Asthma, consistent with its inflammatory nature, associates with an increase in HAT activity in the airways and a partial reduction in HDAC activity, which are restored by glucocorticoid therapy. In COPD, a reduced activity of HDAC is also associated with its reduced expression in the lung parenchyma, induced by oxidative and nitrative stress, caused by cigarette smoking. The reduction of HDAC may account for the amplified inflammation and glucocorticoid

resistance, conditions similarly observed in severe asthmatics and smoking asthmatic patients [277].

GR suppression of the activated inflammatory response through a 'tethering' mechanism with promoter-bound transcription factors and protein-protein interactions with coregulators represents the main mechanism of GCs to exert their therapeutic effect. This mechanism is referred to as 'transrepression' [234, 278].

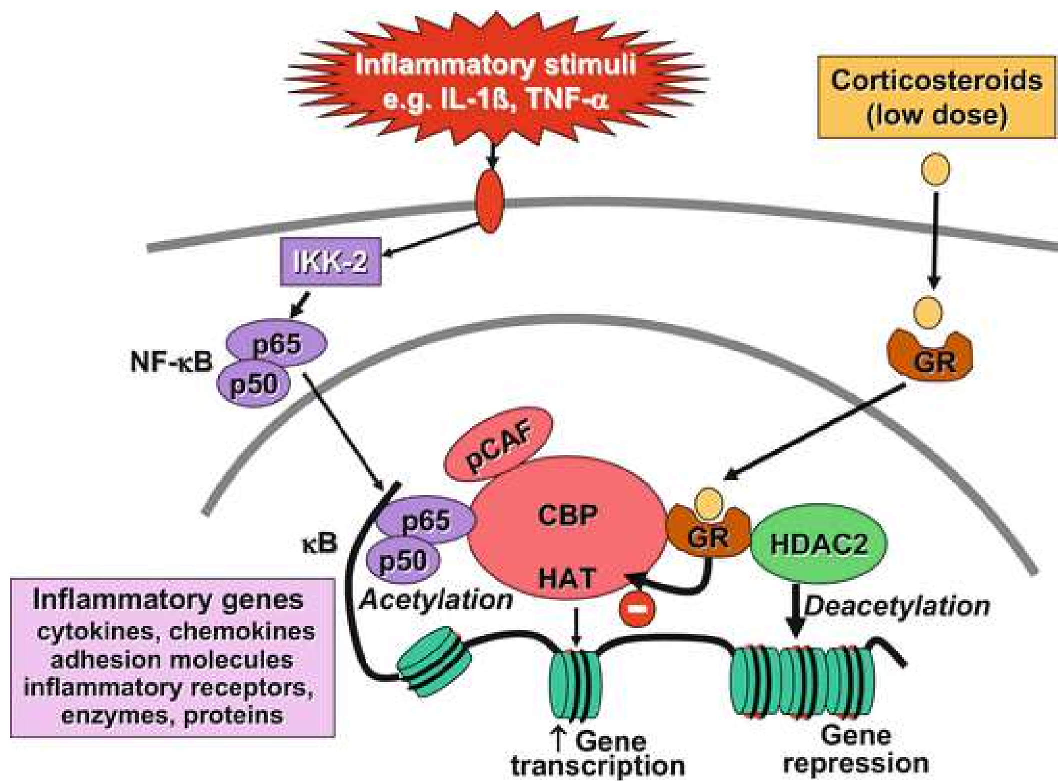


Figure 1.7 Glucocorticoid transrepression of activated inflammatory genes

The anti-inflammatory therapeutic effect of glucocorticoids is largely due to the 'transrepression' pathway. Cell stimulation by pro-inflammatory cytokines such as interleukin-1β (IL-1β) or tumour necrosis factor alpha (TNF-α) induce the activation of IKK2, which phosphorylates the inhibitor of NF-kB (IkB), promoting the dimerisation of NF-kB subunits (p50/p65) and the consequent translocation into the nucleus. Here the dimer binds to specific kB recognition elements within DNA and also interacts with coactivators such as CREB-binding protein (CBP) and p300/CBP-activating factor (pCAF), which have an intrinsic histone acetyltransferase (HAT) activity, required for the activation of multiple genes and consequent production of several pro-inflammatory proteins, including cytokines, chemokines and adhesion molecules. GCs cross the cell membrane and activate cytoplasmic glucocorticoid receptors (GRs) through specific binding, thereby inducing a GR conformational change and consequent nuclear translocation. Once in the nucleus GR tethers with coactivators, directly inhibiting their HAT activity. In addition, GR recruits histone deacetylase-2 (HDAC2), which induces histone deacetylation, resulting in repression of NF-kB activated inflammatory genes.

1.5.2.1.3 Other mechanisms of gene expression regulation

The categorisation of GC action in transactivation and transrepression pathways, even if consolidated, appears too simplistic. In fact, GR-modulated gene expression articulates in various mechanisms, justifying the complexity of GR-mediated GC effects.

1.5.2.1.3.1 GR association with negative GRE (nGRE)

Less commonly, GR interacts with negative GREs (nGREs) or GRE that cross the transcriptional start site, thus suppressing gene transcription [235] through recruitment of corepressors such as NCoR and SMRT, which also recruit histone deacetylases [225]. Monomeric GR most likely binds these elements. In fact, the binding of a second GR monomer was found in the opposite site of the DNA, thereby the two monomers are not in direct contact and their dimerisation is unlikely; in addition, binding of one monomer dramatically reduces the propensity of a second monomer to bind [279]. This mechanism of cis-repression involving GR binding at nGREs is linked to certain side effects of GCs. Osteoporosis, for instance, is linked to the GC-induced inhibition of osteocalcin, which is involved in the synthesis of the bones [280]. Moreover, the suppression of HPA-axis is associated with the GC repression of CRH receptor corticotropin releasing factor 1 (CRF-1) [281, 282].

1.5.2.1.3.2 GR recruitment of the corepressor GRIP1

A further model of suppression is GR recruitment of the coregulator GRIP1. It has been observed a competition for GRIP1 by GR and the transcription factor IRF3. GRIP1 interacts with IRF3 to activate the transcription of target genes such as IFN-β, IP-10, RANTES and ISG15, thereby acting as a coactivator. GRIP1 sequestration by GC-activated GR reduces IRF3-induced gene expression [283]. In other words, GR blocks the recruitment of the crucial coactivator GRIP1 by the promoter-bound IRF3. In these terms GRIP1 functions as a corepressor for GR. GRIP1 (SRC-2) was also reported to interact with GR to repress AP-1 and NF-kB dependent transcription, suggesting the presence of a specific repressor domain which is absent in other SRC members of the p160 coactivators family [276]. A genome-wide profiling through ChIP and high throughput sequencing (ChIP-seq) showed GRIP1 binding to GR target genes in macrophages, following treatment with dexamethasone and TLR4 stimulation by LPS, and was interestingly reported that GRIP1 was equally recruited to activated and repressed genes. GR transcriptional activation involves different coactivators and GRIP1 seems not to be an essential factor, whereas GR-dependent repression of inflammatory genes selectively involves GRIP1, blocking other transcription factors activity, rather than their DNA binding. In fact, it was observed that IRF3 enhancer binding is not reduced in presence of GR

ligand. Further, GRIP1 increases the recruitment of HDACs at repressed loci in response to dexamethasone, leading to chromatin deactivation [278].

1.5.2.1.3.3 GR binding to GREs and interaction with other transcription factors

GR can also repress, or even enhance, transcription in a 'composite' manner by direct binding to GREs on the promoter of target genes, which also contain binding sites for other transcription factors that interact with GR [225, 247].

1.5.2.1.3.4 Alternative mechanisms of GR

GCs therapy may also be beneficial in allergic asthma thanks to the ability to suppress the expression of Th2 cytokines such as IL-4, IL-5 and IL-13 via GR competitive inhibition of importin-α-mediated nuclear translocation of the transcription factor GATA3, which regulates Th2 cytokine transcription. In addition, GATA3 activation by p38 MAPK-phosphorylation is inhibited by GC induction of the kinase phosphatase MKP-1 [284].

Moreover, GCs enhance the activity and expression of indoleamine-2,3-dioxygenase (IDO), an enzyme which increases the secretion of the anti-inflammatory cytokine IL-10 from macrophages and is negatively correlated with sputum eosinophils in asthmatics [285].

1.5.2.2 Non-genomic signalling

Emerging evidences suggest that GCs can also exert their actions through non-genomic mechanisms that does not require GR nuclear translocation and transcriptional modulation of gene expression [286]. Noteworthy, whereas GCs usually take a few hours to exert their genomic actions, the non-genomic effects of GCs are much more rapid and take only minutes [287]. For instance, GCs negatively regulate the MAP kinase c-Jun N-terminal kinase (JNK). This inhibitory effect takes less than one minute, suggesting a GR direct interaction, independent from de novo protein synthesis. In addition, a GR mutant, which failed to dimerise and efficiently activated gene transcription, was reported to inhibit JNK activity as well as wild type GR. The contextual inhibition of c-Jun phosphorylation by GCs suggests that this non-genomic effect represents an additional mechanism for the repression of the transcription factor AP-1 [276].

These non-genomic effects are thought to be mediated through several mechanisms, including: membrane-bound GRs; classic cytosolic or membrane-bound GRs that target signalling proteins; classic GR that translocate into the mitochondria; direct physicochemical interactions of GCs with the cell membrane [288, 289].

To date, a classic GR and a non-classic GR have been identified to be involved in non-genomic effects. They can interact with MAP kinases or activate cAMP and Ca^{2+}-dependent pathways, respectively. The classic membrane GR has similar characteristics compared to the cytosolic GRα, though it presents a different localisation, molecular weight and binding specificity to GCs. Differently, the non-classic membrane GR is a G protein-coupled acidic glycoprotein with different pharmacological characteristics, which shows high affinity for corticosterone but not for other hormones that classically bind GR [233]. To validate the functional activity of the classic membrane GR, Strehl and colleagues demonstrated that bovine serum albumin (BSA)-conjugated dexamethasone (DEX-BSA), which is unable to penetrate the cell membrane, while retaining its GR affinity, can induce the phosphorylation of p38 MAPK in human monocytes [290].

In asthmatic patient with stable asthma or acute exacerbation, the airway mucosal blood flow is significantly increased in comparison to healthy subjects. The inhalation of fluticasone significantly decreases blood flow in both groups, with a greater extent in asthmatics. The maximal effect is observed in 30 minutes and is thought to be due to the rapid inhibition, within 5 minutes, of the extraneuronal monoamine transporter (EMT)-mediated uptake of norepinephrine by bronchial arterial smooth muscle cells, a mechanism involved in neurotransmitter metabolism, thereby facilitating the noradrenergic neuromuscular signal transmission in vascular smooth muscle cells, consequently leading to local vasoconstriction. This is an important non-genomic effect in the treatment of asthma [291]. Anion transport in the lung is mediated trough the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel and is important in the formation of low-viscosity mucus, thereby maintaining a conductive and aseptic environment. In fact, the production of thick mucus plugs is a consequence of CFTR dysfunction, which is a typical condition in cystic fibrosis, provoking serious respiratory damage. Inhaled corticosteroids such as fluticasone and budesonide, which are applied directly to the apical side of airway epithelial cells, stimulate the cAMP-dependent CFTR-mediated anion transport in a non-genomic fashion, thus showing a therapeutic effect for mucus congestive airway diseases, such as asthma and COPD [292].

Overall, on one hand the transcription-dependent genomic effects of GCs are mediated by GR direct interaction with GRE or nGRE within DNA, or indirectly by GR interaction with transcription factors. On the other hand, the less well-known transcription-independent non-genomic effects of GCs may be specific when GR interacts with other proteins such as members of the MAPK family, while other non-specific effects may occurs in a GR-independent way by physicochemical interactions of GCs with the plasma membrane.

Thus, these non-genomic mechanisms certainly contribute to the physiological and pharmacological effects of GCs [293].

1.6 Selective GR modulation

Point mutation studies of GR and choice of different GCs brought to the observation that transactivation and transrepression can be dissociated. Some mutations resulted in the failure of GR to dimerise and bind GRE-dependent promoter to activate gene transcription, whilst the ability to suppress gene expression through interaction with transcription factors such as AP-1 or NF-kB remained unaffected compared with wild-type GR [294-296].

Major efforts have been undertaken to develop new classes of glucocorticoids that have anti-inflammatory efficacy and reduced side effects, thereby favouring the transrepression pathway, while having a reduced capacity to induce transactivation. Thus, theoretically they should have less side effects (Fig. 1.8) [297]. These glucocorticoids are referred as dissociated compounds, selective glucocorticoid receptor agonists (SEGRAs) or selective glucocorticoid receptor modulators (SEGRMs). Steroidal scaffold compounds, which often exhibited a partial agonistic activity on GR transactivation pathway, were historically defined using the term SEGRA. With the advent of newer non-steroidal compounds, the term SEGRMs has started to be used and may identify molecules that not classically bind the GR ligand-binding pocket, so interacting with different contact point in the LBD of GR, or that induce a GR conformational change in a poorly investigated different way [289]. In a mouse model of asthma it has been demonstrated the effect of the selective compound A (CpdA), one of the more extensively studied SEGRMs, in reducing airway inflammation and hyperresponsiveness, thereby confirming *in vivo* its potential therapeutic efficacy in the absence of transactivation observed *in vitro*. However, CpdA is very liable and has a narrow therapeutic range, thereby although it is inappropriate for therapy, it is yet considered excellent for research purposes [298].

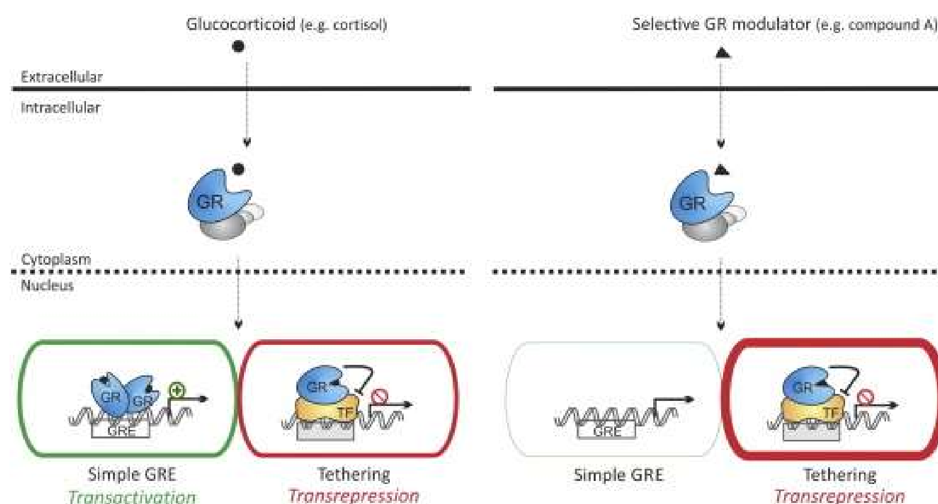


Figure 1.8 Differential modulation of GR by selective compounds

Glucocorticoids enter the cell and bind to the glucocorticoid receptor (GR). Upon ligand binding, activated GR modulate gene transcription via various mechanisms. Although it is not an absolute distinction, it is generally accepted that anti-inflammatory effects of GCs are largely due to GR 'tethering' with promoter-bound transcription factors and coregulators through the so defined 'transrepression' pathway, while several side effects are associated with the so called 'transactivation' pathway, which acts through direct GR binding to GRE within DNA. Considering this dual mechanism of action, a great amount of selective glucocorticoid receptor agonists (SEGRAs) or modulators (SEGRMs) have been developed. The principle of their selectivity underlie in their ability to differentially induce a conformational change of GR, thus favouring 'transrepression' rather than 'transactivation'.

A proposed model to explain the differential ability of SEGRM bound GR to activate only a subset of target genes, compared to standard GC bound GR, suggests an impairment in the recruitment of coactivators linked to side effects, whilst not affecting the recruitment of the coregulator GRIP1. In fact, the latter is considered one of the current model of gene suppression by GCs [299]. In addition, the mechanism of transrepression involving NF-kB or AP-1 is typically unaffected by SEGRMs, suggesting the potential of selective compounds to repress transcription independently from their ability to interact with at least some coactivators [300].

The general reason of the differential effects of selective compounds in modulating gene expression underlies in the differential conformational change that GR undergoes on ligand binding, which explain why receptor agonists induce the recruitment of coactivators and receptor antagonists facilitate corepressor recruitment. Other variants, such the relative concentrations of these factors in cells, the nature of GR interaction with DNA or with multiple chromatin remodelling enzymes, rather than the possible interaction of a

ligand with other GR isoforms, add complexity to the system, thereby determining the variability of GR-dependent responses. In addition, in discriminating between transrepression and transactivation in designing new GR ligands to optimize the anti-inflammatory therapy, experimental evidences that some GC-induced anti-inflammatory genes have a role in the therapeutic efficacy should be considered. Thus, it has been proposed the need to identify differential compounds that display the most favourable functional profiles [294].

Overall, research shed new light on the ability of selective glucocorticoid receptor agonists and modulators (SEGRAMs) in differentially modulating GR conformation and activation, favouring the protein-protein interaction dependent transrepression pathway rather than the DNA-binding dependent transactivation pathway. In fact, it is assumed and therefore it is a common opinion that anti-inflammatory effects are largely due to transrepression, while several side effects are associated with transactivation. Despite this general assumption, additionally experimental evidences, as described above, also correlate anti-inflammatory effects of glucocorticoids with gene activation and other side effects with direct gene suppression or even both activation and suppression. Nevertheless, the potentiality of improving the anti-inflammatory-based GC therapy led to the development of several new selective compounds with the purpose of reducing unwanted side effects associated with the long-term use of GCs [289, 294, 297, 301]. The research is still going on this direction and some selective compounds, such as GW870086X [302, 303] and AZD7594 [304], which have a steroidal and non-steroidal structure, respectively, are currently in clinical trial for the treatment of asthma inflammation.

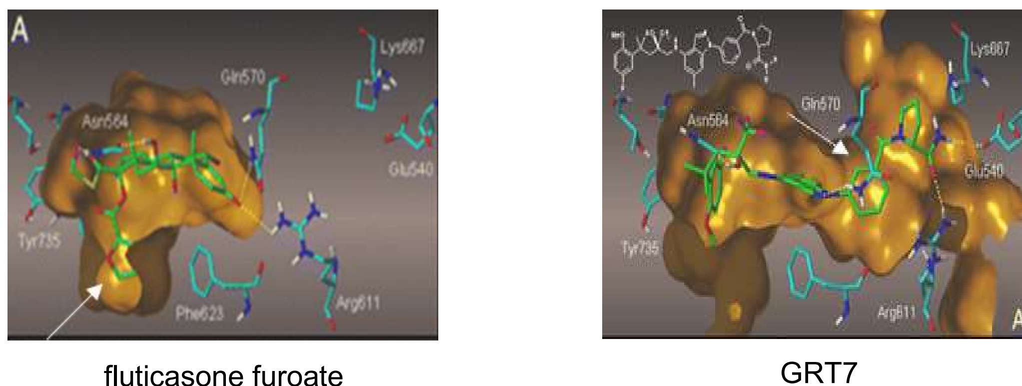
In recent years, research on selective compounds boomed and great amount of new molecules have been reported. Thus, in a future perspective, even if further investigation in clinical studies is needed to evaluate their clinical advantage and safety, in comparison with standard steroids, these compounds are very promising in order to improve the therapeutic index and the lung function. In addition, developing and studying new selective compounds is greatly helpful in elucidating various molecular actions of GRs.

1.6.1 Selective compounds

Studying the interactions between steroids and their receptor GR revealed an intriguing structural malleability of GR within the LBD. Crystal structures of GR LBD complexed with a ligand showed that the endogenous ligand cortisol specifically binds GR but fails to fill the binding pocket. Similarly, despite strong binding specificity, the exogenous dexamethasone occupies only around 65% of the GR ligand-binding pocket. The additional volume within the pocket can potentially be occupied with alternative

modulatory ligands, included the antagonist RU-486. These observations boosted the development of a wide range of ligands with the feature to fill only a portion of the binding pocket or to alter the shape of the pocket, thus conferring different allosteric changes, resulting in ligand specific alterations in GR signalling and gene transcription regulation. Therefore, both steroidal and non-steroidal ligands can bind GR. In the last years, a great amount of selective glucocorticoid receptor agonists (SEGRAs) or modulators (SEGRMs) have been developed with the purpose to preserve the anti-inflammatory action of standard glucocorticoids and at the same time avoiding adverse effects associated with chronic use of GCs [279, 305].

Fluticasone propionate (FP) is a successful standard synthetic glucocorticoid agonist developed maintaining the steroidal scaffold and manipulating the 17α substituent. FP has a simple propionate ester at this position. Successively, the replacement of propionate ester with 2-furoate ester brought to the compound fluticasone furoate (FF), which shows an enhanced affinity for GR and improved nasal and lung tissue affinity. This compound has been developed as a once-daily inhaled medication, administered alone (asthma) or in combination with vilanterol, a long acting β2 agonist (asthma and COPD) [91, 306]. Importantly, despite these two compounds have both the same active principle fluticasone they are completely different drugs with different properties. In fact, the ester moieties are stable and are not detached from their fluticasone backbone during metabolism [307, 308]. X-ray crystallography showed that 2-furoate ester of FF occupies the GR binding pocket much more completely than the smaller propionate ester of FP, accommodating in a lipophilic portion called 17α pocket, unoccupied by dexamethasone, which has a 17α hydroxyl group (Fig. 1.9 left side) [309].



fluticasone furoate GRT7

Figure 1.9 Differential occupation of the GR binding pocket

[left side] The glucocorticoid fluticasone furoate (FF) binds the GR binding pocket. However, unlike classical standard steroids, its furoate group is completely accommodated in the 17α pocket, conferring enhanced affinity for GR. **[right side]** GRT7 is a non-steroidal agonist which partially occupies the 17α pocket of GR, while interacting with an extended *'meta'* channel within the pocket in the opposite direction. Together, these figures clearly reveal a great malleability of the nuclear receptor GR, also showing that non-steroidal ligand-bound GR conformation may differ from that of GC-bound GR and that this versatility may be exploited to differentially modulate the GR signalling pathways, thereby conferring selectivity of action. The narrows indicate the 17α pocket (left) and the *'meta'* channel (right) of GR.

So, as FF shows enhanced GC activity compared to FP and as the analysis of ligand bound GR crystal structure revealed that FF occupies an unexplored portion of GR, also displaying a 60% higher affinity for GR, alternative 17α ester moieties have been studied to fully explore the effects of this substituent on GR pharmacology. These investigations led to identify a tetramethylcyclopropyl ester moiety, which refers to compound GRT10, a novel molecule showing transrepression versus transactivation selectivity [310]. Following encouraging results achieved with GRT10, keeping the 17α tetramethylcyclopropyl ester moiety, further modification of the 17β substituent was explored. Switching from the fluoromethyl thioester to the cyanomethyl carboxylate, a molecule referred to compound GW870086 has been identified and is now in clinical development for the treatment of asthma (Fig. 1.10). Interestingly, while showing a GR affinity comparable to FP, *in vitro* experiments using reporter gene assays displayed a reduced activity of GW870086 at related steroid hormone receptors, such as progesterone receptor and mineralcorticoid receptor. Furthermore, comparing GW870086 to dexamethasone, GW870086 was observed to be accommodated in the 17α pocket of GR and to reflect the classical behaviour of a partial agonist. Notably, GW870086 is a selective compound that retains the ability to repress key pro-inflammatory genes, while activating only a subset of those

genes normally activated by standard GCs and usually associated with adverse effects. In addition, GW870086 substantially retains the ability to up-regulate MKP1, which as explained above participates to the anti-inflammatory response [306].

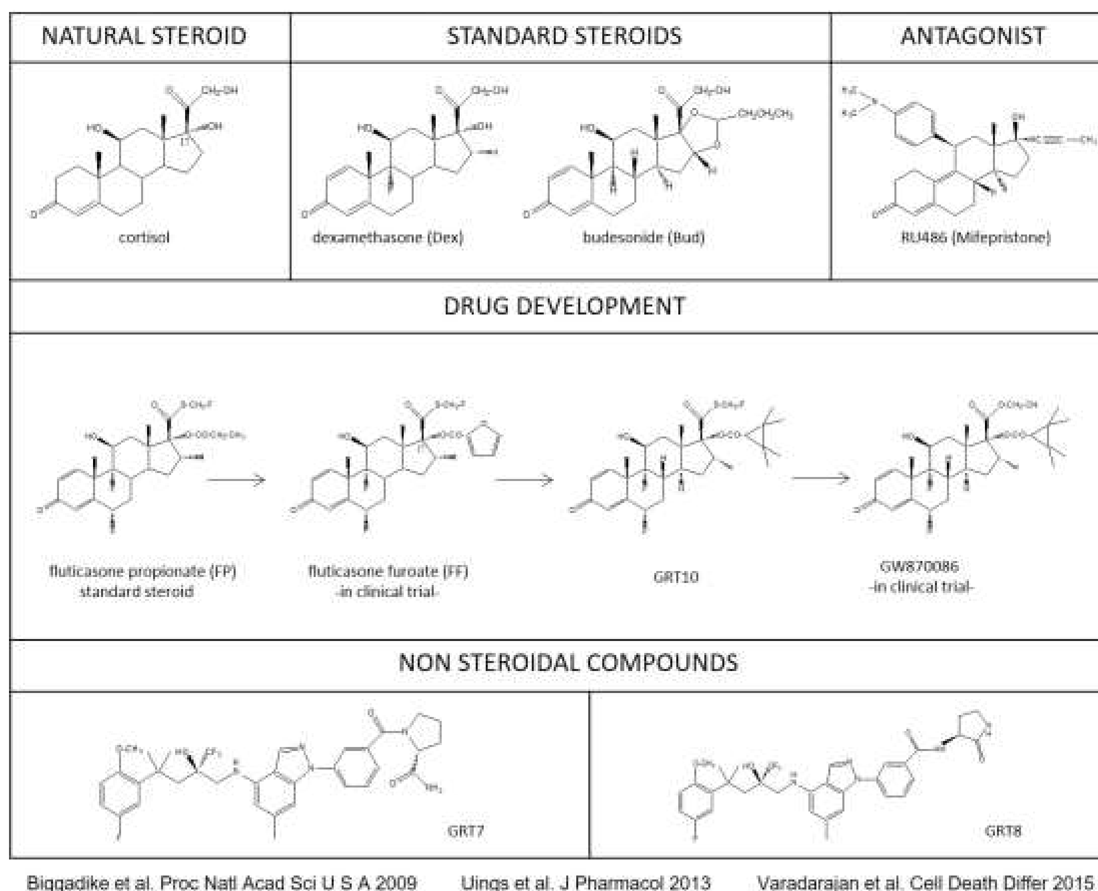


Figure 1.10 Chemical structures of steroidal and non-steroidal compounds

Starting from the natural cortisol, several steroidal compounds have been developed. Dexamethasone (Dex), budesonide (Bud) and fluticasone propionate (FP) are standard steroids, while RU486 (Mifepristone) is a glucocorticoid antagonist. Fluticasone furoate (FF) has been developing in clinical trials as a once-daily intranasal drug in combination with vilanterol, a long acting β2 agonist (LABA). FF shows a greater affinity for the glucocorticoid receptor (GR). FF modifications in 17α led to the novel compound GRT10, which has a tetramethylcyclopropyl ester moiety and shows transrepression over transactivation selectivity. Further modifications of the 17β substituent were explored. Switching from the fluoromethyl thioester to the cyanomethyl carboxylate gave the compound GW870086, a molecule with reduced activity versus other non-GR related steroid hormone receptors. GW870086 is also in clinical trial as an inhaled formulation. Non-steroidal ligands can also bind GR. Molecules with an aryl-indazole scaffold have been identified for their ability to differently bind GR, relative to steroidal-compounds, offering the chance to develop new selective compounds, which can show selectivity for GR versus other hormonal receptors, such as GRT7, or greatly reducing the transactivation activity, such as GRT8.

In recent years has also been increasing interest in the identification of novel selective non-steroidal ligands for GR. In parallel has been achieved a better understanding of the mechanisms of GC activity. These efforts led to the discovery of highly potent ligands with an aryl-indazole scaffold occupying a previously unexplored *'meta'* channel of the GR (Fig. 1.9 right side). The binding pocket opens up to accommodate non-steroidal ligands in a new extended region. Unlike FF, which completely occupies the 17α pocket, indazole derivative compounds force the LBD of GR to open in the opposite direction. This newly identified channel offered the chance to develop molecules with increased potency and/or selectivity. The compound GRT7, which has a D-prolinamide linked to the aryl-indazole scaffold, is a potent agonist showing full efficacy for both GR transactivation and transrepression compared to FF and dexamethasone. GRT7 also shows a significant reduced progesterone receptor (PR) activity, resulting in a GR/PR selectivity and no significant activity over other steroid receptors, such as mineralcorticoid receptor, androgen receptor and estrogen receptor. GRT7 also shows very low oral bioavailability and sufficient aqueous solubility, so it would be an ideal compound for development as an intranasal or inhaled formulation. The Manipulation of the chemical structure to optimise interactions within the GR channel gave the additional compound GRT8, which has a (3S)-2-pyrrolidinone amide linked to the aryl-indazole scaffold. GRT8 is less potent than GRT7, FF and dexamethasone, although it retains much of the desired transrepression activity, while greatly reducing the transactivation activity, showing evidence for a partial agonist response. Thus, GRT8 shows a very different pharmacological profile compared to standard glucocorticoids. Reasonably, this compound has yet to be fully explored and it may have the potentiality for further development, with the hope to reach the unmet goal to find novel selective anti-inflammatory drugs with reduced risks associated with prolonged use of GCs during the treatment of chronic inflammatory disease, included asthma and COPD [311].

In summary, x-ray crystallography displays that natural and synthetic glucocorticoids, besides optimised selective non-steroidal ligands, have high binding affinity for GR. The natural compound cortisol binds the GR similarly to the synthetic standard steroids dexamethasone and FP, without fill the binding pocket. Differently, the furoate group of the glucocorticoid FF and the tetramethylcyclopropyl group of GRT10 completely occupy the 17α pocket of GR, which appears enlarged. Furthermore, non-steroidal compounds such as GRT7 and GRT8 also interact with an extended *'meta'* channel within the pocket in the opposite direction. Therefore, these analyses clearly reveal a great malleability of the nuclear receptor GR, also showing that non-steroidal ligand-bound GR conformation may differ from a GC-bound GR, thereby exposing different cofactor binding surfaces,

leading to alternative binding to different coactivators, or corepressors, as it is the case of the steroidal antagonist RU-486-bound GR.

Overall, accumulating research, focussed on the identification of novel steroidal or non-steroidal selective GR agonists with better therapeutic profiles, which means have anti-inflammatory properties and reduced adverse effects, may bring to improve the GC therapy. Furthermore, these selective compounds represent important tools for investigating GR signalling pathways as their modulatory mechanisms are not fully elucidated. Therefore, further research is needed.

1.7 Experimental rationale

Exacerbations of obstructive lung diseases such as asthma and COPD are among the main causes of mortality and morbidity around the world. Exacerbation healthcare costs have a strong impact on the global economic burdens. Respiratory viral infections are the most common cause of obstructive lung disease acute worsening and rhinovirus (RV) is the most frequently detected virus in adults and children in asthma and COPD exacerbations. However, no anti-viral drugs or vaccines are currently available for the treatment or prevention of RV infections. ICS are the major anti-inflammatory medications in asthma and are widely prescribed in COPD to prevent exacerbations. ICS use is associated with several long-term adverse effects, including the inhibition of the innate immune response. However, not many studies on glucocorticoids and rhinovirus interactions have been made so far.

Airways epithelial cells represent the first site of RV infection and the innate immune response is the first line of host defence against the virus. Following virus uptake via endocytosis, different pathogen associated molecular patterns (PAMPs) are recognised by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and RIG-like receptors (RLRs). Single-stranded (ss) and double-stranded (ds) viral RNA respectively trigger the membrane-located TLR7/8 and TLR3 in the endosomal vesicle, promoting the translocation into the nucleus of the nuclear factor kB (NF-kB) and consequent gene activation of pro-inflammatory cytokines and chemokines such as IL-6, IL-8, RANTES and IP-10. TLR3 also activates the cytoplasmic receptors retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated protein 5 (MDA5), which increase type I (IFN-β), type II (IFN-γ) and type III (IFN-λ1/IL-29) interferon production. In an autocrine and paracrine fashion, once released in the extracellular compartment, type I IFNs (type I IFNs) bind to the interferon-alpha receptor (IFNAR) on the apical surface of nearby epithelial cells, activating the proteins tyrosine kinase 2 (TYK2) and Janus kinase 1

(JAK1), respectively associated with IFNAR subunits IFNAR1 and IFNAR2. IFN binding promotes the recruitment of the latent cytoplasmic signal transducer and activator of transcription (STAT) proteins to the receptor and their subsequent phosphorylations by the two kinases TYK2 and JAK1, thereby activating the IFN-induce JAK/STAT signalling pathway. Tyrosine phosphorylations on STAT1 (pSTAT1 Y701) and STAT2 (pSTAT2 Y690) induce STAT1-STAT2 dimerisation and association with the interferon regulatory factor 9 (IRF9) to form a complex called interferon stimulated growth factor 3 (ISGF3). The translocation of STAT1-STAT2-IRF9 into the nucleus induces interferon stimulated gene (ISG) expression. This complex recognise interferon-stimulated response elements (ISRE) within the promoter of target genes, activating the production of antiviral proteins such as the virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible (viperin) and 2',5' oligoadenylate synthetase (OAS).

Glucocorticoids (GCs) diffuse across the cell membrane to the cytosol and bind to the glucocorticoid receptor (GR), inducing its translocation into the nucleus, thereby modulating the expression of several genes through a general accepted genomic dual mechanism, transactivation and transrepression. The latter is largely involved in the therapeutic action of GCs. In addition, the GR exerts other non-genomic effects without involving gene expression. This thesis examines the effects of a set of GCs and selective compounds on the innate antiviral immunity and virus replication in bronchial epithelial cells following RV infection. Moreover, since GCs mediate their actions through several mechanisms, investigations of their modulatory activity in different molecular pathways such as JAK/STAT by measuring ISG expression and targeting STAT1 and STAT2 phosphorylations have been explored. Additionally, as ICS combinations with a long acting β2-agonist (ICS/LABA) are widely used both in asthma and in COPD, the assessment of the effect of this combination on RV-induced ISG expression and RV replication has been carried out.

1.7.1 Hypothesis

This thesis focuses on GC modulation of antiviral innate immunity. The main hypothesis is that GCs impair the host response to RV infections. Thus, the use of GCs may have implications in virus-induced asthma and COPD exacerbations.

1.7.2 Aims

The experimental model of asthma or COPD exacerbations consists in *in vitro* cultures of bronchial epithelial cells (BEAS2B) infected with RV. Biological techniques were applied to explore the effects of glucocorticoid (GC) treatments on the modulation of the innate

immune response. The interactions of GCs with the JAK/STAT signalling pathway were investigated through cell stimulation with recombinant IFN-β.

1.7.2.1 Aim n.1: Anti-inflammatory and immune-suppressive effects of GCs

To investigate the effect of glucocorticoid receptor (GR) ligands on the modulation of pro-inflammatory cytokines and IFNs production and on the anti-viral gene expression. To this aim, RV-1B infected BEAS2B cells were treated with the standard steroid fluticasone propionate (FP) and a set of selective glucocorticoid receptor agonists (SEGRAs) or modulators (SEGRMs): GRT7, GRT8 and GRT10. Samples were analysed by qRT-PCR and/or ELISA. In addition, the effect on RV replication by qRT-PCR and viral titre determination ($TCID_{50}$) has been assessed.

1.7.2.2 Aim n.2: GC modulation of type I IFN signalling pathway

To explore the interactions of GR ligands with the type I IFN signalling pathway in recombinant IFN-β stimulated BEAS2B cells. To this aim, interferon stimulated gene (ISG) expression and phosphorylations of the signal transducer and activator of transcription (STAT) proteins STAT1 and STAT2 were assessed in cells treated with FP, GRT7, GRT8 and GRT10 by qRT-PCR and western blotting (WB).

1.7.2.3 Aim n.3: Effect of ICS/LABA combination on innate antiviral response

To evaluate the effect of the glucocorticoid Budesonide (Bud), clinically administered as an inhaled corticosteroid (ICS), alone or in combination with Formoterol, a long acting β2-agonist (LABA), on ISG expression and viral replication in BEAS2B cells infected with RV-1B. In addition, to evaluate the effect of dexamethasone, clinically administered as systemic corticosteroid. Samples were analysed by qRT-PCR.

Chapter 2: Materials and methods

2.1 Compounds

The standard steroids or glucocorticoids (GCs) assessed in this thesis, fluticasone propionate (FP), budesonide (Bud) and dexamethasone (Dex), in addition to the β2 adrenergic agonist Formoterol (Form) are commercially available powder (Sigma-Aldrich).

Compounds GRT7, GRT8 and GRT10 were provided by GSK under a Materials Transfer Agreement (MTA). Under the terms of the agreement public disclosure of any data generated requires approval of GSK.

All compounds were solubilised in DMSO for cell treatments.

2.2 Cell culture

Human bronchial epithelial cells (BEAS2B) from European Collection of Authenticated Cell Cultures (ECACC cat. 95102433) were cultured in RPMI 1640 medium (Sigma-Aldrich) with L-glutamine supplemented with 10% fetal bovine serum (FBS), 2% HEPES buffer solution and 1% sodium bicarbonate.

Reagent	Information	Supplier
Collagen	0.1% solution	Sigma-Aldrich
DMSO	Filtered sterile	TOCRIS
fetal bovine serum (FBS)	Heat inactivated FBS	Thermo Fisher Scientific
HEPES buffered solution	1M HEPES	Life Technologies
Phosphate buffered saline (PBS)	pH 7.4, Ca^{2+} and Mg^{2+} free	Life Technologies
RPMI 1640	With L-glutamine and sodium bicarbonate. Refer to manufacturers' medium formulation	Sigma-Aldrich
Sodium bicarbonate	7.5% $NaHCO_3$ solution	Life Technologies
Trypan Blue	Used for cell counting at 0.01% in PBS	Thermo Fisher Scientific
Trypsin/EDTA	0.05%	Life Technologies

Table 2.1 Medium and reagents for cell cultures

2.3 Virus culture and propagation

A stock of human rhinovirus 1B (HRV-1B) genus *Enterovirus* was obtained from the American Type Culture Collection (ATCC VR-1645) and propagated in HeLa H1 cells (ATCC CRL-1958). In 175 cm^2 flasks, cells were grown to approximately 90% confluence in DMEM medium supplemented with 10% FBS, 2% HEPES buffer solution and 1% sodium bicarbonate. Cells were washed twice and infected with HRV-1B in 2% FBS DMEM medium. To allow virus attachment to cells, flasks were incubated at room temperature (RT) for 1h with gentle shaking. Cells were then incubated in humidified atmosphere at 37°C in 5% CO_2 for 18-24h. Once a cytopathic effect (CPE) was observed, cells were detached by gentle tapping of the flask and harvested, comprising the media, in two further 175 cm^2 flasks before storage at -80°C. Following three repeated freeze-thaw cycles to break membranes and allow virus collection, the lysed cells were centrifuged at 1,400 x g for 15 minutes at 4°C to remove cellular debris and supernatant filtered through a 0.2 µm syringe filter before storage as 3ml aliquots of HRV-1B containing medium at -80°C. The virus was titrated in HeLa Ohio cells (ECACC 930021013) seeded in DMEM medium supplemented to determine the tissue culture infective dose ($TCID_{50}$/ml). Virus stocks were stored at -80°C and thawed only once as needed.

2.4 Viral endpoint titre determination ($TCID_{50}$)

Virus infectivity was assessed in HeLa Ohio cells seeded in 96-well plates and grown in DMEM medium supplemented with 2% FBS, 2% HEPES buffer solution, 1% sodium bicarbonate and 1% Penicillin/Streptomycin (P/S). HRV-1B stocks were serially diluted to give concentrations from 10^{-1} to 10^{-8} in six replicates. Cells were incubated at 37°C in 5% CO_2 for 5 days. HRV-16 was used as a positive control. The viral endpoint titre determination was obtained by CPE observation by light microscopy and the $TCID_{50}$/ml calculated using the Spearman-Karber formula.

Reagent	Information	Supplier
Dulbecco’s modified Eagle’s medium (DMEM) – high glucose	With 4,500 mg/L glucose and L-glutamine. Refer to manufacturers’ medium formulation	Thermo Fisher Scientific
Penicillin/Streptomycin (P/S)	5,000 units/ml penicillin, 5,0000 µg/ml streptomycin	Life Technologies

Table 2.2 Medium for HeLa H1 and HeLa Ohio (P/S added)

2.5 Infection experiments

BEAS2B cells cultured in 10% FBS RPMI 1640 medium were seeded (1.5 x 10^5 cells/ml) in 12-well plates and incubated at 37°C in 5% CO_2. The day after, 10% FBS medium was replaced with 0% FBS medium overnight. Cells at a confluence of approximately 90% were infected with the virus (HRV-1B) at a MOI of 1 for 1h at RT with gentle shaking to allow cell attachment. To discard the unbound virus, cells were washed before adding fresh medium.

Drugs or compounds were added before and after infection, to assess their effect in RV infected cells. To inactivate the virus (UV-RV) as a control for certain experiments, HRV-1B was left under UV-light exposure for 40 minutes.

2.6 IFN stimulation experiments

BEAS2B cells were seeded at 1.5 x 10^5 cells/ml in 12-well plates. After incubation overnight to a confluence of approximately 90% at 37°C in 5% CO_2, the medium was replaced with serum free medium and the day after cells were stimulated with recombinant IFN-β at 30 U/ml. Drugs were added before and/or after stimulation, depending on the protocol.

Reagent	Information	Supplier
Recombinant IFN-β1α	In 10 mM acetic acid. Used at a final concentration of 30 U/ml.	R&D System

Table 2.3 Recombinant IFN-β for cell stimulation

2.7 Enzyme-linked immunosorbent assay (ELISA)

Cell supernatants, diluted as required, were collected to quantify cytokines release. Analyses were carried out in 96-well plates using reagents from Duoset ELISA kits (R&D Systems) according to manufacturers' instructions. Plates were washed with the wash buffer three times between steps. Plates were covered with the capture antibody at room temperature overnight. After blocking with reagent diluent (1% BSA in PBS) for at least 1h, a volume of 100 µL of sample or standard (prepared using 2-fold serial dilutions) was incubated for 2h. The biotinylated detection antibody was added for a further 2h. The plate was then covered with streptavidin-HRP diluted in reagent diluent for 20 minutes. A substrate solution obtained mixing H_2O_2 and tetramethylbenzidine (TMB) was then added

for not more than 20 minutes. Colour development was observed after addition of the stop solution (2N H_2SO_4), followed by optical density measurement immediately after, using a Spectramax Plus plate reader. Plates were analysed at 450 nm with correction at 540 nm for plate and background absorbance. Data were analysed using Pro software and concentrations calculated from the standard curve.

Reagent	Information	Supplier
Duo Set ELISA Human CXCL8/IL-8	Human IL-8 Capture Antibody , Human IL-8 Detection Antibody, Human IL-8 Standard, Streptavidin-HRP	R&D System
Duo Set ELISA Human IL-29/IL-28B (IFN-λ1/3)	Human IL-29/IL-28B Capture Antibody, Human IL-29/IL-28B Detection Antibody, Human IL-29/IL-28B Standard, Streptavidin-HRP	R&D System
Duo Set ELISA Human IL-6	Human IL-6 Capture Antibody , Human IL-6 Detection Antibody, Human IL-6 Standard, Streptavidin-HRP	R&D System
DuoSet Ancillary Reagent Kit 2	ELISA Plate-coating Buffer, Reagent Diluent Concentrate 2 (10x), Stop Solution, Color Reagent A, Color Reagent B, Wash Buffer Concentrate, Clear microplates, ELISA plate sealers	R&D System

Table 2.4 Reagents and kits for ELISA

2.8 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

At predetermined harvest times, cells were lysed with RLT buffer supplemented with β-mercapto-ethanol at a 1:100 dilution. RNA extraction was performed using the RNeasy Minikit (Qiagen) according to the manufacturers' instructions in a final volume of 30 µL and DNA removed by DNAse incubation. Then, reverse transcription in cDNA of 1 µg RNA template was obtained by incubation at 37°C for 1h using the Omniscript RT kit (Qiagen). Amplifications of 1 µL cDNA or appropriately diluted standard plasmid DNA were carried out by quantitative PCR.

Reagent	Information	Supplier
Omniscript reverse transcription (RT) kit	Components: dNTP mix (contain 5 mM each dNTP), 10X buffer RT, RNase-free water, Omniscript reverse transcriptase.	Qiagen
RNAse-free DNase set	1500 U RNase-free DNase I, RNase-free buffer RDD	Qiagen
RNeasy Mini Kit	Buffer RLT, Buffer RW1, Buffer RPE, RNase-free water, RNeasy mini spin columns, collection tubes	Qiagen
B-mercapto-ethanol	14.4 M $C_2H_6O_5$, added at 1:100 RLT lysis buffer for RNA extraction	Sigma-Aldrich

Table 2.5 Reagents and kits for RNA extraction and reverse transcription in cDNA

2.8.1 Taqman qRT-PCR

The amplification of cDNA was performed in a reaction mix containing Quantitect Probe PCR Mastermix (Qiagen) with primers (Invitrogen) and FAM-TAMRA-labelled probes (Eurofins) specific for RV, 18S ribosomal RNA, viperin, OAS and IP-10 (table) in a total volume of 12.5 µL per well of a 96-well plate. Analyses were performed using a LightCycler 480 II real-time PCR system (Roche). The reactions were run with the following conditions: 1 pre-incubation cycle of 95°C (10 minutes), 45 cycles at 95°C (10 seconds), 60°C (30 seconds) and 72°C (2 seconds), 1 cooling cycle at 40°C (1 minute). To get absolute values of quantification, each gene quantification was normalised to the 18S rRNA level and copy number calculated by comparison with a standard curve generated by amplification of plasmid DNA. All samples were analysed in duplicate.

Reagent	Composition	Supplier
QuantiTect Probe PCR Master Mix	HotStarTaq DNA polymerase, Quantitect probe PCR buffer (Tris-HCl, KCl, $(NH_4)_2SO_4$, 8 mM $MgCl_2$ pH 8.7) dNTP mix, ROX (passive reference dye)	Qiagen

Table 2.6 Reagent for Taqman qRT-PCR

Gene	Concentration [nM]	Sequence (5'-3')
18S forward	300	cgccgctagaggtgaaattct
18S probe	100	FAM-accggcgcaagacggaccaga-TAMRA
18S reverse	300	cattcttggcaaatgctttcg
IP-10 forward	900	
IP-10 probe	100	FAM-ctgactctaagtggcattcaaggagtacctctctc-TAMRA
IP-10 reverse	900	
OAS forward	900	ctgacgctgacctggttgtct
OAS probe	100	FAM-cctcagtcctctcaccacttttca-TAMRA
OAS reverse	900	ccccggcgatttaactgat
RV forward	50	gtgaagagccscrtgtgct
RV probe	100	FAM-tgagtcctccggcccctgaatg-TAMRA
RV reverse	300	gctscagggttaaggttagcc
viperin forward	900	cacaaagaagtgtcctgcttggt
viperin probe	100	FAM-cctgaatctaaccagaagatgaaagactcc-TAMRA
viperin reverse	900	aagcgcatatatttcatccagaataag

Table 2.7 Taqman qRT-PCR primers and probes

2.8.2 SYBRGreen qRT-PCR

Analyses were performed with by SYBRGreen detection following the manufacturers' instructions in a reaction mix containing QuantiFast SYBRGreen PCR Master Mix with ready to use QuantiTect primer assays (Qiagen), which include both forward and reverse primers for viperin (RSAD2), OAS1 and GAPDH in a total volume of 20 µL per capillary strip tube. Separate primers (Biomers) were used for HRV-1B RNA amplification. Analyses were performed using a Rotor-Gene (Qiagen). The reactions were run for 40 cycles with the following conditions: 1 pre-incubation cycle of 95°C to activate the DNA polymerase (5 minutes), 45 cycles at 95°C for denaturation (10 seconds) and 60°C for combined annealing and extension (30 seconds). Each gene was normalised to the GAPDH level and comparative quantitation relative to a fixed calibrator value of gene expression performed. All samples were analysed in duplicate.

Gene	Concentration [nM]	Sequence (5'-3')
RV forward	50	gtgaagagccscrtgtgct
RV reverse	300	gctscagggttaaggttagcc

Table 2.8 Custom primers for RV used in SYBRGreen qRT-PCR

Reagent	Composition	Supplier
QuantiFast SYBRGreen PCR Master Mix	HotStarTaq Plus DNA polymerase, QuantiFast SYBRGreen PCR buffer, SYBRGreen I dye, dNTP mix (dATP , dCTP , dGTP, dTTP), ROX (passive reference dye)	Qiagen
QuantiTect Primer Assay GAPDH	Hs_GAPDH_2_SG, mix of lyophilized forward and reverse primers	Qiagen
QuantiTect Primer Assay OAS	Hs_OAS1_vb.1_SG mix of lyophilized forward and reverse primers	Qiagen
QuantiTect Primer Assay viperin	Hs_RSAD2_1_SG mix of lyophilized forward and reverse primers	Qiagen
TE buffer (100X)	for primers resuspension (1X) TE buffer pH 8.0	SERVA

Table 2.9 Reagents for QuantiTect Primer Assay

2.9 Western blotting

At endpoint incubation times with recombinant IFN-β, cell extracts were collected with ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with protease and phosphatase inhibitors, followed by protein quantification by BCA assay and measuring through the plate reader FLUOstar OMEGA, BMG Labtech. Vertical electrophoresis runs were performed in 4 to 12% Bis-Tris SDS-PAGE gels loaded with equal amounts of proteins. Proteins were transferred into polyvinylidene difluoride (PVDF) membranes and blocked by immersion in TBS, supplemented with 5% BSA and 0.1% Tween 20, for 1h at RT. Following incubation with primary antibodies at 4°C overnight, secondary antibodies were added for 1h at RT before ECL reagent addition and subsequently reading using Fusion FX7 image analyser (Vilber Lourmat).

Antibody	Isotype	Clone	Working dilution	Supplier
pSTAT1 S727	Rabbit IgG	Polyclonal	1:1000	Cell Signalling
pSTAT1 Y701	Rabbit IgG	D4A7	1:1000	Cell Signalling
pSTAT2 Y690	Rabbit IgG	D3P2P	1:1000	Cell Signalling
STAT1	Rabbit IgG	Polyclonal	1:2000	Cell Signalling
STAT2	Rabbit IgG	D9J7L	1:2000	Cell Signalling

Table 2.10 Antibodies used for western blotting analyses

Reagent	Information	Supplier
Blocking buffer and antibody incubation buffer for western blotting	TBS containing 5% BSA and 01% Tween-20	Sigma-Aldrich
Bovine serum albumin (BSA) powder	1% BSA in 0.15M NaCl pH 7.0	Sigma-Aldrich
complete™, Mini, EDTA-free Protease Inhibitor Cocktail	Inhibits a broad spectrum of serine and cysteine proteases. Use 1 tablet per 10ml	Roche
Invitrolon™ PVDF/Filter Paper Sandwich	For protein transfer 0.45 µm pore size, 8.3 x 7.3 cm, used for mini gels	Life Technologies
Laemmli buffer (2X)	For 40 ml w/o DTT: 20 ml SDS 10%, 10 ml glycerol, 6 ml 1 M Tris (pH=6.8), 4 ml of H_2O. To make 1 ml of L2 with DTT: 800 µL of L2X w/o DTT + 200 µL of DTT 1M + Bromophenol blue	In house
Novex™ Tris-Glycine Transfer Buffer (25X)	Used at 1X, for 1L: dH_2O containing 40 ml of the 25X buffer	Thermo Fischer Scientific
NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.5 mm	A neutral pH environment of these gels minimises protein modifications	Life Technologies
NuPAGE™ MES SDS Running Buffer (20X)	Used at 1X, for 1L: dH_2O containing 50 ml of the 20X buffer	Thermo Fischer Scientific
Phosphatase inhibitor cocktail 3	Vehicle: DMSO. Sage at a final concentration of 1% (v/v) Components: Cantharidin, (-)-p-	Sigma-Aldrich

	Bromolevamisole oxalate, Calyculin A	
Pierce^{TM} Bicinchoninic acid assay (BCA) Protein Assay Kit	For protein quantification measuring absorbance in a microplate reader. Components: BCA Reagent A, 2 x 500 ml, BCA Reagent B, 25 ml, Albumin Standard Ampules, 2 mg/ml, 10 x 1 ml	Thermo Fischer Scientific
Radioimmunoprecipitation assay (RIPA) buffer	50 mM Tris-HCl, pH 8.0, with 150 mM NaCl, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate	Sigma-Aldrich
SeeBlueTM Plus2 Pre-stained Protein Standard	10 polypeptides resolved into 8 blue and 2 coloured bands in the range of 3-198 kDa with MES SDS running buffer	Thermo Fischer Scientific
Tris-buffered saline (TBS)	dH_20 containing 20 mM Tris and 0.9% NaCl	Sigma-Aldrich
Tween-20	Polyoxetylene-sorbitan monolaureate (2 ethylene oxide units, 1 sorbitol unit, 1 lauric acid unit)	Sigma-Aldrich
Washing buffer	TBS containing 0.1% Tween-20	Sigma-Aldrich

Table 2.11 Materials and reagents for western blotting

2.10 Statistical analysis

Data are represented as means ± standard error of the mean (SEM) of at least 2 independent experiments performed in duplicate. The statistical analysis was carried out by using GraphPad Prism 7 software and one-way analysis of variance (ANOVA) with Dunnett's post hoc multiple comparison test. Statistically significance is indicated as * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Chapter 3: Results – Anti-inflammatory and immune-suppressive effects of GCs

3.1 Introduction

Obstructive lung diseases such as asthma and COPD are characterised by chronic inflammation of the airways. Glucocorticoids (GCs) are the most effective anti-inflammatory drugs available and are widely prescribed as maintenance therapy in both asthma [234, 238] and COPD [88, 90] as inhaled corticosteroids (ICS). Acute worsening of respiratory symptoms are frequently triggered by viral infections of the airways [40-42] and rhinovirus (RV) is the most frequently detected virus associated with diseases exacerbations [43-45, 78, 81, 82]. Although the efficacy of GCs in the attenuation of symptoms is well documented [237, 238], randomised controlled trials have reported reduce COPD exacerbation frequency by only 20-25% [235]. In addition, the clinical use of GCs correlates with the onset of several long-term adverse effects [239, 240]. For these reasons, there have been significant efforts to separate the therapeutic action of GCs from their unwanted side effects and a great number of selective compounds developed [297], including selective steroidal glucocorticoid receptor (GR) agonists (SEGRAs), and modulators (SEGRMs) with a non-steroidal structure [289]. Although there is some *in vitro* evidence supporting their development, further research is needed to confirm their clinical advantages. However, these compounds are excellent research tools to better understand GR modulation at molecular level [298]. Overall, there are speculations about GCs safety in asthma and COPD treatments [243] and their effect on the innate immune response against respiratory virus infections remains a relevant and questioned argument [241, 242].

Nasal and bronchial epithelial cells are the first sites of viral infection of the airways. In order to control virus infectivity, both anti-viral and pro-inflammatory responses cooperate to eliminate the pathogen [61, 131]. RV uptake and endosomal internalisation trigger the host innate antiviral response [136]. Toll-like receptors include vesicle membrane-located TLR3 and TLR7/8, which sense single stranded (ss) and double stranded (ds) viral RNA, respectively, and TLR2, which recognises the viral capsid on the cell surface membrane, thereby inducing the activation of NF-kB and upregulation of cytosolic RIG-1 and MDA5 [99, 137, 138]. The result is an increased production of pro-inflammatory cytokines such as IL-6 and IL-8 and anti-viral cytokines such as type I (IFN-β) and type III (IL-29/IFNλ1, IL-28A/IFN-λ2, IL-28B/IFN-λ3) IFNs [140, 141], which in turn induce the expression of interferon stimulated genes (ISGs) such as viperin and OAS in nearby cells [148, 212-216,

221, 222]. IFN-λs may be the primary IFNs produced by the innate immune response at the lung mucosal barrier [205, 206].

Cell production of several mediators also induces the recruitment of other immune cells such as macrophages, eosinophils and neutrophils *in situ*, which contribute to airway inflammation and respiratory symptoms, orchestrating an effective link between innate and adaptive immune responses [132-135].

3.2 Hypothesis

1. Do selective GR ligands suppress RV-induced pro-inflammatory cytokines production as standard GCs do?
2. Do GCs and selective GR ligands suppress the innate immune response to RV infection?
3. Do GCs and selective GR ligands affect RV replication?

3.3 Aims

For this set of aims the experimental model of asthma or COPD exacerbations consists in *in vitro* cultures of bronchial epithelial cells (BEAS2B) infected with human rhinovirus 1B (HRV-1B).

1. To evaluate the effects of the standard steroid FP and to investigate the effect of selective GR agonists or modulators (GRT7, GRT8 and GRT10) on pro-inflammatory IL-6 and IL-8 cytokines production.
2. To investigate the effects of fluticasone propionate (FP) and selective GR agonists or modulators (GRT7, GRT8 and GRT10) on IL-29/IFNλ1 production and ISG expression.
3. To determine the effects of FP and selective GR agonists or modulators (GRT7, GRT8 and GRT10) on RV replication.

3.4 Results

3.4.1 Set up of experimental conditions

At first, experimental conditions of rhinovirus (RV) infection in bronchial epithelial cells were set up (Figure 3.1). BEAS2B cells were infected with HRV1B at an MOI of 1 for 1h to allow virus adhesion and subsequently washed before fresh media addition. Following cell incubation at 37°C for up to 24h, cells were harvested at different time points post infection and viral RNA measured by Taqman qRT-PCR.

Starting from time 0, which represents the viral genome immediately after infection, a gradual reduction of RV RNA was observed at 2 and 4 hours post infection, an effect probably related to host cellular anti-viral response by action of endogenous RNAses. Then, this decreasing trend reverted and quantification at 8 and 24h post infection revealed a viral genome increase. As a control for viral replication, virus was inactivated by UV-light irradiation exposure. This is included in the graph as UV-RV and will be used as a negative control in experiments described in chapter 5.

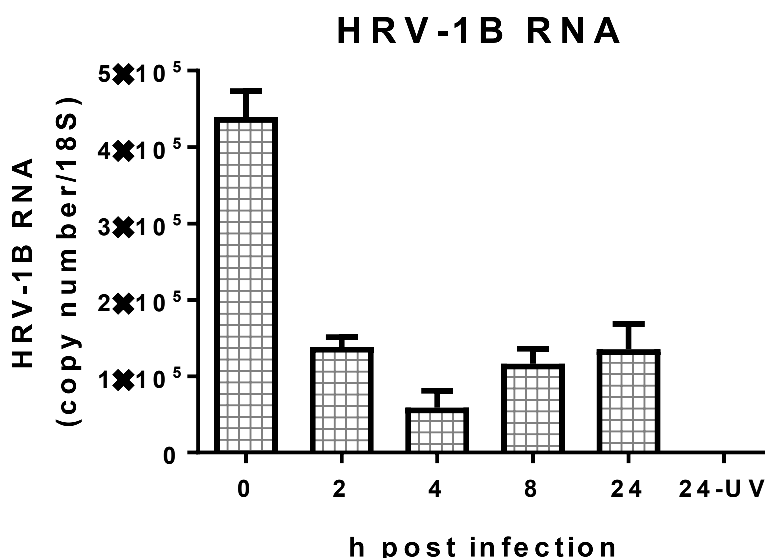


Figure 3.1 Virus time-course

Set up of experimental conditions. BEAS2B cells were infected with HRV-1B at an MOI of 1 for 1h to allow virus adhesion, followed by cell washing and fresh media addition. Cells were incubated at 37°C and cell lysates collected at different time points post infection to measure viral RNA by Taqman qRT-PCR. Data are illustrated as mean (±SEM) of four independent experiments performed in duplicate and normalised to the housekeeping gene 18S. The level of viral genome immediately after infection is shown as time 0 and represent the “input” of virus to the cells. UV represents the virus killed by UV-light irradiation exposure and is a control of virus inactivation. HRV-1B, human rhinovirus 1B.

3.4.2 Experimental conditions

As a result of setting up the experimental conditions described in figure 3.1, the protocol for RV infections was designed (Fig 3.2).

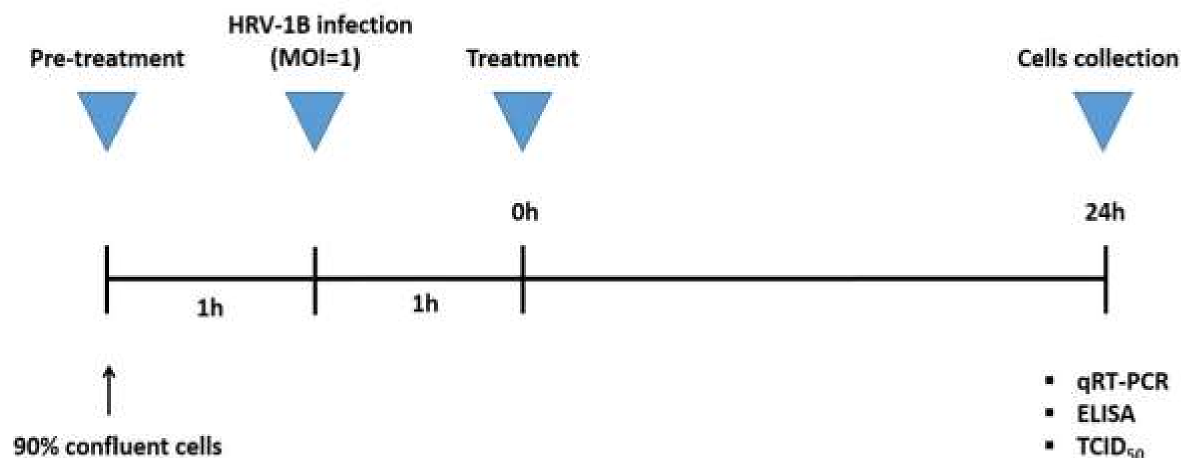


Figure 3.2 Experimental conditions for HRV-1B infections

BEAS2B cells were pre-treated with drugs for 1h and subsequently infected with HRV-1B at an MOI of 1 for a further 1h. After the 1h infection cells were washed of unbound virus and further incubated for 24h with fresh medium containing drug. At 24h post infection, cells were collected for Taqman qRT-PCR, ELISA or $TCID_{50}$ analyses.

3.4.3 RV-induced pro-inflammatory response and GCs

The experimental protocol of RV infection and treatments in BEAS2B cells followed the scheme reported in figure 3.2. Analyses were performed on cell supernatants by ELISA assays. The anti-inflammatory action of fluticasone propionate (FP) was assessed by measuring the level of IL-6 and IL-8, pro-inflammatory cytokines whose production is stimulated in response to viral infections. Comparisons between the effects of the standard steroid FP and novel selective GR agonists or modulators (GRT7, GRT8 and GRT10) were made, in order to investigate the effects of different GR ligands and to estimate the potential therapeutic action of these compounds. Cells were treated with increasing concentrations of FP, GRT7, GRT8 or GRT10 and dose-response curves were produced to show the dose-dependent effect on pro-inflammatory cytokines production (Fig 3.3/a-b). DMSO was used as vehicle control, so the reference point on graphs is expressed as 100% and refers to the effect of HRV-1B on DMSO treated cells. The effect

of the treatment for each compound is presented relative to control, thus single values are illustrated as percentage of DMSO (% of DMSO). Results clearly demonstrated an inhibitory effect on IL-6 and IL-8 with all tested compounds in a dose-dependent way.

FP significantly suppressed RV-induced IL-6 (IC_{50} = 0.4 nM) release (Fig 3.3/a). GRT7 suppressed IL-6 (IC_{50} = 0.3 nM) release in a similar way, while GRT10 had a comparable effect on IL-6 (IC_{50} = 1 nM) with slightly less potency relative to FP and GRT7. GRT8 suppressed IL-6 (IC_{50} = 106 nM) release at higher doses and with lower efficacy compared to FP, GRT7 and GRT10.

The assessment of IL-8 cytokine showed a pattern analogous to IL-6. FP significantly suppressed RV-induced IL-8 (IC_{50} = 0.2 nM) release (Fig 3.3/b) similarly to GRT7 (IC_{50} = 0.2 nM), while GRT10 had the same suppressive trend on IL-8 (IC_{50} = 0.5 nM) release compared to FP and GRT7 but slightly less potency. GRT8 suppressed IL-8 (IC_{50} = 29 nM) release with less potency and lower efficacy relative to FP, GRT7 and GRT10.

In summary, FP had the expected suppressive effect on RV-induced IL-6 and IL-8 pro-inflammatory cytokines production, while selective compounds GRT7, GRT8 and GRT10 also demonstrated a significant anti-inflammatory effect, with a differential pharmacological profile exhibited by GRT8.

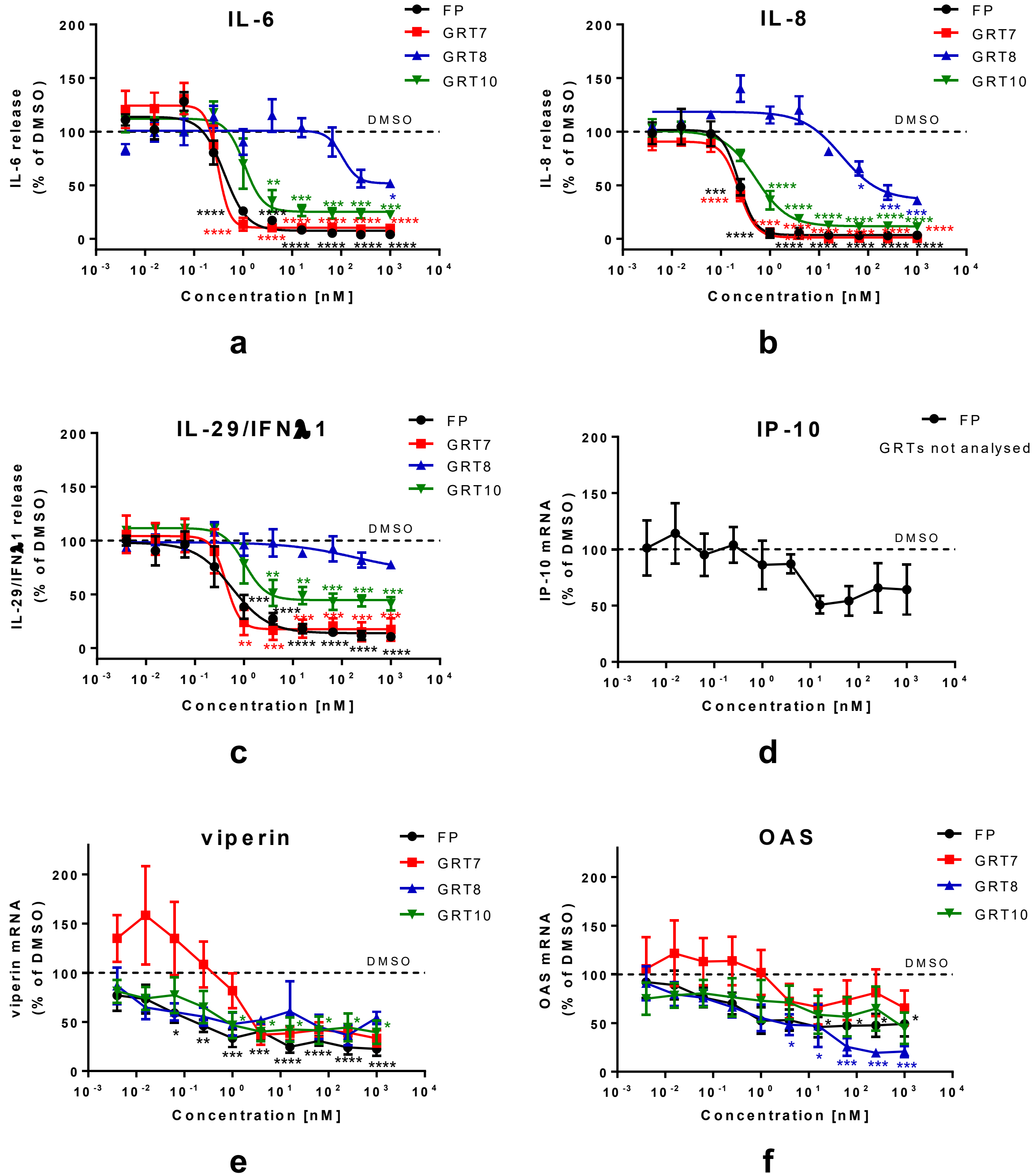


Figure 3.3 GC suppression of RV-induced pro-inflammatory and anti-viral responses

BEAS2B cells were pre-treated with increasing concentrations of fluticasone propionate (FP) or selective glucocorticoid receptor (GR) agonists and modulators (GRT7, GRT8 and GRT10) for 1h and subsequently infected with HRV-1B at an MOI of 1 for 1h, followed by media replacement and treatment. Supernatants and cell lysates were collected at 24h post infection. The production of **(a)** IL-6, **(b)** IL-8 and **(c)** IL-29/IFNλ1 proteins was measured by ELISA assays. **(d)** IP-10, **(e)** Viperin and **(f)** OAS gene expression was measured by Taqman qRT-PCR, using 18S rRNA for normalisation. DMSO is the vehicle control, whose effect in cells is marked with a dotted line, corresponding to 100%. All data are illustrated as percentage of DMSO (% of DMSO) and represent mean (±SEM) of **(a-c)** four independent experiments replicated in duplicate, except for GRT8 that was performed once. In **(d-f)** results were obtained from four to six independent experiments. All data were analysed by one-way ANOVA with Dunnett's post hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. MOI, multiplicity of infection; HRV-1B, human rhinovirus 1B.

3.4.4 Effect of GCs on RV-induced IL-29/IFNλ1 production and ISG expression

In order to investigate the effects of GCs on the innate immune response to respiratory viruses, HRV-infected BEAS2B cells were treated with FP and a set of novel selective GR agonists or modulators (GRT7, GRT8 and GRT10) following the scheme reported in figure 3.1. Supernatants were collected and the production of IL-29/IFNλ1, which exerts a major role in the anti-viral response, assessed by ELISA assays (Fig 3.3/c). In parallel, cell lysates were analysed by Taqman qRT-PCR to quantify the expression of interferon stimulated genes (ISGs) such as viperin, OAS and IP-10 (Fig 3.3/d-f). Cell responses to HRV-1B infection and treatments with vehicle DMSO are expressed as 100% and single values illustrated as percentage of DMSO (% of DMSO).

The standard steroid FP showed a significant dose-dependent suppression of RV-induced IFNλ1 (IC_{50} = 0.6 nM) release (Fig 3.3/c). GRT7 similarly suppressed IFNλ1 (IC_{50} = 0.4 nM), while GRT10 supressed IFNλ1 (IC_{50} = 1 nM) release with the same trend but slightly reduced efficacy and potency in comparison to FP and GRT7. By contrast, GRT8 had a completely different scenario. In fact, even high doses of GRT8 showed no effect or an irrelevant effect on IFNλ1 production, suggesting a better anti-viral response in RV-infected cells treated with GRT8.

In bronchial epithelial cells, released IFNs activate the expression of ISGs in nearby cells. Therefore, ISGs were also measured to investigate the effect of GCs on the innate immune response to viral infections. All tested compounds demonstrated a suppressive effect on ISG expression. Dose-response curves were produced to show the dose-dependent effects. At a concentration of 15 nM viperin gene expression was significantly suppressed (Fig 3.3/e) by FP (75%), GRT7 (62%), GRT8 (40%) and GRT10 (58%).

In addition, a significant suppressive effect was observed on OAS gene expression (Fig 3.3/f) by FP (54%), GRT7 (34%), GRT8 (53%) and GRT10 (41%) at the same concentration of 15 nM. OAS was also suppressed at a greater extension by GRT8 (81%) at 250 nM.

Moreover, the expression of IP-10 (Fig 3.3/d), an additional ISG, was suppressed by FP (49%).

In summary, FP had a suppressive effect on the innate immune response to RV-infections, revealed by assessment of IFNλ1 protein release and viperin, OAS and IP-10 gene expression. Further, GRT7, GRT8 and GRT10 had the same effect of FP on viperin and OAS, while GRT7 and GRT10, but not GRT8, had a suppressive effect on IFNλ1 production, suggesting the inhibition of the innate immune response by GCs but also by

the investigated selective GR agonists or modulators, with an ambivalent interpretation of results for GRT8.

3.4.5 GC effect on RV replication

GCs impaired the innate immune response in RV-infected bronchial epithelial cells. Therefore, to investigate whether this effect had implications on viral replication, BEAS2B cells were treated with FP and compounds GRT7, GRT8 and GRT10, novel selective GR agonists or modulators, following HRV-1B infection, as schematised in the protocol reported in figure 3.1. Cell lysates were harvested and RV genome replication assessed by Taqman qRT-PCR. In experiments conducted in parallel, cell lysates plus supernatants were collected to determine the endpoint viral titre by $TCID_{50}$ (Fig. 3.4/a-b). Data are represented as percentage of genome replication relative to control (% of DMSO) and the effect of DMSO on HRV-1B viral replication is referred as 100%.

The result of cell treatment with the standard steroid FP was a significant dose-dependent increase of HRV-1B RNA, with an increase of at least 2-fold at a concentration of 62 nM (Fig. 3.4/a). GRT7 and GRT10 had a similar trend and showed a comparable effect at this concentration. Differently, GRT8 had no effect on HRV-1B genome replication, suggesting a safer pharmacological profile of this compound.

In order to estimate whether the increase of HRV-1B RNA corresponds to the production of mature virions, viral titre was also determined. At a concentration of 250 nM FP induced viral replication of 2.5-fold and GRT7 of around 1.7-fold (Fig. 3.4/b). At the same concentration of 250 nM, GRT8 and GRT10 did not affect the viral titre.

In summary, the effect of FP and GRT7 on HRV-1B genome replication appeared to be dose-dependent, with an increase of HRV-1B RNA of at least 2-fold at 62 nM concentration and an increase of HRV-1B titre of 2.5 fold and 1.7-fold at 250 nM, respectively. GRT10 increasing effect on HRV-1B genome replication also showed dose-dependence with an increase of around 2-fold at 62 nM, in contrast to the lack of effect on HRV-1B load at 250 nM. Compound GRT8, more consistently with the unaltered effect on HRV-1B RNA replication, did not affect the viral titre.

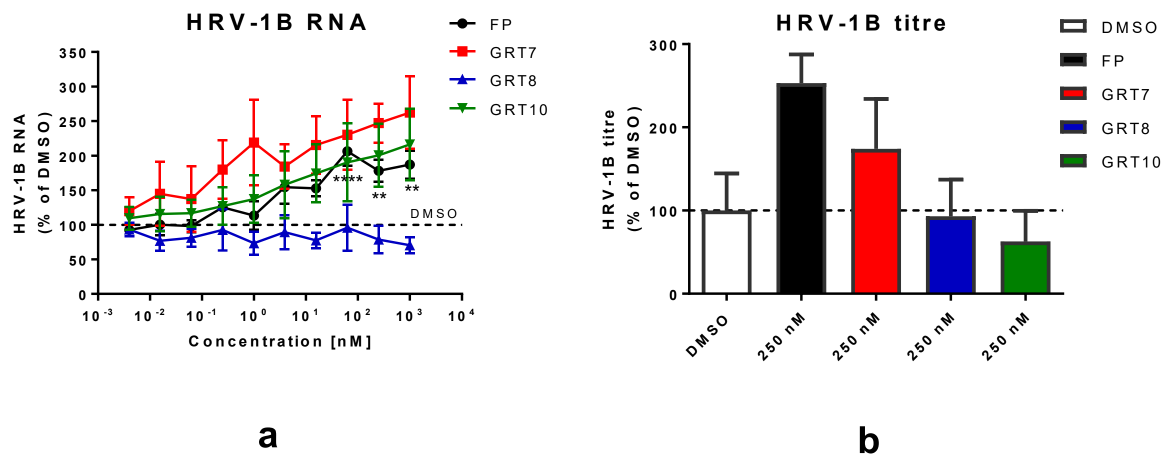


Figure 3.4 GC effect on RV replication

Following 1h pre-treatment with fluticasone propionate (FP) or selective glucocorticoid receptor (GR) agonists and modulators (GRT7, GRT8 and GRT10), BEAS2B cells were infected with HRV-1B at an MOI of 1 for 1h. After washing and media replacement, cells were treated for 24h at **(a)** increasing concentrations of compounds or **(b)** single dose of 250 nM. DMSO is the vehicle control. The dotted line at 100% corresponds to viral genome or viral titre level in DMSO-treated cells. Virus replication was measured **(a)** in cell lysates through HRV-1B RNA quantification by Taqman qRT-PCR using 18S rRNA to normalise data, or **(b)** in cell lysates plus supernatants through endpoint viral titre determination by $TCID_{50}$. Data represent mean (±SEM) comprising **(a)** at least four or **(b)** three independent experiments. Data analysis was carried out by one-way ANOVA with Dunnett's multiple comparison test. ** $p<0.01$, **** $p<0.0001$. MOI, multiplicity of infection; HRV-1B, human rhinovirus 1B.

3.5 Discussion

As GC safety in asthma and COPD treatment is a matter of debate and there are contrasting opinions from the scientific community [243], in this thesis *in vitro* studies of the balance between beneficial and unwanted side effects have been conducted at a molecular level in the context of acute infections by respiratory viruses. Human rhinovirus (HRV) is the virus of the common cold and is the main responsible for airway tract infections [107].

At first, a time-course analysis of HRV-1B infection in bronchial epithelial cells (BEAS2B) was performed and experimental conditions set up for GC treatment for 24h, following 1h pre-treatment and 1h infection.

The anti-inflammatory action of GCs was assessed quantifying by ELISA IL-6 and IL-8 pro-inflammatory cytokines release. As expected, low doses of the standard glucocorticoid FP, clinically administered as inhaled corticosteroid (ICS), suppressed IL-6 and IL-8 release in HRV-1B infected BEAS2B cells, consistently with ICS use as first-line therapy in asthma [237, 238] and as exacerbation-preventive treatment in COPD [235]. The

therapeutic effect of GCs in these chronic inflammatory pulmonary diseases is due to the activation of glucocorticoid receptors (GRs) and it is generally accepted that the transrepression pathway, which leads to NF-kB and AP-1 transcription factors repression, is the predominant mechanism of action involved [297, 300]. This is the reason why a great amount of selective glucocorticoid receptor agonists (SEGRAs) or modulators (SEGRMs) that favour the transrepression versus the transactivation pathway have been developed in the last years [289], as the transactivation pathway is commonly associated with side effects such as diabetes and muscle wasting [274, 275]. Although this is not a net distinction [294], the identification of differential compounds with the most favourable functional profile is an ambitious goal of relevant scientific interest [301-304, 306]. Therefore, a comparative analysis of the effects of selective compounds such as GRT7, GRT8 and GRT10 on pro-inflammatory cytokine release relative to the standard glucocorticoid FP was carried out. GRT7 and GRT10 had an effect comparable to FP, whilst GRT8 had a different pharmacological profile on IL-6 and IL-8 release, related to FP and also to GRT7 and GRT10, showing a strong reduction in potency and in efficacy, too. GCs, comprising selective compounds, modulate their action through GR. Different GR conformational changes may be induced by different ligands, resulting in different effects [311]. This concept may explain the diversified behaviour observed for GRT8. In fact, in the transrepression pathway GR directly interacts with coactivators of transcription factors, inhibiting their histone acetyltransferase (HAT) activity. GR also recruits histone deacetylase 2 (HDAC2) to induce chromatin remodelling in a closed conformation [235]. A hypothesis of the differential effect of GRT8 may be a reduction of protein-protein interactions (tethering) between GR and coactivators of NF-kB and AP-1 or between GR and HDAC2. In particular, GRT8-bound GR may have a reduced affinity for the coregulator GRIP1/SRC2/TIF2/NCOA2, as GR was specifically reported to interact with GRIP1 to repress NF-kB and AP-1 [276]. However, dissociated properties of GRT8 were also reported in literature [311] where it is referred as compound 12. Other cytokines such as TNF-α and IL-1β [312] or RANTES [313] could also be targeted as markers of inflammation. However, IL-6 and IL-8 are abundantly produced by airway epithelial cells in response to viral infections and represent reliable indicators of an acute inflammatory state [99, 140, 141, 314]. Thus, the suppressive effect of all selective compounds tested on IL-6 and IL-8 release demonstrated their potential to be further developed as anti-inflammatory agents.

Emerging evidence of GC impairment of antiviral immunity have been reported [241, 242]. The implication of RV infections as primary causative agents of asthma and COPD exacerbations have also been well documented [43, 45-47, 78, 82]. In the context of unwanted side effects, GC modulation of the innate immune response against RV

infections in bronchial epithelial cells was considered in this thesis. As IFNs are major orchestrators of cellular anti-viral response, GC effect on IL-29/IFNλ1 production was measured by ELISA assays. Low doses of the standard steroid FP suppressed IFNλ1 release, suggesting GC inhibition of the innate immune response in RV-infected BEAS2B cells. GRT7 had a dose-responsive effect overlapping with FP, and GRT10 also had a similar trend. Interestingly, the production of IFNλ1 was unaffected by GRT8, revealing a completely different scenario. This suggests GRT8 may have a potential therapeutic advantage towards RV infections in terms of reduced side effects. Thinking about an explanation for this diversified effect, the involvement of the coregulator GRIP1 was speculated. As a further GC model of suppression is GR recruitment of GRIP1, which is competitively recruited by the transcription factor IRF3 [283] to regulate type I (IFN-β) and type III (IFN-λs) IFN expression in response to respiratory virus infections [315], a reduced affinity of GRT8-bound GR for GRIP1 may explain the lack of effect on IFNλ1 production. Previous results also demonstrated that FP treatment suppressed RV induction of IFN-β and IFNλ2/3 both *in vitro* and *in vivo*. FP suppressed TLR3 and RIG-1-mediated, but not MDA5-mediated, IFN responses [97]. Double stranded (ds) viral RNA generated during RV replication activates the PRRs such as TLR3, RIG-1 and MDA5 [137, 140, 141]. Summarising, results of these thesis demonstrated an inhibitory effect on antiviral immunity by suppression of IL-29/IFNλ1 production by FP, GRT7 and GRT10, but not GRT8, thereby giving evidence of an unwanted side effect of GCs besides their therapeutic anti-inflammatory effect.

To better investigate this inhibitory effect of GCs on the innate immune response, interferon stimulated gene (ISG) expression was also measured by Taqman qRT-PCR in RV infected BEAS2B bronchial epithelial cells. Virus-induced IFN release involves autocrine and paracrine loops, activating cognate IFNAR (type I IFNs) and IFNLR1 (type III IFNs) receptor binding on the surface of nearby cells [144, 145], thereby promoting expression of several ISGs such as viperin and OAS, which are antiviral agents of the immune system [212, 213, 215, 222]. FP had a dose-responsive effect towards both viperin and OAS mRNA induction. Selective compounds GRT7, GRT8 and GRT10 had all comparable results on viperin and OAS relative to the standard steroid FP. Furthermore, FP had a suppressive effect on expression of IP-10, an additional ISG. These data clearly revealed an inhibition of the innate immunity and strongly suggested GC interference with IFN signalling pathways, as whether in presence or absence of IFN impairment, ISG expression was significantly suppressed. FP, GRT7, GRT10 and further GRT8, which differently from the previous ones appeared to not have a suppressive effect on IFNλ1 production, impaired RV-induced viperin and OAS mRNA induction. In fact, while FP, GRT7 and GRT10 suppression of viperin and OAS could be a direct consequence of IFN

suppression, the same effect by GRT8 was unexpected. These data suggested an unknown mechanism of GC inhibition of antiviral immunity involving the JAK/STAT signalling pathway. The same hypothesis also raised from data collected by testing selective GR ligands.

To investigate the consequence of GC and selective GR ligand inhibition of the innate antiviral immune response, HRV-1B replication in BEAS2B cells was assessed. FP increased HRV-1B RNA and viral titre in a dose-dependent way, suggesting an unwanted side effect of GCs on viral replication. Similarly, GRT7 showed a comparative trend relative to FP towards both HRV-1B RNA and viral titre. Among the other selective compounds tested, GRT10 had the same dose-dependent increasing effect on HRV-1B RNA, but no effect on RV titre. Although this need to be further clarified, a consideration could be that even if HRV-1B replication is increased in terms of HRV-1B RNA, genome assembly into the viral capsid to give mature virions may be blocked. Once HRV-1B is internalised inside the host cell, the viral RNA is translated into a large polyprotein, which is subsequently cleaved by viral proteases to produce structural proteins that compose the external capsid [108-111]. The inhibition of one of these proteases may be responsible for GRT10 blockage of virions maturation. More consistently and differently from FP, GRT7 and GRT10, the selective compound GRT8 had no influence on both HRV-1B RNA and titre, thus suggesting a differentiated effect in line with the controversial interpretation of results towards inhibition of the innate antiviral immunity.

3.6 Summary

Consistent with clinical use of inhaled corticosteroids (ICS) in asthma and COPD, glucocorticoid treatment resulted in pro-inflammatory cytokine suppression in bronchial epithelial cells, as demonstrated targeting IL-6 and IL-8 in BEAS2B cells treated with the standard steroid fluticasone propionate (FP) following RV infection. However, FP also suppressed the innate antiviral immune responses *in vitro*, including IFN λ1 (type III IFN) production and interferon stimulated gene (ISG) induction, comprising viperin and OAS. This was associated with increased viral replication, measured by RV genome (RV RNA) quantification and viral titre determination. Thus, besides the anti-inflammatory therapeutic effect, the inhibition of antiviral immunity is an unwanted side effect of GCs that may have implications in disease exacerbations, as RV is a major cause of acute worsening of asthma and COPD.

Selective GR agonists (SEGRAs) or modulators (SEGRMs), designed with the purpose to improve the GC therapy, were also tested. Although not necessarily these selective

compounds have the predicted effects in terms of reduced side effects, they are excellent research tools to study glucocorticoid receptor (GR) modulation. Compounds GRT7 and GRT10 had similar effects compared to FP, except for GRT10, which increased viral genome replication but did not affect the viral load. Differently, results obtained on both pro-inflammatory (IL-6 and IL-8) and antiviral (IFNλ1) cytokines release oriented the selective compound GRT8 towards a differential pharmacological modulation of the innate immune response against viral infections, with a partial retaining of the anti-inflammatory activity of GCs and a potential safer profile, shown by a lack of effect on RV replication. By contrast, ISG suppression by GRT8 questioned this interpretation of results.

Chapter 4: Results – GC modulation of type I IFN signalling pathway

4.1 Introduction

The host antiviral response is mediated by IFN induction of a number of interferon stimulated genes (ISGs), including virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible (viperin) and 2'-5' oligoadenylate synthetase (OAS), which are important agents of the innate immune system [150]. Viperin and OAS play a key role against several viral infections [217, 222] including human rhinovirus (HRV) [221]. Viperin localises to lipid droplets in the cytosolic face of the endoplasmic reticulum (ER) [216], which together with Golgi membranes are remodelled by positive-strand RNA viruses, including HRV, to build membranous vesicles with a unique lipid composition, associated with viral replication and so named viral replication organelles (ROs) [112, 113]. Viperin appears to be involved in cellular lipid metabolic pathways [212] and to alter plasma membrane fluidity [219], thereby inhibiting virus replication and release. Differently, OAS senses double-stranded RNA and catalyses ATP-dependent formation of 2'-5'-oligoadenylates, which activate the latent ribonuclease L (RNase L) to induce viral RNA degradation. Thus, OAS blocks viral replication and protein synthesis [223].

Activated type I IFN (IFN-α/IFN-β) receptor signalling pathways regulate viperin and OAS gene expression [215, 220] in neighbouring non-immune cells such as epithelial cells and fibroblasts. Innate immune cells, including macrophages and dendritic cells, also produce type I IFNs as first-line defence against viruses [148]. IFN-α and IFN-β, the most well defined type I IFNs, bind to interferon-alpha receptor (IFNAR) on the cell surface. IFNAR is expressed by nearly all cell types and is composed of IFNAR1 and IFNAR2 subunits, respectively associated with the cytoplasmic receptor proteins tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1), which shift from a latent inactive state to an active one, undergoing a reciprocal phosphorylation upon IFN binding [149-151]. In turn, these two kinases phosphorylate IFNAR, which functions as docking site for signal transducer and activator of transcription (STAT) proteins. In the canonical pathway, the recruitment of STAT1 and STAT2 induces STAT1 Y701 (pSTAT1 Y701) and STAT2 Y690 (pSTAT2 Y690) phosphorylations on tyrosine residues. These phosphorylations are crucial to promote STAT1-STAT2 dimerisation and further association with IRF9 to form a complex called interferon stimulated gene factor 3 (ISGF3) [152-154]. Once activated, STAT1-STAT2-IRF9 complex translocates into the nucleus, where it recognises IFN-stimulated response elements (ISREs) within promoter sequences of target ISGs, thus inducing gene

transcription [155, 156]. For full transcriptional activity and biological function, STAT1 must also be additionally phosphorylated on serine S727 residue [157-159].

GCs, widely prescribed medications in both asthma and COPD, have been reported to impair host antiviral responses [241, 242]. In addition, the primary role of respiratory virus infections in disease exacerbations has been well documented [43, 45-47, 78, 82]. However, the mechanisms of GC modulation of the innate immunity have not been well investigated so far.

4.2 Hypothesis

4. Do GCs and selective GR ligands impair the type I IFN signalling pathway?
5. Do GCs and selective GR ligands interfere with the JAK/STAT pathway?

4.3 Aims

The experimental model for this set of aims consists in *in vitro* cultures of bronchial epithelial cells (BEAS2B) stimulated with recombinant IFN-β.

1. To assess the effects of the standard steroid fluticasone propionate (FP) and selective GR agonists or modulators (GRT7, GRT8 and GRT10) on IFN-β stimulated gene (ISG) expression.
2. To investigate the effects of FP and selective GR agonists or modulators (GRT7, GRT8 and GRT10) on STAT1 and STAT2 phosphorylations.

4.4 Results

4.4.1 Experimental conditions

BEAS2B cells stimulated with recombinant IFN-β represented the model for studying GC effects on type I IFN signalling pathway (Fig 4.1). To better investigate the effects of GCs on the JAK/STAT pathway and to explore the kinetics of cell response, further protocols were designed (Fig 4.2).

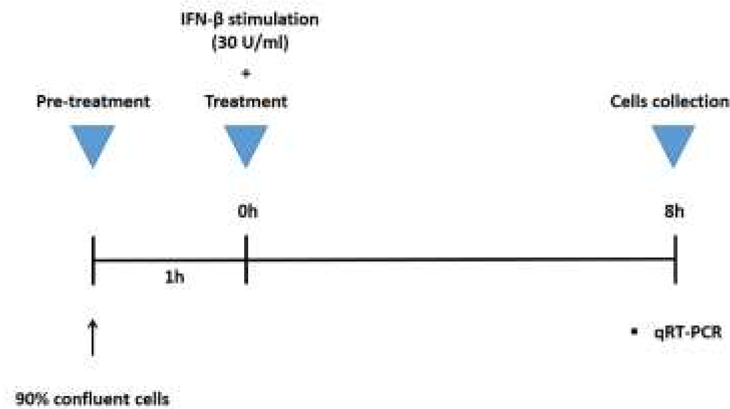


Figure 4.1 Experimental model to assess IFN-β induced ISG expression

Following 1h pre-treatment, BEAS2B cells were further incubated with fresh media containing drug and recombinant IFN-β at 30 U/ml. At 8h post IFN-β stimulation, cells were harvested for Taqman qRT-PCR analysis.

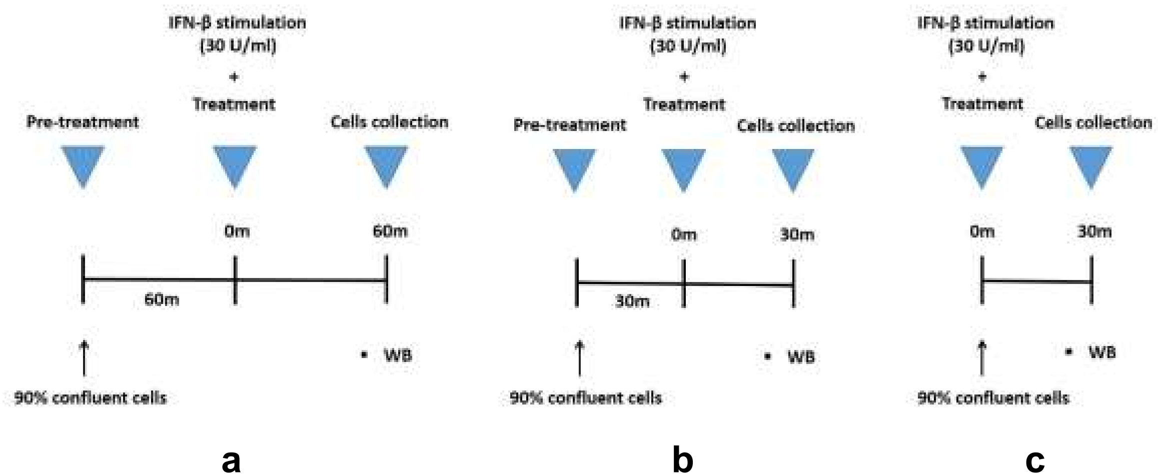


Figure 4.2 Experimental conditions to explore the kinetics of GC effects on the JAK/STAT pathway

Different protocols were designed for western blotting analyses in BEAS2B cells **(a)** Cells were pre-treated for 60mins and subsequently stimulated with recombinant IFN-β at 30 U/ml dissolved in fresh media also containing drug. **(b)** The pre-treatment time was reduced to 30mins and the period of IFN-β stimulation and treatment also reduced to 30mins. **(c)** Cells were incubated with IFN-β and drug for 30mins without pre-treatment.

4.4.2 Effect of GCs on IFN-β stimulated gene (ISG) expression

As GCs were observed to suppress the innate antiviral response to RV infections (chapter 3), investigations on GCs interaction with the type I IFN signalling pathway have been carried out. BEAS2B cells were stimulated with recombinant IFN-β to activate the pathway. Treatments with FP and a set of novel selective compounds (GRT7, GRT8 and GRT10) have been assessed following the scheme reported in figure 4.1. Cell lysates were collected and expression of ISGs such as viperin and OAS measured by Taqman qRT-PCR (Fig 4.3/a-b). Cell treatment with DMSO vehicle, without stimulation with IFN-β, represents the negative control. Additional controls of tested compounds effect on viperin and OAS gene expression, in absence of IFN-β stimulation, were also added.

At a concentration of 50 nM, FP suppressed viperin (46%) transcription (Fig 4.3/a). Comparable results were obtained with selective compounds, showing viperin suppression by GRT7 (42%), GRT8 (53%) and GRT10 (41%) treatments.

In addition, 50 nM concentration of FP significantly suppressed OAS (51%) transcription (Fig 4.3/b). Similar suppressive responses were observed by treatments with GRT7 (38%), GRT8 (42%) and GRT10 (34%).

In summary, the standard steroid FP suppressed gene expression of viperin and OAS in bronchial epithelial cells stimulated with recombinant IFN-β at a concentration of 50 nM, suggesting implications of the type I IFN signalling pathway with GC impairment of the innate antiviral response. Selective GR agonists and modulators such as GRT7, GRT8 and GRT10 had an analogous effect on both viperin and OAS gene expression.

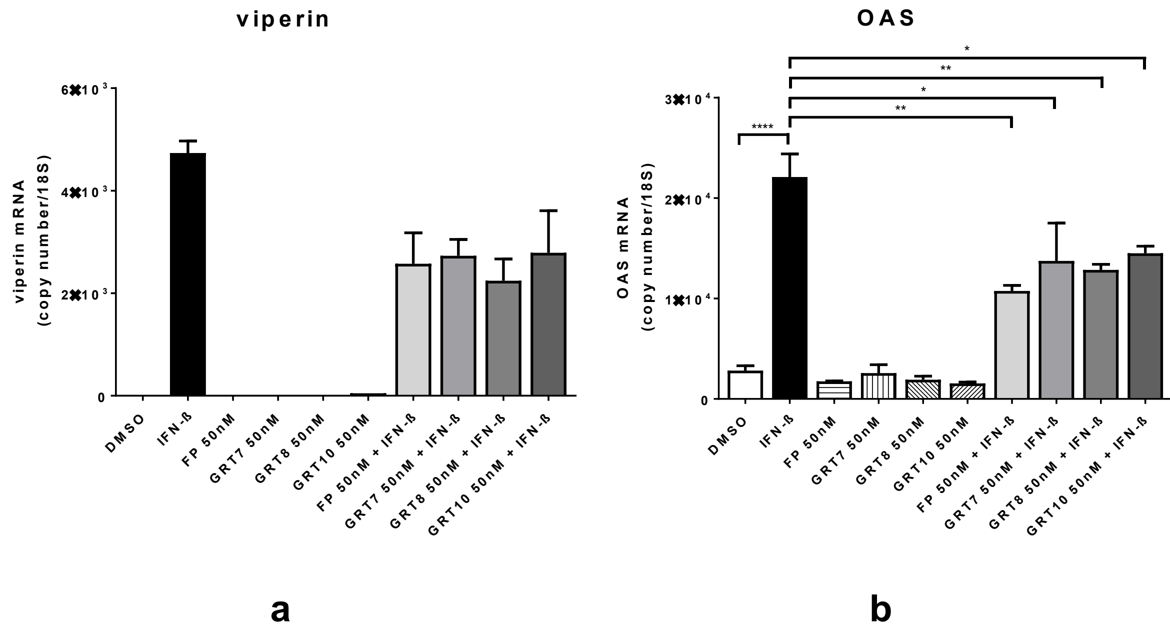


Figure 4.3 GC impairment of type I IFN signalling pathway and ISG expression

BEAS2B cells were treated with fluticasone propionate (FP) or selective glucocorticoid receptor (GR) agonists and modulators (GRT7, GRT8 and GRT10) at a concentration of 50 nM for 8h in presence or absence of recombinant IFN-β at 30 U/ml, following 1h pre-treatment. **(a)** Viperin and **(b)** OAS gene expression was measured by Taqman qRT-PCR as copy number normalised to 18S rRNA (copy number/18S). DMSO vehicle, without IFN-β stimulation, represents the negative control. Data represent mean (±SEM) of two independent experiments performed in duplicate, analysed by one-way ANOVA with Dunnett's post hoc test. * $p<0.05$, ** $p<0.01$. IFN, interferon.

4.4.3 GC effect on IFN-β induced STAT1 and STAT2 phosphorylations

Having shown that GC treatment suppressed IFN induced ISG expression, I examined the activation of the JAK/STAT signalling pathway at the molecular level. BEAS2B cells were treated with FP and a set of selective GR agonists and modulators (GRT7, GRT8 and GRT10) following stimulation with recombinant IFN-β. The negative control consists in DMSO treatment without IFN-β stimulation. Controls of compounds effect without IFN-β stimulation were also added. Different protocols (Fig. 4.2/a-c) of IFN-β stimulation with different pre-treatments and/or treatments times were designed in order to explore the kinetics of GC effects on STAT1 and STAT2 proteins, the molecular targets for this set of experiments. Cell extracts were collected to perform western blotting (WB) analyses on equal amounts of loaded proteins. Antibodies were used to detect the totality of STAT1 and STAT2 proteins, as indicators of equal loading, and specific phosphorylations of STAT1 (pSTAT1 Y701 and pSTAT1 S727) and STAT2 (pSTAT2 Y690) on tyrosine (Y) or serine (S) amino acidic residues.

Using the 60 minutes pre-treatments plus 60 minutes of IFN-β stimulation and treatment (60m+60m) protocol (Fig. 4.4/a), FP inhibited STAT1 Y701 phosphorylation (pSTAT1 Y701) at concentrations of 10, 50 and 250 nM, suggesting GC interference with the activated JAK/STAT signal. GRT7, GRT8 and GRT10 similarly inhibited pSTAT1 Y701, suggesting that also tested selective GR ligands interfere with the JAK/STAT pathway. In parallel, no effect of GCs in non IFN-β stimulated cells was also observed.

In order to investigate the kinetics of this effect, the treatment time was reduced to 30 minutes and a comparison between 30 minutes pre-treatment (30m+30m) or even no pre-treatment (0m+30m) carried out (Fig. 4.4/b). Additionally, in these experiments the concentrations were reduced at 0.1, 1 and 10 nM. FP showed an inhibitory effect on IFN-β induced pSTAT1 Y701 at the concentrations of 1 and 10 nM in both 30 minutes pre-treatment and no pre-treatment conditions. All selective compounds, GRT7, GRT8 and GRT10, also inhibited pSTAT1 Y701 without distinction between short pre-treatment and no pre-treatment at all tested concentrations. These results revealed that GC inhibition of pSTAT1 Y701 phosphorylation is a rapid event that occurs within 30 minutes and that GR activation before IFN-β stimulation is not required for this effect. Moreover, nuclear interactions between DNA and GR is unlikely to be required, thus suggesting this may be a non-genomic effect of GCs.

To clarify the effect of the standard steroid FP on the modulation of the JAK/STAT pathway, in parallel experiments using both (60m+60m) and (0m+30m) protocols were performed (Fig. 4.4/c) and specific phosphorylations of both STAT1 and STAT2 proteins assessed. The concentration-dependent inhibition of IFN-β stimulated pSTAT1 Y701 was confirmed. This effect was visible at 10, 50 and 250 nM (Fig. 4.4/c left side) and also at 0.1, 1 and 10 nM scalar concentrations (Fig. 4.4/c right side) of FP. In addition, FP strongly inhibited pSTAT2 Y690 following stimulation with IFN-β at all experimental conditions, but had no effect on pSTAT1 S727, which also appeared not induced by IFN-β stimulation.

In summary, the standard steroid FP rapidly inhibited IFN-β induced phosphorylation of STAT1 at tyrosine residue Y701 in a dose-dependent way, independently from cells pre-treatment. Selective GR agonists or modulators such as GRT7, GRT8 and GRT10 also had a similar inhibitory effect on pSTAT1 Y701. In addition, FP inhibited pSTAT2 Y690. Differently pSTAT1 S727 appeared not stimulated by IFN-β and not affected by FP. Data suggest GCs interference with the activated JAK/STAT pathway in bronchial epithelial cells.

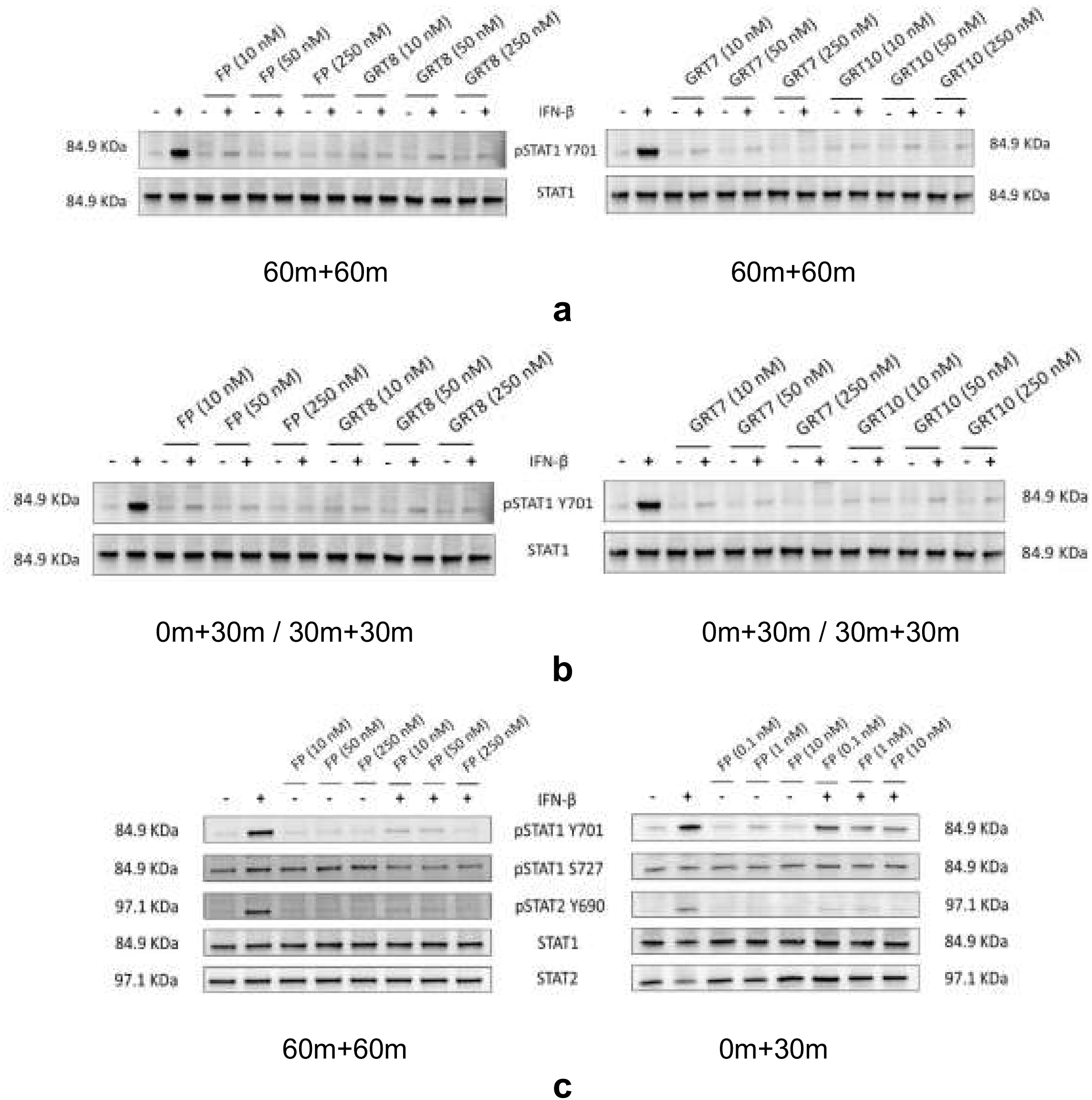


Figure 4.4 GC inhibition of IFN-β induced STAT1 and STAT2 phosphorylations

Different protocols were designed in BEAS2B cells, comprising pre-treatment with fluticasone (FP) or selective glucocorticoid receptor (GR) agonists and modulators (GRT7, GRT8 and GRT10), plus stimulation with recombinant IFN-β (30 U/ml) and treatment with the compounds. The negative control consists in DMSO treatment without IFN-β stimulation. Cell extracts were collected and analysed by western blotting using antibodies to detect specific phosphorylations (pSTAT1 Y701, pSTAT1 S727, pSTAT2 Y690) and the totality (STAT1 and STAT2) of signal transducer and activator of transcription proteins. **(a)** Pre-treatment for 60 minutes plus IFN-β stimulation and treatment for other 60 minutes (60m+60m). Inhibition of pSTAT1 Y701 at 10, 50 and 250 nM concentrations of FP, GRT8, GRT7 and GRT10. **(b)** Comparison between different pre-treatment times. Pre-treatment for 30 minutes plus IFN-β stimulation and treatment for 30 minutes (30m+30m) or even no pre-treatment plus 30 minutes IFN-β stimulation and treatment (0m+30m). Inhibition of pSTAT1 Y701 at FP, GRT8, GRT7 and GRT10 reduced concentrations of 0.1, 1 and 10 nM. **(c)** Evaluation of the kinetics of STAT proteins phosphorylations at scalar concentrations of FP. Overview between the 60m+60m protocol with FP concentrations of 10, 50, 250 nM and the 0m+30m protocol with FP concentrations of 0.1, 1, 10 nM. Inhibition of pSTAT1 Y701 and pSTAT2 Y690, but not pSTAT1 S727. All results are representative of three independent experiments. Y701, tyrosine 701; S727, serine 727; Y690, tyrosine 690.

4.5 Discussion

Inhaled corticosteroids (ICS) are the major treatment in asthma [234, 238] and are widely prescribed to prevent exacerbations in COPD [88, 90]. However, emerging evidence of GC impairment of the antiviral immune response have been reported [241, 242]. In chapter 3 of this thesis, immune-suppressive effects of GCs in the contest of human rhinovirus (HRV) infection of bronchial epithelial cells were discussed. The main observation towards unwanted side effects was the inhibition of innate antiviral response related to the use of GCs and consequent increase of viral replication. The hypothesis of GC impairment of type I IFN signalling pathway was formulated. Thus, the modulatory effects of GCs on this pathway comprising specific interactions with JAK/STAT pathway activation were investigated at molecular level.

The experimental model was represented by *in vitro* cultures of BEAS2B cells stimulated with recombinant IFN-β in order to specifically explore the effects of GCs on IFNAR activated signal transduction. Herein, cells were not infected with HRV, which triggers the innate immune response starting from virus binding on the cell surface with subsequent induction of IFNs amplification and release in bronchial epithelial cells, thereby activating IFNAR in a paracrine fashion. Stimulating cells with recombinant IFN-β experimentally recreates the conditions of the triggered antiviral immune response without taking in consideration the activation of other molecular targets such as PRR, including TLRs, RIG-I and MDA5.

To assess the effects of GCs and selective GR agonists or modulators (GRT7, GRT8 and GRT10) on IFN signalling the pathway was activated with recombinant IFN-β and ISG expression measured for viperin and OAS at 8h post stimulation by Taqman qRT-PCR, following 1h pre-treatment. At a concentration of 50 nM, the standard glucocorticoid FP suppressed both viperin and OAS gene expression. Similarly, all selective compounds tested had a comparable effect relative to FP, revealing the involvement of the type I IFN signalling pathway in GC impairment of the innate antiviral response.

To further investigate the effects of GCs on the activated JAK/STAT pathway, different experimental protocols of IFN-β stimulation were designed. At first, a pre-incubation time of 1h followed by 1h treatment and IFN-β stimulation was considered. Cell extracts were collected and immunoblotting assessment performed to specifically detect STAT1 phosphorylation on tyrosine residue Y701 (pSTAT1 Y701), which is indicative of IFNAR signalling pathway activation. FP appeared to have a dose-responsive inhibitory effect on pSTAT1 Y701. This observation suggested a novel mechanism of GC inhibition of the innate antiviral immunity in bronchial epithelial cells. At our knowledge, at this level of

regulation no evidence of GC interference with the JAK/STAT signalling pathway have been reported in literature. A comparative analysis with a set of selective GR agonists or modulators (GRT7, GRT8 and GRT10) displayed a similar trend relative to FP. All compounds clearly inhibited pSTAT1 Y701 starting from a concentration of 10 nM. These data revealed a modulatory effect of ligand-bound GR in the direction of type I IFN signalling pathway inhibition.

Experimental protocols were designed in order to further explore the kinetics of GC inhibition of STAT1 phosphorylation on tyrosine Y701 residue. The treatment time, comprising stimulation with recombinant IFN-β, was reduced to 30 minutes. To define whether GR pre-activation was needed, the pre-treatment time was also reduced to 30 minutes or even to 0 minutes (no pre-incubation). In addition, to investigate the dose-responsiveness towards this effect, lower concentrations of compounds were assessed.

In parallel analyses demonstrated an inhibitory effect of FP on pSTAT1 Y701 at 30 minutes post IFN-β stimulation starting from a concentration of 1 nM, in both short (30 minutes) and even none (0 minutes) pre-incubation conditions. Selective compounds GRT7, GRT8 and GRT10 similarly inhibited pSTAT1 Y701 in both conditions starting from low concentrations (0.1 nM). The observations that pSTAT1 Y701 inhibition occurs in a short (within 30 minutes) period of time and that this effect does not require GR activation prior to IFN-β stimulation revealed the rapidity by which GCs may have a relevant pharmacological effect, thus inhibiting the innate antiviral immune response.

Focusing on the effects of the standard glucocorticoid FP, further investigations were conducted on IFN-β induced pSTAT1 Y701 and on additional key phosphorylations for pathway activation involving STAT1 and STAT2 proteins. A rapid dose-responsive inhibitory effect on pSTAT1 Y701 was clearly visible with or even without pre-treatment, using scalar concentrations of FP. In addition, a rapid strong inhibition on IFN-β stimulated pSTAT2 Y690 was also observed starting from low concentration of FP (0.1 nM). Once again, pre-activation of GR was not required. Differently, STAT1 phosphorylation on serine S727 residue appeared not induced by IFN-β and not affected by FP. Results confirmed GC inhibition of innate immune response involving GC interference with the JAK/STAT pathway at the level of STAT1 and STAT2 phosphorylations, important steps of the signal regulation.

The stimulation of type I IFN signalling by IFN-α/IFN-β binding to IFNAR on the cell surface induces STAT1 and STAT2 recruitment to the receptor and their subsequent activation through JAK1 and TYK2-mediated phosphorylations of STAT1 (pSTAT1 Y701) and STAT2 (pSTAT2 Y690) tyrosine residues. Once activated, STAT1 and STAT2 dimerise and associate with IRF9 to form ISGF3 complex (STAT1-STAT2-IRF9), which

translocates into the nucleus to induce ISG expression. Alternatively, STAT1 can also form STAT1-STAT1 homodimers that activate the transcription of a different subset of ISGs without involving IRF9, through interaction with promoter gamma-activated sequences (GAS) within DNA [148-154]. Type I IFN induced suppressor of cytokine signalling (SOCS) proteins such as SOCS1 and SOCS3 have been described as inhibitors of JAK activity through protein binding and subsequent ubiquitin-mediated proteasomal degradation [180, 181]. In addition, a competition between SOCS proteins and STAT proteins for IFNAR binding has also been reported [182] and glucocorticoid induction of SOCS1 [316] and SOCS3 [317, 318] expression has been proposed. GC suppression of ISGs including STAT1 and IRF9 has also been established [188, 319].

It was herein demonstrated that the standard glucocorticoid FP and the selective compounds GRT7, GRT8 and GRT10 suppressed the expression of ISGs viperin and OAS at 8h post stimulation with recombinant IFN-β. A potential explanation of type I IFN signalling inhibition could be the increased expression of putative IFN-signalling inhibitors such as SOCS1 and SOCS3 by the activated GR. It would also be interestingly to assess if GC treatments could downregulate IFN response by IFNAR internalisation, even if it is unlikely in absence of inflammatory stimuli.

STAT1 Y701 and STAT2 Y690 phosphorylations are crucial cytoplasmic events for STAT1 homo (STAT1-STAT1) or hetero (STAT1-STAT2) dimerisation and nuclear localisation sequences activation, with consequent accumulation into the nucleus [163, 164].

Results reported here from further investigations conducted on the JAK/STAT pathway revealed GC inhibition of pSTAT1 Y701 and pSTAT2 Y690, observations that could be consistent with the hypothesis that increased expression of SOCS proteins may inhibit IFNAR activation. However this is unlikely the driving mechanism by which the glucocorticoid FP or selective compounds GRT7, GRT8 and GRT10 impaired the type I IFN signalling, as the inhibition of pSTAT1 Y701 and pSTAT2 Y690 were detected at 60 and 30 minutes from recombinant IFN-β stimulation and within these short periods of time a genomic effect seems not a plausible explanation. In addition, pSTAT1 Y701 inhibition occurred even without pre-treatment, which means that GR were not activated prior to stimulation with IFN-β, a relevant detail that strongly orientates towards non-genomic effects of GCs that do not require GR interaction with DNA and generally occur rapidly [286, 287]. Thus, GC modulation of type I IFN signalling pathway seems to be mediated by a previous undemonstrated mechanism. In fact, there are evidence of GC inhibition of the innate antiviral immunity [195, 241], but no evidence of GC interference with STAT1 or STAT2 phosphorylations have been described at our knowledge. By contrast, the

impairment of type I IFN signalling was reported to be not dependent from GC interference at the level of STAT proteins phosphorylations [97, 188].

Data reported herein contrast with these findings and are supported by a precaution adopted for all experiments that were conducted, which consists in the constant cell treatment with a standard GC or selective compound pre and/or post stimulation with recombinant IFN-β. This means that GR were activated during all the duration of the experiments and that their rapid activation may result in rapid effects, which not necessarily involve a transcriptional modulation of gene transcription. Non-genomic mechanisms of GCs contribute to their physiological and pharmacological effects [293].

These non genomic effects are thought to be mediated through several mechanisms including classic cytosolic or membrane-bound GR but also non-classic GR activation or physicochemical interactions of GCs with the cell membrane [288, 289].

Non-classic GR, for instance, are G-protein-coupled receptors that act through cAMP and Ca^{2+}-dependent pathways. Although non-classic membrane GR affinity for FP or selective compounds needs to be confirmed, it would be interesting to assess whether they could be involved in GC induced JAK/STAT inhibition. In fact, an additional G-protein coupled receptor not related to GCs have been reported to inhibit IFNAR1 signalling pathway, in particular by inhibition of pSTAT1 Y701 [320]. Otherwise, classic membrane GR may be involved. Bovine serum albumin (BSA)-conjugated steroids, with lost cell membrane penetration ability due to BSA conjugation but retained GR affinity [290], could be used to assess classic membrane GR involvement in IFNAR signalling inhibition.

To determine whether GC inhibition of IFN signalling was a cytoplasmic or nuclear event is another good question. The speculation that pSTAT1 Y701 and pSTAT2 Y690 inhibition does not require GR interaction with DNA could suggest that this was exclusively a cytoplasmic event due to GC inhibition of one of the two IFNAR associated protein kinases JAK1 or TYK2. Although STAT1 and STAT2 phosphorylations occur in the cytoplasmic compartment, the hypothesis that GC inhibition of the JAK/STAT pathway may be subsequent STAT1 and STAT2 nuclear translocation could not be excluded.

It has been demonstrated that promoter occupancy of pSTAT1 Y701 enables the transcriptional machinery to initiate the expression of ISGs, whereas STAT1 Y701 de-phosphorylation causes STAT1 loss of DNA-binding ability and its relocation to the cytoplasm, thereby inactivating STAT1 in both ISGF3 complex and STAT1 homodimer. The dissociation of STAT1 from the promoter represents the regulatory step for transcriptional cessation, followed by STAT1 de-phosphorylation. The regulatory process is similar for STAT2 [167]. TC45 is an identified protein tyrosine phosphatase (PTP)

responsible for the de-phosphorylation of STAT1 in the nucleus [168]. SHP2 has been described as an additional nuclear phosphatase involved in STAT1 de-phosphorylation at both Y701 and S727 residues [169].

The observation that GCs inhibit pSTAT1 Y701 and pSTAT2 Y690 in 30-60 minutes could be interpreted as a lack of transcriptional complex association at the promoter level and a rapid initiation of STAT1 and STAT2 de-phosphorylation.

However, IRF9 is the major protein of the complex involved in DNA binding and STAT2 is not able to directly bind the DNA and its participation in transcriptional regulation through the recruitment of coactivators such as p300/CBP with a histone acetyltransferase (HAT) activity has been described [155,156]. The depletion of the coactivator GRIP1, which associates with ISGF3 complex to induce expression of at least a subset of ISGs has been described as a glucocorticoid mechanism of type I interferon signalling pathway inhibition [188].

An interpretation of the observed GC inhibition of pSTAT1 Y701 and pSTAT2 Y690 may be that glucocorticoids interact with the coactivator GRIP1, thus interfering with promoter-bound ISGF3 activity and reducing induction of ISG expression. STAT2 reduced interaction with GRIP1 may induce a rapid dissociation of ISGF3 complex from the promoter, thereby inducing STAT1 and STAT2 de-phosphorylations by action of phosphatases. Moreover, GR may enhance phosphatase activity by direct interaction.

In literature it has also been reported that STAT1 phosphorylation at Y701 residue, its nuclear translocation and DNA binding are necessary for IFN-induced STAT1 S727 additional phosphorylation within the COOH-terminal amino acidic sequence, which is needed for STAT1 full transcriptional activity and biological function. [157-159]. IFN-β induced pSTAT1 S727 seems to occur after pSTAT1 Y701 with a delay of around 15 minutes and pSTAT1 S727 also seems to have a role in the disassembly of the transcriptional complex once the transcription has started [157]. Protein kinase CDK8 [170, 321] and PKC-δ [171] have been described as responsible for STAT1 phosphorylation at S727 residue.

Differently, in the model used here, a basal level of pSTAT1 S727 in unstimulated cells and further no-increase following IFN-β stimulation were observed. Furthermore, no inhibition by FP was noticed. The basal phosphorylation of STAT1 at S727 may indicate a basal nuclear localisation of STAT1 and, as pSTAT1 S727 was unchanged following pathway activation, STAT1 phosphorylation at S727 residue may be not strictly necessary for STAT1 transcriptional activity. This would be in line with a reported evidence that STAT1β, lacking the C-terminal portion, suffices for transcriptional activation or that

S727A mutation retains the ability to form functional ISGF3 in cells [322]. Moreover, pSTAT1 S727 could be differently regulated in bronchial epithelial cells than by simple activation of the pathway by type I IFNs.

4.6 Summary

As demonstrated in chapter 3, glucocorticoids (GCs) such as the standard fluticasone propionate (FP) or selective compounds (GRT7, GRT8 and GRT10) inhibited the innate antiviral immune response in RV-infected bronchial epithelial cells (BEAS2B). To further investigate the effects of these compounds on the type I IFN signalling pathway, uninfected cells were stimulated with recombinant IFN-β. FP, similarly to GRT7, GRT8 and GRT10 suppressed the expression of the ISGs viperin and OAS by qRT-PCR measurements, revealing the impairment of the activated pathway.

Going through the mechanism involved in GC inhibition of the innate antiviral immunity, specific phosphorylations on proteins STAT1 (pSTAT1 Y701 and pSTAT1 S727) and STAT2 (pSTAT2 Y690) were investigated and western blotting analyses carried out in order to explore the effects on crucial events of the JAK/STAT pathway activation at a molecular level. Data clearly showed a dose-dependent inhibition of pSTAT1 Y701 by FP and also a strong inhibition of pSTAT2 Y690, whereas no effect was observed for pSTAT1 S727. GRT7, GRT8 and GRT10 also inhibited pSTAT1 Y701. All together results demonstrated that GCs, including selective compounds, interfere with the JAK/STAT pathway through rapid events that occur in 30-60 minutes, thus suggesting this may due to non-genomic effects that do not require GR interaction with DNA.

Chapter 5: Results – Effect of ICS/LABA combination on innate antiviral response

5.1 Introduction

Inhaled corticosteroids (ICS), alone or in combination with long acting β2-agonists (LABA) represent the mainstay of asthma management. ICS/LABA therapy is effective in a large proportion of asthmatic patients [76, 77]. However, around 10% of them with a clinical picture of severe asthma are steroid-resistant and do not respond to ICS treatments even at high doses or with the use of oral glucocorticoids [22, 96]. The same GC-based therapy, including ICS/LABA combination, is common in COPD as an exacerbation preventive strategy [35, 95], although clinical trials have reported little improvement in lung function or symptoms in a modest 20-25% of COPD subjects [235]. Thus, the efficacy of ICS in COPD inflammation control is a controversial question. Clinical trials have reported that airway sputum inflammatory cells are reduced with prolonged use of ICS. By contrast, short period of treatment with GCs did not demonstrate beneficial effects in COPD [30].

The anti-inflammatory action of corticosteroids and the bronchodilator action of β2-agonists are the therapeutic effects linked to their pharmacological use in obstructive lung diseases such as asthma and COPD. Inhaled formulations of these two classes of drugs are also administered as ICS/LABA combinations, including fluticasone propionate (FP), budesonide (Bud) or beclometason with formoterol (Form) or salmeterol [75] and the more recent once-daily inhaled medication fluticasone furoate with vilanterol [91]. The beneficial interaction of ICS/LABA in reducing inflammation and reversing bronchoconstriction is based on GC ability to increase the expression of cell surface β2-adrenergic receptors (β2ARs), which are G protein-coupled receptors (GPCRs), thereby protecting against receptor down-regulation after long-term administration [235]. Additionally, Form increases GC-activated GR translocation from the cytoplasmic compartment into the nucleus [273]. GCs can also increase β2AR coupling [272]. Hence, ICS/LABA combinations have a greater efficacy compared to increased doses of ICS. It has been reported that Bud/Form combinatorial therapy suppresses eosinophilic inflammation and alleviates airway hyperresponsiveness, while Form additionally inhibits other important cells of acquired immune response such as neutrophils and mast cells, which contribute to disease symptoms [270].

However, the beneficial effects of GCs represent only one side of the coin. Unfortunately, the use of GCs is associated with several long-term adverse effects, including diabetes, muscle wasting, osteoporosis and HPA-axis suppression [239, 240]. An emerging adverse

effect associated with the use of ICS is the increase of respiratory infections [241, 242]. Experimental evidence reported the impairment of the antiviral immune response, which also determines the reduced host ability to efficaciously clear the virus [79, 97]. It is because of these side effects that GC safety in clinical practice is widely debated.

5.2 Hypothesis

6. Do standard GCs and β2-adrenergic agonists suppress the innate antiviral response to RV infections?
7. Do the combination ICS/LABA suppress the innate antiviral response to RV infections?

5.3 Aims

The experimental model for this set of aims consists in *in vitro* cultures of bronchial epithelial cells (BEAS2B) stimulated with rhinovirus 1B (RV-1B).

3. To investigate the effects of Formoterol (Form), a long acting β2 adrenergic agonist (LABA), and budesonide (Bud), an inhaled corticosteroid (ICS), or dexamethasone (Dex), a systemic steroid, on ISG expression and RV replication.
4. To define the effects of Bud/Form (ICS/LABA) combination on ISG expression.

5.4 Results

5.4.1 Experimental conditions

To experimentally recreate the conditions of respiratory virus-induced asthma or COPD exacerbations in a GC-based therapeutic regimen, BEAS2B cells were pre-treated with drug for 1h and subsequently infected with HRV-1B at an MOI of 1 for a further 1h to allow virus adhesion before continued treatment with drugs for 24h (Fig. 5.1).

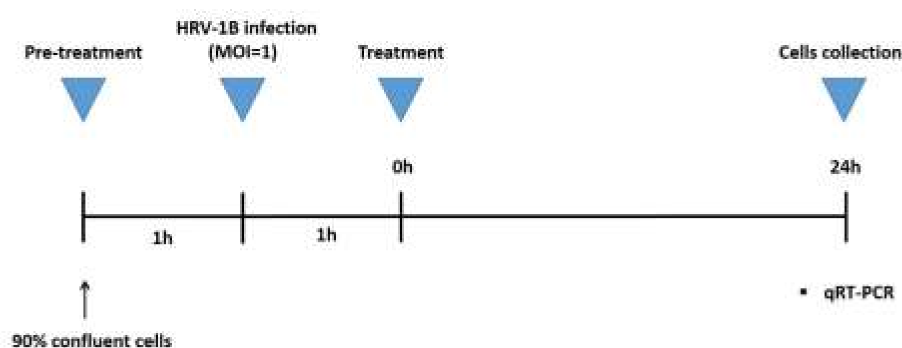


Figure 5.1 BEAS2B cells infection with HRV-1B

BEAS2B cells were pre-treated with drug for 1h before infection with HRV-1B for a further 1h. After washing of unbound virus and media replacement, cells were treated for a further 24h with drug. Then, cells were harvested for SYBRGreen qRT-PCR analyses.

5.4.2 GC and β2-adrenergic agonist effects on ISG expression and viral replication

The major aim of this thesis is to investigate the effects of GCs on the innate immune response to RV infections. In order to add experimental evidence of the immune-suppressive effects of GCs described in chapter 3 of this thesis, further experiments using standard steroids such as budesonide (Bud) and dexamethasone (Dex) were carried out. In addition, the effects of formoterol (Form), a β2 adrenergic agonist clinically administered as inhaled bronchodilator were also assessed. The experimental steps of RV infection and treatments in BEAS2B cells are schematised in figure 5.1. Infected cells were treated with increasing concentrations of compounds to measure expression of ISGs such as viperin and OAS or RV genome replication (Fig. 5.2/a-c). Dose-response curves were produced and data presented as fold induction compared to a DMSO vehicle control, following analysis by SYBRGreen qRT-PCR. Untreated cells and cells infected with UV-inactivated virus (UV-RV) are both negative controls.

The standard steroid Bud showed a suppressive effect on viperin (92%) gene expression at a concentration of 1 nM (Fig. 5.2/a). Similarly, Dex suppressed viperin (75%) at 1nM and even at a greater magnitude (95%) at 10 nM. To a lesser extent, the β2 adrenergic agonist Form suppressed viperin (60%) at 10 nM concentration, with a higher effect (76%) at 250 nM.

At 1 nM Bud, suppressed OAS (82%) and also Dex suppressed OAS (45%) gene expression (Fig. 5.2/b). Once again, the effect of Dex was higher (86%) at 10 nM. To a weaker magnitude, Form suppressed OAS (45%) at 10 nM, up to 67% at 1μM. Consistently, these effects on ISGs expression had a counterbalanced response on RV replication, at least for GCs (Fig. 5.2/c). In fact, while Form had no effect on HRV-1B RNA levels, an increase of RV genome was detected with Bud (1.5-fold) at 10 nM and Dex (1.9-fold) at 100 nM.

In summary, results clearly demonstrated a trend of GCs to suppress RV-induced viperin and OAS gene expression and thus to inhibit the innate immune response to viral infections in a concentration-dependent manner. To a lesser extent, the same trend was observed with the β2 adrenergic agonist Form. However, only with GCs was observed a dose-dependent increase in RV replication.

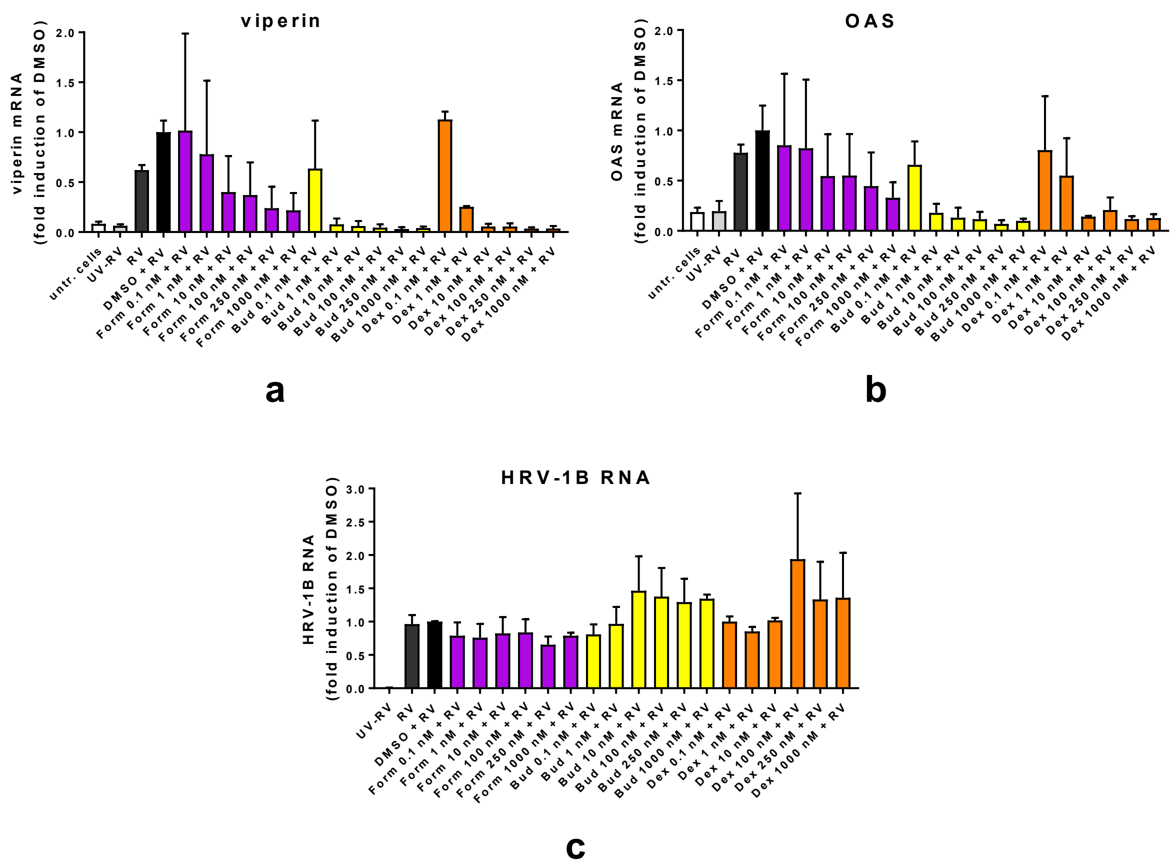


Figure 5.2 GC and β2-adrenergic agonist suppression of the innate antiviral response to HRV-1B

BEAS2B cells were treated with increasing concentrations of formoterol (Form), a long-acting β2-adrenergic agonist (LABA), or with the glucocorticoids budesonide (Bud) and dexamethasone (Dex), following 1h pre-treatment and 1h infection with HRV-1B at an MOI of 1. Cells were harvested at 24h post infection and **(a)** viperin and **(b)** OAS gene expression and **(c)** HRV-1B RNA replication assessed by SYBRGreen qRT-PCR. Data were normalised to GAPDH housekeeping gene and represented as fold induction of DMSO. RV alone is a positive control and DMSO + RV is an additional control that include the effect of the vehicle. UV-RV is the replication-deficient virus, killed by UV-light irradiation exposure and is a negative control such as untreated cells. Data represent mean (±SEM) of two independent experiments performed in duplicate. HRV-1B, human rhinovirus 1B.

5.4.3 Effects of ICS/LABA combination on ISG expression

As the expression of antiviral genes was suppressed by the two standard GCs Bud and Dex and, to a lesser extent, by the long acting β2 adrenergic agonist (LABA) Form, a combination of the inhaled corticosteroid (ICS) Bud with Form was investigated. The aim was to define whether this suppressive effect on the innate antiviral immunity may have clinical implications in asthma and COPD treatment. The experimental design in BEAS2B cells refers to the conditions of RV infection and treatments reported in figure 5.1. Untreated cells and cells infected with UV-inactivated rhinovirus (UV-RV) are both

negative controls, while RV stimulation and RV + DMSO represent positive controls, the latter including the vehicle. Additional controls of tested compounds effect in absence of RV infection were also added. To the set of experiments herein, relative low concentrations of Form and Bud were selected from results reported in figure 5.2 and further investigation towards their suppressive effects on targeted ISGs viperin and OAS carried out (Fig. 5.3/a-b). Data were analysed by SYBRGreen qRT-PCR and presented as fold induction of DMSO.

First of all the suppressive effect of Form at 10 nM (80%) and Bud at 0.1 nM (75%) and 0.5 nM (95%) on RV-induced viperin gene expression was confirmed (Fig. 5.3/a). The combinations of Form 10 nM with Bud 0.1 (Form 10 nM / Bud 0.1 nM) or 0.5 nM (Form 10 nM / Bud 0.5 nM) were also tested. Both combinations completely suppressed viperin of the same magnitude (97%) relative to DMSO control. Moreover, (Form 10 nM / Bud 0.1 nM) had a potentiated effect relative to both Form 10 nM and Bud 0.1 nM alone. By contrast, the effect of (Form 10 nM / Bud 0.5 nM) was higher relative to Form 10 nM but equal to Bud 0.5, suggesting that the suppression of viperin in RV-infected BEAS2B cells was due to the effect of the glucocorticoid budesonide and not to its combination with Form at these concentrations.

In parallel, the suppressive effect of Form at 10 nM (53%) and budesonide at 0.1 nM (56%) and 0.5 nM (85%) on RV-induced OAS gene expression was also confirmed (Fig. 5.3/b). In addition, OAS was significantly suppressed by both (Form 10 nM / Bud 0.1 nM) and (Form 10 nM / Bud 0.5 nM) combinations relative to DMSO control by an extent of 89% and 87%, respectively. Moreover, (Form 10 nM / Bud 0.1 nM) had a potentiated effect relative to both Form 10 nM and Bud 0.1 nM alone. The combination (Form 10 nM / Bud 0.5 nM) also had a higher suppressive effect on OAS expression relative to Form 10 nM but an equal effect relative to Bud 0.5 nM alone. Once again, data suggest that at these concentrations the effect of Bud, and not its combination with Form, is responsible for the enhanced suppression of ISG expression, such as OAS, in RV-infected BEAS2B cells.

In summary, both Form and Bud had a suppressive effect on ISG expression in RV-infected BEAS2B cells, with Bud showing an increased effect at 0.5 nM relative to 0.1 nM. (Form 10 nM / Bud 0.1 nM) and (Form 10 nM / Bud 0.5 nM) combinations of the ICS Bud and the β2 adrenergic agonist Form also suppressed ISG expression, suggesting a key role of GCs. In addition, (Form 10 nM / Bud 0.1 nM) had an increased effect relative to Bud 0.1 alone, suggesting a potentiated suppressive action of Bud in combination with Form on viperin and OAS gene expression. Differently, with a little increase of Bud concentration (Form 10 nM / Bud 0.5 nM) no difference was observed relative to Bud 0.5

alone, giving evidence of the prevalent effect of Bud on viperin and OAS gene expression relative to Form. Thus, ICS/LABA combinations seem to have a suppressive and also potentiated effect on the innate antiviral immune response. These observations may have important implications in asthma and COPD treatment.

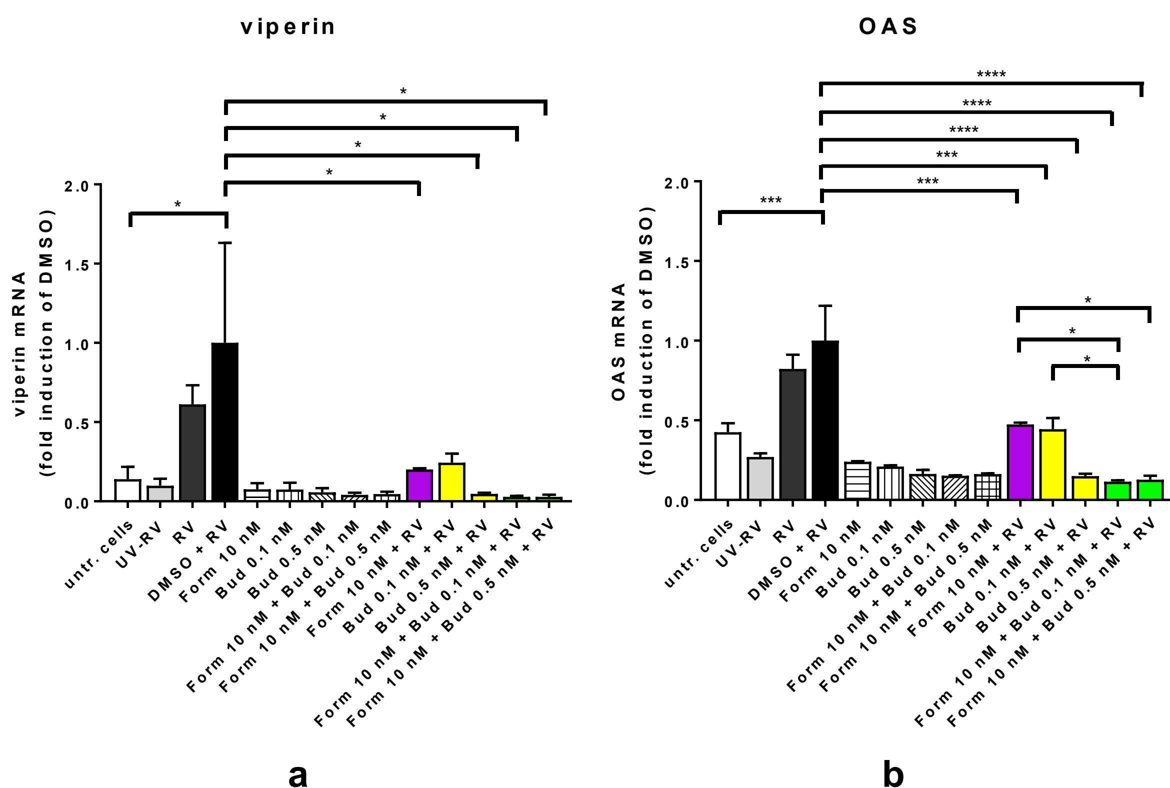


Figure 5.3 Potentiated suppression of ICS/LABA combination on the innate antiviral response to RV

BEAS2B cells were pre-treated for 1h with formoterol (Form) at 10 nM and with the glucocorticoid budesonide (Bud) at 0.1 and 0.5 nM, or with a combination of Form plus Bud, and infected with HRV1B at an MOI of 1 for 1h. Subsequently, cells were treated for 24h. **(a)** viperin and **(b)** OAS gene expression was measured by SYBRGreen qRT-PCR analysis, using GAPDH for normalisation. Data are represented as fold induction of DMSO. RV alone is a positive control and DMSO + RV is an additional control of DMSO effect. UV-RV is the replication-deficient virus, killed by UV-light irradiation exposure and is a negative control, such as untreated cells. Data represent mean (±SEM) of two independent experiments performed in duplicate and analysed by one-way ANOVA with Dunnett's multiple comparison test. * $p<0.05$, *** $p<0.001$, **** $p<0.0001$. ICS, inhaled corticosteroid; LABA, long acting β2 adrenergic agonist; HRV1B, human rhinovirus 1B.

5.5 Discussion

In chapter 3 of this thesis the effects of the standard glucocorticoid fluticasone propionate (FP) and selective GR ligands the innate immune response against the respiratory virus HRV-1B were investigated at the molecular level in bronchial epithelial cells (BEAS2B).

Glucocorticoid (GC) suppression of IL-29/IFNλ1 release and ISG expression was shown. In particular, important antiviral agents of the immune system such as viperin and OAS were assessed. The result of the impaired host defence was the increase of the viral replication. RV infections are the primary cause of asthma and COPD exacerbations [78]

Inhaled corticosteroids (ICS) are the most effective treatments to control inflammation in asthma [237, 238] and are also widely used to prevent exacerbations in COPD [235]. Emerging evidence of GC inhibition of the innate immune response has been reported [241, 242]. The unwanted side effects are the major limiting factors that concern the scientific community about the long-term use of GCs, which is justified by their important therapeutic action.

To widely investigate the effects of GC therapy on the innate antiviral response, budesonide (Bud) and dexamethasone (Dex), respectively administered as inhaled or systemic corticosteroid in clinical practice, were also investigated. The experimental model of asthma or COPD exacerbation used here was the same described in chapter 3 of this thesis: BEAS2B cells infected with human rhinovirus 1B. The pharmacological approach in asthma [323, 324] and COPD [325, 326] includes ICS administration, alone or in combination with long acting β2 adrenergic agonists (LABA), an additional class of drugs used to reverse bronchoconstriction. Thus, the effect of Formoterol (Form) was also tested.

Treatments with low doses of both Bud and Dex showed a strong dose-responsive suppression of viperin and OAS gene expression. The consequent reduced level of these two antiviral ISGs resulted in an increase of HRV-1B RNA replication, quantified by SYBRGreen qRT-PCR. To a lesser extent, Form also suppressed viperin and OAS but viral replication was not affected. Then, to assess the effect of ICS/LABA combination, additional experiments were carried out using selected concentrations of the glucocorticoid Bud and the β2 agonist Form. The suppression of viperin and OAS gene expression was confirmed by cell treatment with Bud or Form alone, while no effect was observed on HRV replication at these low concentrations of drugs (data not shown). The combinations of formoterol 10 nM with budesonide 0.1 nM (Form 10 nM / Bud 0.1 nM) or 0.5 nM (Form 10 nM / Bud 0.5 nM) had an additive effect on viperin and OAS suppression in comparison with Form alone without distinction between the two. However, the concentration of the glucocorticoid (0.1 or 0.5 nM) was determinant. In fact, while the suppressive effect was increased with both (Form 10 nM / Bud 0.1) nM and Form (10 nM / Bud 0.5 nM) combinations relative to Form alone, only (Form 10 nM / Bud 0.1) had a potentiated effect relative to Bud 0.1 nM alone. By contrast, the effect of (Form 10 nM / Bud 0.5) nM was unchanged relative to Bud 0.5 nM alone, suggesting that a slightly

increased concentration of Bud in the Bud/Form combination suffices to determine viperin and OAS increased suppression, independently from its combination with Form. This latter observation also suggests that is unlikely that increased suppression of ISGs by (Form 10 nM / Bud 0.1 nM) combination relative to Bud 0.1 nM alone was caused by the effect of Form.

Several studies on ICS/LABA combinatorial therapy have been conducted. Davies and colleagues demonstrated that in TLR7-activated peripheral blood mononuclear cells (PBMC) from healthy donors, Bud alone reduced the production of the type I IFN-induced ISG IP-10 at concentrations of 100 nM. Form also reduced IP-10 at 1000 nM. The effect of the combination Bud/Form was also investigated at reported pharmacologically relevant concentrations of 10 nM each. A reduced production of IP-10 was observed only with the combination of the two drugs but not when tested individually. In RV-16 infected cells from healthy donors the production of IP-10 was reduced by Bud at a concentration of 10 nM. A further reduction was observed by addition of 10 nM Form. In cells from asthmatics, the effect was complete with both Bud alone or Bud/Form combination. However, with a reduced concentration of Bud to 1 nM, the addition of Form 10 nM showed a further inhibition of IP-10 synthesis, while Form alone had no effect at 10 nM. A complete suppression of OAS and MxA, two important antiviral agents downstream of type I IFN, by the Bud/Form combination was also observed. In addition, RV-16 induced production of IFN-α in cells from both healthy and asthmatic donors was abolished by Bud alone and also in combination with Form, but only modestly reduced with Form alone [327]. Thus, Bud and Form reduced early innate antiviral immune response *in vitro*, also providing evidence of a potentiated effect by the combination Bud/Form. However, whether this has implications in viral clearance *in vivo* remains to be determined. Other evidence of ICS and LABA suppression of the type I IFN response have been reported. Kuo and colleagues demonstrated that in TLR-activated plasmacytoid dendritic cells (pDCs) Bud and Form alone suppressed IFN-α and IFN-β at 1, 10 and 100 nM concentrations. The addition of Form at 1 and 10 nM further enhanced this suppressive effect of Bud at 10 nM concentration. The involvement of the β2-adrenoreceptor-cAMP-Epac-Ca^{2+} pathway on the suppressive effect of Form was proposed. Authors observed this effect of Form was also partly mediated via inhibition of the MAPK-p38/ERK and IRF-3/IRF-7 pathways. Additionally, they reported Form inhibition of WDR5 translocation from the cytoplasm to the nucleus. WDR5 is a metyltransferase specifically directed to the H3K4 site within the promoter of IFN-α and IFN-β, which event is important for gene expression [328]. Edwards and colleagues reported that combination therapy ICS/LABA suppressed RV-induced chemokines in bronchial epithelial cells (BEAS2B) in a synergistic and additive way and

that this effect was greater for lymphocyte- (CCL5, CXCL10/IP-10) than for neutrophil- (CXCL5, CXCL8) related chemokines [329].

Results were herein obtained by assessing the effects of ICS and LABA on HRV-1B activated type I IFN response in *in vitro* cultured BEAS2B cells. Experiments were carried out in order to provide further evidence of GC impairment of the innate antiviral immune response reported in chapters 3 and 4 of this thesis and additionally to assess GC effects in ICS/LABA combinations, which is of relevant clinical interest. Although no investigations at mechanistically level were conducted, Barnes PJ reported that ICS and LABA have a reciprocal interaction, hence glucocorticoids increase the expression of β2-adrenergic receptors (β2ARs) whereas β2 adrenergic agonists enhance GR nuclear translocation and GRE binding [235].

Transferring our and other experimental observations to real life and to the use of ICS/LABA therapy in asthma and COPD treatment, an important consideration arises from the interpretation of results. In fact, the immune-suppressive effect of corticosteroids and the potentiated effect that ICS/LABA combination may have on the innate antiviral response suggest potential clinical implications. In fact, a lack of host efficacy to clear the virus may have detrimental consequences and cause disease exacerbations. However, as the beneficial effects of this therapy in terms of reduced inflammation and reversed bronchoconstriction are well documented, ICS/LABA withdrawal would probably not be the best solution. What would be relevant for physicians in evaluating the therapeutic regimen and in prescribing drugs as an inhaled ICS/LABA formulation was to be conscious of the real or potential risk/benefit balance and to consider a reduction of GC doses. Other authors concluded that inhaled glucocorticoids could be reduced when combined with β2 agonists, minimising the side-effects of the drugs [273]. In fact, beneficial effects may be obtained with low doses of GCs, while a slight increase of drug concentration may result in increased detrimental effects and enhanced risk of exacerbations, triggered by respiratory viral infections such as rhinovirus in particular.

5.6 Summary

In order to investigate the effect of inhaled corticosteroids (ICS), clinically administered alone or in combination with LABA, on the innate antiviral immune response, BEAS2B cells were treated with the glucocorticoid (GC) budesonide (Bud) and the β2 adrenergic agonist formoterol (Form) or a combination of Bud/Form following HRV-1B infection. Additionally, the effect of dexamethasone (Dex), clinically administered as systemic corticosteroid, was assessed.

The viral infection induced the activation of viperin and OAS gene expression, whereas treatments with the glucocorticoids Bud or Dex suppressed these ISGs, with the consequent increase of viral replication. To a lesser extent, the β2 adrenergic agonist Form suppressed both viperin and OAS, but had no effect on HRV-1B RNA. Furthermore, the co-treatment with Bud/Form had an additional effect on viperin and OAS gene suppression compared to Form alone. Importantly, Bud at the lower concentration tested in association with Form had a potentiated effect compared to both Bud and Form alone, whereas a slightly increased concentration of Bud in the combination Bud/Form had no differential effect compared to Bud alone, independently from Form. Taken together, data questioned the use of higher doses of GCs in the ICS/LABA combinatorial therapy.

Chapter 6: Discussion and future work

6.1 Contextualisation and discussion

Glucocorticoids (GCs) are the most effective anti-inflammatory and immune-suppressive drugs currently available for the treatment of many autoimmune and inflammatory disorders including chronic obstructive lung diseases such as asthma [234, 238, 282] and COPD [88, 90]. Viral infections are the main aetiological causes of acute worsening of respiratory symptoms, a condition known as exacerbation, which results in increased airway inflammation and reduced lung function in both adults and children [40-42, 48-50]. These severe episodes are a major cause of hospitalisation and increased healthcare costs at global level, also associated with high mortality and morbidity [2, 34, 38, 39]. Rhinovirus (RV), the virus of the common cold, is responsible for respiratory tract infections that are usually self-limited, but in asthmatics and COPD patients can trigger disease exacerbations [43-45, 78, 82]. Rhinovirus is the most frequently detected virus during hospital admissions for asthma and CPOD exacerbations [46, 47, 81]. Although Rhinovirus was discovered in the 1950s, no anti-RV treatment nor vaccine is currently available. Around 170 RV-serotypes have been identified so far and the high mutability of the virus explicates the lack of an approved prevention strategy [99, 107].

Asthma and COPD are non-curable diseases, hence the pharmacological approaches are pointed towards symptoms control, thus increasing patient quality of life by reducing airways inflammation or improving shortness of breath [1, 4]. GCs are clinically administered as inhaled corticosteroids (ICS) alone or in combination with bronchodilators, long acting β2 receptor adrenergic agonists (LABA). This is the mainstay of asthma therapy [22, 235, 237, 238, 330] and a maintenance treatment to prevent exacerbations in COPD [34, 35, 95]. Although ICS/LABA combination is an effective therapy [76, 77, 270], the use of ICS alone missed the exacerbation preventive effect in almost half of asthmatic patients in clinical trials [78]. Furthermore, in around 10% of cases, associated with severe asthma and a steroid-resistant profile, ICS are not effective even at high doses or with the use of oral glucocorticoids [22, 96]. In COPD, the efficacy of GCs to control inflammation is controversial [35] as clinical trials reported GCs improve lung function or symptoms by only a 20-25% frequency. However, although this intersubjective variability, GC efficacy has been established and is well documented in asthma, whereas despite low therapeutic adherence, GCs are also widely prescribed in COPD to prevent exacerbations [235]. The use of ICS and ICS/LABA in asthma and COPD was discussed in chapter 5.

The dark side related to the use of GCs as anti-inflammatory drugs is the onset of several long-term adverse effects, including suppression of HPA-axis, reduction in growth velocity, osteoporosis, diabetes [239, 240] and pneumonia [244]. An emerging adverse effect of GCs is the increase of respiratory infections [241, 242]. Thus, there are speculations towards safety of GCs in asthma and COPD management [243]. Glucocorticoid impairment of the host innate antiviral immune response, associated with a potential reduced host ability to efficaciously clear the virus, is a relevant matter of debate [79, 97]. To investigate towards this argument is the principal aim of this thesis.

The classical mechanism of glucocorticoids involves glucocorticoid receptor (GR) activation in the cytoplasmic compartment and subsequent modulation of gene expression through GR translocation into the nucleus. The therapeutic effect of GCs is due to a dual action, the repression of many activated pro-inflammatory genes (transrepression) and the activation of other anti-inflammatory genes (transactivation). Despite this, it is generally accepted that low doses of GCs activate the DNA-independent transrepression pathway, which mainly accounts for the clinical efficacy, whilst high doses of GCs are needed to activate the DNA-dependent transactivation pathway, responsible for both therapeutic and adverse effects [234, 235, 264]. For these reasons, there have been significant efforts to separate the therapeutic action of GCs from their unwanted side effects and thus favouring transrepression over transactivation [294, 297]. The identification of safer drugs with the most favourable functional profile is an ambitious goal of relevant scientific interest. A great number of selective GR agonists (SEGRAs) with a steroidal structure or selective GR modulators (SEGRMs) with a non-steroidal structure have been developed [289] and some compounds are in clinical trials as inhaled formulations for asthma treatment [302, 303, 304]. In general, selective compounds are excellent research tools to better understand how GR can be differentially modulate by different ligands [298]. Thus, there is a need for further research. A comparison between a set of selective GR ligands, in particular two SEGRMs (GRT7 and GRT8) and one SEGRA (GRT10), and the standard glucocorticoid fluticasone propionate (FP) on the modulation of pro-inflammatory and innate antiviral immune responses was carried out in this thesis, as discussed in chapter 3 and 4.

6.1.1 Anti-inflammatory effect of GCs and selective GR ligands

Several inflammatory cell types have been detected in the respiratory tract of both asthma and COPD subjects, which include eosinophils, T-lymphocytes, mast cells, dendritic cells and macrophages. A complex network of chemotactic mediators orchestrates an effective link between innate and adaptive immune responses, leading to the recruitment and activation of these inflammatory cells, which drive the chronic inflammatory state of the

airways. GCs suppress cell production of cytokines, chemokines and adhesion molecules, thereby reducing inflammation in the lungs [133-135, 235]. A correlation between eosinophilic asthma phenotype and GC responsiveness has been established. Eosinophilic inflammation has also been associated with an increased risk of RV-induced exacerbations [20, 70, 71]. Conversely, in COPD subjects the prevalence of neutrophils in the airways has been related to the typical resistance to GC therapy [21].

Wide investigations of GCs ability/failure to suppress inflammation in chronic obstructive pulmonary diseases led to a better understanding of their underlying mechanisms of action. Inflammatory stimuli, including viral infections, induce the activation of transcription factors NF-kB and AP-1, which translocate into the nucleus and recruit coactivators such as CBP, pCAF and SRC family members to promote the expression of multiple genes including pro-inflammatory cytokines IL-6 and IL-8. The coactivators have an intrinsic histone acetyltransferase (HAT) activity, crucial for acetylation of core histones and recruitment of chromatin remodelling factors such as SWI/SNF, with subsequent DNA association of RNA polymerase II and activation of transcription. The activated GC-bound GR translocates from the cytoplasm to the nucleus and directly interacts with coactivators of NF-kB and AP-1 inflammatory gene complex, thereby inhibiting their HAT activity. In addition, GR recruitment of histone deacetylase-2 (HDAC2) induces chromatin remodelling in a closed conformation, thus inhibiting the transcription of inflammatory genes. This is the transrepression mechanism of GCs [235, 265, 276].

The theory towards the development of selective or differential compounds is to identify promising molecules that retain the anti-inflammatory activity of standard GCs but with reduced side effects. This may be possible because of the structural malleability of GR within its ligand-binding domain (LBD). Crystal structure analyses of GR revealed that the endogenous ligand cortisol specifically bind GR but fails to fill the binding pocket. The exogenous dexamethasone similarly occupies only 65% of the GR LBD. Further explorations of GR showed the potentiality of alternative modulatory ligands to bind different areas and confer different allosteric changes, thus altering GR signalling and gene transcription regulation. Both steroidal and non-steroidal ligands can bind GR [279, 305]. For instance, starting from the standard glucocorticoid fluticasone propionate (FP) and maintaining the steroidal scaffold, the substitution of the propionate ester at the 17α position with a 2-furoate ester gave the compound fluticasone furoate (FF) [306]. This compound occupies a lipophilic portion of GR called 17α pocket much more completely than FP. Interestingly, the same 17α pocket is unoccupied by dexamethasone, which has a 17α hydroxyl group. FF also showed a 60% higher affinity for GR and an enhanced GC activity compared to FP. The clinical advantage of FF as an inhaled formulation is its

potential administration at lower doses or as once-daily treatment [309]. FF is currently approved as an inhaled formulation for asthma and COPD treatments [91]. Further drug development, oriented to explore the effects of 17α substituents on GR pharmacology, led to the identification of compound GRT10, a novel 17α tetramethylcyclopropyl ester, which transrepression arm antagonises endoplasmic reticulum (ER) stress-induced upregulation of growth differentiation factor 15 (GDF15) and consequent GDF15-mediated apoptosis, thereby demonstrating the potential of selective GC therapy in treating neurodegenerative diseases [310]. GRT10, also reported in literature as compound 5, showed a transrepression (81%) versus transactivation (15%) selectivity, differently from transrepression (102%) and transactivation (120%) values for FP, quantitative measured by reporter gene assays, compared to dexamethasone. Replacement of the β fluoromethyl thioester in 17β with a cyanomethyl carboxylate group gave the additional compound GW870086, with a transrepression (68%) over transactivation (2%) selectivity, compared to dexamethasone. GW870086 also has little activity at other steroid hormone receptors and has a comparable efficacy to FP. GW870086 is in clinical trials as an inhaled formulation for asthma treatment [306]. In recent years, novel selective non-steroidal GR ligands were identified. Biggadike and colleagues described aryl-indazole compounds GRT7 and GRT8 (referred in literature as compounds 11 and 12, respectively) occupying a previously unexplored *meta* channel of GR. These compounds force the GR LBD to open in the opposite direction to the 17α pocket. GRT7 has a D-prolinamide moiety linked to an aryl-indazole scaffold and is a potent agonist with full transrepression (107%) and transactivation (132%) activity, compared to dexamethasone. GRT7 is selective for GR, with reduced or irrelevant selectivity to other steroid receptors and is an ideal compound for development as an intranasal or inhaled formulation. Further manipulation of GRT7 to optimize interactions within GR channel gave the compound GRT8, which has a (3S)-2-pyrolidinone amide linked to an aril indazole scaffold. GRT8 is less potent than GRT7 but has greater transrepression (79%) over transactivation (32%) selectivity, compared to dexamethasone. Thus, GRT8 has a very different pharmacological profile [311].

In a future perspective, though a net separation between anti-inflammatory and side effects of GCs is likely a utopia, the development of new selective compounds that differentially modulate GR may bring to improve the GC therapy and optimistically overcome the GC-resistance. Moreover, systemic absorption of inhaled corticosteroids may have detrimental effects. Thus, there has been a search for safer steroids. In addition, studying the effects of novel selective compounds, with both steroidal and non-steroidal structure, is greatly helpful to better understanding the modulatory mechanism of GR transcriptional activity.

In this thesis, the experimental model of asthma and COPD exacerbations consists in *in vitro* cultures of bronchial epithelial cells (BEAS2B) infected with human rhinovirus 1B (HRV-1B). Cells were treated with increasing concentrations of FP and a set of novel selective compounds (GRT7, GRT8 and GRT10) to measure their anti-inflammatory activity. The RV-induced release of pro-inflammatory cytokines in the supernatants was assess. As expected, low doses of the standard glucocorticoid FP suppressed IL-6 and IL-8 release, consistently with its use as ICS in asthma and COPD. Compound GRT7 had the same overlapping dose-responsive inhibitory effect on IL-6 and IL-8 release in comparison to FP, whereas GRT10 had a similar trend but slightly less potency and efficacy relative to FP and GRT7. Interestingly, GRT8 showed a different pharmacological profile. GRT8 suppressed IL-6 and IL-8 release with significantly reduced potency and efficacy, compared to all the other compounds. Thus, all tested compounds showed anti-inflammatory activity.

The mechanism of suppression of pro-inflammatory cytokines involves the activation of GR transrepression pathway and the inhibition of transcription factors such as NF-kB and AP-1 is widely documented. Data reported in literature towards transrepression by GRTs compounds confirm the full GR activity of FP and GRT7 and the reduced GR activity of GRT8 and GRT10. The malleability of the nuclear receptor GR allows different conformational changes depending on GR binding to different ligands, which may result in differentiated binding to different coregulators. As GR specifically interacts with GRIP1/SRC2/TIF2/NCOA2 to repress NF-kB and AP-1 transcriptional activity, a hypothesis of the distinct effect of GRT8 may be a reduced affinity of GRT8-bound GR for this coactivator. Besides the predicted inhibition of pro-inflammatory cytokines production by the standard steroid FP, the anti-inflammatory effect of novel selective compounds GRT7, GRT8 and GRT10 was demonstrated.

6.1.2 GC inhibition of the innate antiviral immune response

Nasal and bronchial epithelial cells are the first sites of respiratory virus infections, which trigger both host pro-inflammatory and anti-viral responses that cooperate to eradicate the pathogen [61, 131]. Rhinovirus (RV) binding on the cell surface and subsequent internalisation inside endosomal vesicles trigger the host innate antiviral response [102, 103].

The endosomal membrane-located pattern recognition receptors (PRRs) TLR3 and TLR7/8 respectively sense single stranded (ss) and double stranded (ds) viral RNA, thereby inducing the activation of NF-kB and the upregulation of RIG-1 and MDA5, other cytosolic PRRs [99, 137, 138]. Besides pro-inflammatory cytokines, this results in an

increased production of antiviral cytokines such as type I (IFN-α/IFN) and type III (IFN-λs) interferons (IFNs), which are released in the extracellular space [140, 141]. In the canonical pathway, released type I IFNs bind to the interferon-alpha receptor (IFNAR) on the surface of nearby cells. IFNAR is composed of the two subunits IFNAR1 and IFNAR2, which are associated with the cytoplasmic receptor proteins tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1), respectively [148-151]. In turn, these two kinases recruit and phosphorylate STAT1 and STAT2 on specific tyrosine (Y) residues. Once activated, pSTAT1 Y701 and pSTAT2 Y690 dimerise and associate with IRF9 to form a complex called interferon stimulated gene factor 3 (ISGF3), which translocates into the nucleus to promote the expression of interferon stimulated genes (ISGs) [152-160].

Among these ISGs, virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible (viperin) and 2'-5' oligoadenylate synthetase (OAS) have an important role in host antiviral response [150, 217, 221, 222]. Positive-strand RNA viruses, including RV, build membranous vesicles with a unique lipid composition, known as replication organelles (ROs), by remodelling endoplasmic reticulum (ER) and Golgi membranes [112, 113, 216]. Viperin participates in cellular lipid metabolism [212] and alter the fluidity of the plasma membrane [219], thereby inhibiting virus replication and release. The role of OAS is to sense ds RNA and to catalyse the formation of 2'-5'-oligoadenylates, which induce viral RNA degradation by action of the ribonuclease L (RNaseL). Thus, OAS inhibits viral replication and protein synthesis [223].

Increased expression of viperin and OAS were observed in nasal epithelial cells from naturally acquired human rhinovirus (HRV) infections, while viperin knockdown increased rhinovirus replication in primary cultures of human bronchial epithelial cells [221]. Several studies demonstrated a reduced production of IFN-β and IFN-λs in asthmatic [51-57] and COPD [85-87] subjects. However, to establish whether the impairment of the innate immune response is due to the disease *per se* or as an effect of a GC-based therapy is an unmet goal.

There are contrasting opinions from the scientific community towards GC safety in asthma and COPD treatments [243]. The observation that eosinophilic inflammation increase the risk of RV-induced exacerbations and is predictive of GC responsiveness [20, 70, 71] may also support the idea that GCs inhibit the innate antiviral immune response, which is the main hypothesis formulated in this thesis.

In the context of acute respiratory virus infections of the airways, bronchial epithelial cells (BEAS2B) were infected *in vitro* with human rhinovirus 1B (HRV-1B) and investigations towards molecular aspects of GC modulation of the innate antiviral response carried out. To investigate the effects of scalar doses of FP and novel selective compounds (GRT7,

GRT8 and GRT10) on RV-induced production of IL-29/IFNλ1 release, ELISA analyses were performed. Interestingly, low doses of the standard steroid FP suppressed IFNλ1 production, suggesting the inhibition of cell response to RV infection. The effect of GRT7 was exactly the same of FP, whereas GRT10 had a similar dose-responsive trend but a slightly reduced potency and efficacy. By contrast, the production of IFNλ1 was unaffected by GRT8, showing a completely different scenario. The diversified effect of GRT8, which did not affect IFNλ1 release in RV-induced BEAS2B cells, may be explained considering GR recruitment of GRIP1. In fact, an additional model of GC suppression of gene transcription is GR recruitment of GRIP1, which is competitively recruited by the transcription factor IRF3 to induce type I and type III IFN expression in response to respiratory viral infections [283]. Thus, a reduced affinity of GRT8-bound GR for GRIP1 may explain the lack of effect on IFNλ1 production. This may result in a potential therapeutic advantage of GRT8 in terms of reduced side effects.

Further investigating the effects of FP and selective compounds on RV-induced innate response, interferon stimulated gene (ISG) expression was determined by Taqman qRT-PCR, following cell infection with HRV-1B. FP suppressed the induction of antiviral ISGs such as viperin and OAS in a dose-dependent way. An additional ISG, the T-cell chemokine IP-10 was also assessed for FP and a dose-dependent inhibition was observed. All selective compounds GRT7, GRT8 and GRT10 had comparable results on viperin and OAS gene expression relative to the standard steroid FP. No significant differences were found among tested compounds.

Data clearly revealed a suppressive effect with the standard steroid FP and also with selective compounds GRT7 and GRT10 on the innate antiviral immune response, observed by suppression of IFNλ1 production and downstream expression of ISGs. This latter event may be a direct consequence of the lack of an adequate IFN stimulation on the cell surface. As type I and type III IFNs share a common mechanism of signal transduction, which involves the JAK/STAT pathway, the inhibition of IFNλ1 could be responsible for the suppression of viperin and OAS gene expression by FP, GRT7 and GRT10. However, a different reasoning comes from the observation that GRT8 unexpectedly suppressed viperin and OAS even if IFNλ1 release was unaffected. Taken together, results showed that both in presence or absence of a full IFN stimulation ISG expression was suppressed, suggesting that some molecular events on IFN signalling pathways may be blocked, altering the downstream innate antiviral response. Speculation on the JAK/STAT pathway were made. The possibility of a direct effect of the virus on IFNAR internalisation and consequent pathway inhibition is one other interpretation that was not investigated here. What is evident is that the activation of GR by the

glucocorticoid FP or selective compounds GRT7, GRT8 and GRT10 may have inhibitory effects on the host ability to eliminate the virus, the consequence of which may be an increase of viral replication in infected bronchial epithelial cells.

6.1.3 Effect of GCs on viral replication

An emerging adverse effect associates the use of GCs with the increase of respiratory virus infections [241, 242]. Experimental evidence suggest GC inhibition of the innate antiviral immune response [79, 97].

To investigate whether this has an effect on viral replication, BEAS2B cells were infected with HRV-1B and viral genome and titre measured by Taqman qRT-PCR and $TCID_{50}$, respectively. FP increased both HRV-1B RNA and viral load in a dose dependent way. The effect of GRT7 was similar to FP. Also GRT10 increased the viral RNA but no effect was observed on viral titre. In line with a differential scenario shown by GRT8, this compound had no influence on both HRV-1B RNA and viral titre.

These results demonstrated that the standard steroid FP has an inhibitory effect on antiviral response in bronchial epithelial cells, which in real life means FP may have an unwanted side-effect towards viral replication in the airways, similarly to GRT7 and probably GRT10. The hypothesis of GRT10 inhibition of viral proteases, responsible for the production of structural and non-structural proteins by cleavage of a viral polyprotein, was formulated. This would block the viral genome assembly into the viral capsid. Interestingly, the lack of effect of GRT8 confirm its differentiated pharmacological profile, suggesting a potential to be further developed as a safer drug, although data about inhibition of the innate response were controversial.

6.1.4 GC impairment of type I IFN signalling pathway

There are evidence of GC inhibition of the innate antiviral immune response [195, 241], but the modulatory mechanisms have not been well clarified so far.

Herein, it was hypothesised the involvement of the type I IFN signalling. To explore this, BEAS2B cells were stimulated with recombinant IFN-β, but not infected with HRV-1B, and treated with FP and selective compounds (GRT7, GRT8 and GRT10). The effects on viperin and OAS gene expression was measured by Taqman qRT-PCR. In addition, to investigate the effects of GCs on the activation of IFNAR signal transduction, specific phosphorylations of STAT proteins were detected by western blotting.

The standard steroid FP reduced IFN-β induction of viperin and OAS gene expression. Selective compounds GRT7, GRT8 and GRT10 reduced ISGs in a similar way. These

results validated the hypothesis of the involvement of the type I IFN signalling in GC impairment of the innate antiviral response.

6.1.5 GC interference with JAK/STAT pathway activation

Going into the JAK/STAT pathway, different experimental protocols were designed to investigate the kinetics of IFN-β induced STAT1 and STAT2 phosphorylations at tyrosine (Y) and serine (S) amino acidic residues. FP appeared to have a dose-responsive inhibitory effect on pSTAT1 Y701 and totally inhibited pSTAT2 Y690 at all tested concentrations. However, pSTAT1 S727 was not stimulated by IFN-β and not affected by FP. These results were obtained at low concentration of FP, with or without pre-treatment and following short periods of recombinant IFN-β stimulation (30 or 60 minutes). The assessment of GRT7, GRT8 and GRT10 on pSTAT1 Y701 gave comparable results, apparently without distinction among them. These results suggest GCs interfere with the activated JAK/STAT pathway.

At our knowledge, no evidences of GC interference with the JAK/STAT signalling at the level of STAT proteins phosphorylations have been reported in literature. Differently, contrasting conclusions have been reported [97, 188]. Thus, we conclude this is a previous undemonstrated novel mechanism of GC inhibition of the innate antiviral immune response. Ligand-bound GRs negatively modulate type I IFN signalling pathway.

Furthermore, the observation that these pharmacological effects of GCs on pSTAT1 Y701 and/or pSTAT2 Y690 inhibition are rapid events opened the way to important considerations. Genomic effects of GC inhibition of IFNAR signalling pathway, including the activation of SOCS1 and SOCS3 [182, 316-318], which inhibit JAK activity competing with STAT proteins for IFNAR binding, and the suppression of ISGs such as STAT1 and IRF9, have been proposed [188, 319]. However, genomic events are unlikely the driving mechanisms involved. As pSTAT1 Y701 and pSTAT2 Y690 inhibitions occurred within 30 minutes and even without pre-treatment, genomic effects seem not plausible explanations. More likely, GCs exert these actions in a transcriptional independent way, without GR interaction with the DNA. Results reported here orientate towards non-genomic effects of GCs, which generally occur rapidly. Speculations about them included various hypothesis: the involvement of membrane-bound GR, the inhibition of nuclear phosphatases and the recruitment of GRIP1 from ISGF3 complex.

An additional observation towards the role of pSTAT1 S727 is that this phosphorylation is not strictly necessary for STAT1 transcriptional activation in bronchial epithelial cells, as it was unstimulated by IFN-β and also unaffected by GCs. This is in contrast with the reported crucial role for pSTAT1 S727 on STAT1 full transcriptional activity [157-159].

Understanding the mechanisms of GC modulation of the innate immune response is essential to identify new molecular targets and develop coadjuvant treatments to reverse GC impairment of the innate immune response, which is an emerging side effect.

6.1.6 Effect of GCs and β2 adrenergic agonists on ISG expression and RV replication

To assess the effect of GCs on RV-induced ISG expression at a wider scale, the standard steroids budesonide (Bud) and dexamethasone (Dex), clinically administered as ICS and OCS, respectively, were also investigated in BEAS2B cells, in addition to the β2 adrenergic agonist Formoterol (Form).

Treatments with low doses of both the standard steroids Bud and Dex resulted in a dose-dependent gene suppression of viperin and OAS, quantified by SYBRGreen qRT-PCR. To a lesser extent, Form also suppressed both ISGs. In parallel, an increase of HRV-1B RNA was observed with Bud and Dex, but not with Form. These data further confirm the suppressive effect of GCs on the innate antiviral immune response and suggest a suppressive effect by action of LABA, too.

6.1.7 ICS/LABA combination and antiviral response

Inhaled formulations of ICS/LABA combination are widely prescribed in asthma and COPD treatment to reduce inflammation and reverse bronchoconstriction [30, 35, 76, 77, 95]. These two classes of drugs have beneficial interactions when administered in association. In fact, while GCs increase the expression of β2 adrenergic receptors (β2AR) and protect against receptor downregulation due to long-term use of LABA, the latter increase the translocation of the GC activated GR from the cytoplasm into the nucleus [235, 272, 273]. Thus, the combinatorial therapy ICS/LABA has pharmacological advantages compared to increased doses of ICS [270]. Some examples include Bud/Form, FP/Salmeterol [75] or the more recent combination FF/vilanterol, introduced as once-daily inhaled drugs thanks to the high nasal and lung tissue affinity of FF [91, 306]. Despite the beneficial interactions of ICS/LABA combinations, several studies demonstrated additional inhibitory effects on experimentally induced ISG expression, type I IFN and chemokines production when the two drugs were used together, thus providing evidence of a potentiated effect due to the combination ICS/LABA in reducing early innate antiviral immune response *in vitro* [327-329]. However, whether *in vivo* this may have implication towards the clearance of the virus in the airways remains to be determined.

As the glucocorticoid Bud is administered as an inhaled formulation, alone or in combination with Form, an *in vitro* analysis of their effects on viperin and OAS gene

expression in both situations has been carried out in RV-infected BEAS2B cells. Pharmacological doses of Bud/Form, used as a co-treatment, showed an additional effect on viperin and OAS gene suppression, compared to Form alone. Importantly, the concentration of the steroid Bud was determinant. Bud at the lower concentration tested in combination with Form had a potentiated effect compared to both Bud and Form alone. However, a slight increased concentration of Bud in combination with Form had the same effect of Bud alone, independently from Form, suggesting the suppressive effect was due to the action of the GC alone and not to the ICS/LABA combination.

These observations may have clinical implications related to ICS/LABA therapy in asthma and COPD treatments. Transferring these experimental observations to real life, the potential lack of host ability to clear the virus may cause disease exacerbations with detrimental consequences. Despite these concerns, withdrawal from ICS/LABA would probably not be the best solution. Thus, physicians should consider the real or potential risk/benefit balance in defining the therapeutic regimen and evaluating that a reduction of GC doses in ICS/LABA combinatorial therapy may suffice to retain the anti-inflammatory activity of GCs and reduce the risk to inhibit the innate antiviral response in respiratory epithelial cells.

6.2 Concluding remarks and future work

This thesis demonstrated the main hypothesis by experimental *in vitro* evidence that GCs inhibit the innate antiviral immune response in bronchial epithelial cells. A novel mechanism involving the JAK/STAT signalling pathway is proposed. I can finally conclude that lowering GC doses may have advantages in terms of reduced probability of RV-induced asthma and COPD exacerbations in ICS/LABA therapy.

To deeply investigate on this thesis it will be interesting:

- To assess the effects of the GR antagonist RU486 (mifepristone) on the modulation of the innate immune response to RV.
- To explore the role of GRIP1 on GR modulation of STAT1-STAT2-IRF9 transcriptional activity.
- To quantify the activation of STAT family transcription factors by using TransAM STAT family assay (Active Motif) in RV infected or IFN-β stimulated cells treated with GCs.
- To screen GR ligands effects on the antiviral response by measuring the activation of STAT1 using the cell lines A549 GFP STAT1 (Sigma), in which the genomic STAT1 gene has been tagged with a Green Fluorescent Protein, and JAK/STAT signalling pathway ISRE Reporter - HEK293 (Bioscience).

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Abbreviations

11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1

11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2

AA, aminoacid

AAAAI, American Academy of Allergy, Asthma and Immunology

ACBD3, acyl-coenzyme A binding domain-containing 3

ACO, asthma-COPD overlap

ACTH, adrenocorticotropic hormone

AHR, airways hyperresponsiveness

AP-1, activator protein 1

Arfs, ADP-ribosylation factors

BAL, bronchoalveolar lavage

BC, breast cancer

BECs, bronchial epithelial cells

BFA, brefeldin A

BSA, bovine serum albumin

Bud, budesonide

CAMKII, Ca^{2+}/calmodulin-dependent kinase II

CBP, CREB binding protein

CCL5, chemokine (C-C motif) ligand 5

CDHR3, cadherin-related family member 3

CDK8, cyclin-dependent kinase 8

CFTR, cystic fibrosis transmembrane conductance regulator

ChIP, chromatin immunoprecipitation

CME, clathrin-mediated endocytosis

COAST, Childhood Origins of Asthma

COPD, chronic obstructive pulmonary disease

CpdA, selective compound A

cPLA2, cytosolic phospholipase A2

CRD, chronic respiratory disease

CREB, cAMP response element-binding protein

CRF-1, corticotropin releasing factor 1

CRH, corticotropin-releasing hormone

CRT, calreticulin

CSE, cigarette smoke extract

CXCL10, C-X-C motif chemokine 10

CYS-LTRA, cysteinyl leukotriene receptor antagonists

DBD, DNA binding domain

DCs, dendritic cells

DEX, dexamethasone

dsDNA, double-stranded DNA

dsRNA, double-stranded RNA

eIF4GI, eukaryotic initiation factor 4 gamma-1

eIF4GII, eukaryotic initiation factor 4 gamma-2

EMT, extraneuronal monoamine transporter

ENA78, epithelial-neutrophil activating peptide

ER, endoplasmic reticulum

ERC, endocytic recycling compartment

FENO, fractional exhaled nitric oxide

FEV_1, forced expiratory volume in 1 second

FF, fluticasone furoate

FKBP51, 51 kDa FK506-binding protein

FKBP52, 52 kDa FK506-binding protein

FOXO3, forkhead box O3

FP, fluticasone propionate

FVC, forced vital capacity

GASs, gamma-activated sequences

GBD, global burden of disease

GBF1, Golgi-specific brefeldin-A (BFA) resistance factor 1

GCs, glucocorticoids

GILZ, glucocorticoid-induced leucine zipper protein

GINA, Global Initiative for Asthma

GOLD, Global Initiative for Chronic Obstructive Lung Disease

GPCR, G-protein-coupled receptor

GREs, glucocorticoid response elements

GRIP1, glucocorticoid receptor-interacting protein 1

GRs, glucocorticoid receptors

GS, glutamine synthetase

HAT, histone acetyltransferase

HBECs, human bronchial epithelial cells

HDAC, histone deacetylase

HDM, house dust mite

HPA, hypothalamic-pituitary-adrenal

HR, hinge region

HRV-1B, human rhinovirus 1B

hsp, heat shock protein

ICAM-1, intracellular adhesion molecule-1

ICS, inhaled corticosteroids

IDO, indoleamine-2,3-dioxygenase

IFN, interferon

IFNAR, interferon-alpha receptor

IFNGR, IFN-gamma receptor

IFNLR1, IFN-lambda receptor chain 1

IgE, immunoglobulin E

IkB, inhibitor of NF-kB

IKK, IkB kinase

IKKε, inhibitor of NF-kB kinase-ε

IL, interleukin

IL-10Rβ, IL-10 receptor chain 2

ILCs, innate lymphoid cells

iNOS, inducible nitric oxide synthase

IP-10, interferon gamma-induced protein 10

IPS-1, IFN-β promoter stimulator 1

IRES, internal ribosomal entry subunit

IRF, interferon regulatory factor

ISGF, interferon stimulated gene factor

ISGs, interferon stimulated genes

ISREs, IFN-stimulated response elements

ITAM, immunoreceptor tyrosine-based activation motif

JAK, Janus Kinase

JNK, c-Jun N-terminal kinase

LABA, long acting β2-agonists

LAMA, long-acting muscarinic antagonists

LBD, ligand-binding domain

LDLR, low-density lipoprotein receptor

LPS, lipopolysaccharide

MAP, mitogen-activated protein

MAPK, mitogen-activated protein kinase

MDA5, melanoma differentiation-associated protein 5

MIP-1α, macrophage inflammatory protein

MKP-1, mitogen-activated kinase phosphatase-1

MPO, myeloperoxidase

Mx, myxovirus resistance

MyD88, myeloid differentiation primary-response gene 88

NCOA2, nuclear receptor coactivator 2

NCOR2, nuclear corepressor 2

NE, neutrophil elastase

NEF, nucleotide exchange factor

NETs, neutrophil extracellular traps

NF-kB, nuclear factor-kB

NLSs, nuclear localisation sequences

NOS2, nitric oxide synthase 2

NSPs, non-structural proteins

NTD, N-terminal domain

OAS, 2'-5'-oligoadenylate synthetase

OASL, protein OAS-like

OCS, oral corticosteroids

ORF, open reading frame

OSBP, oxysterol-binding protein

PAMPs, pathogen associated molecular patterns

PBMCs, peripheral blood mononuclear cells

pCAF, p300/CBP-activating factor

pDC, plasmacytoid dendritic cells

PDE4, phosphodiesterase 4

PDE4I, PDE-4 inhibitors

PEF, peak expiratory flow

PI, phosphatidylinositol

PI3K, phosphatidylinositol 3-kinase

PI4KB, phosphatidylinositol 4-kinase IIIβ

PI4P, phosphatidylinositol 4-phosphate

PIAS, protein inhibitors of activated STAT protein

PKC-δ, protein kinase C-delta

PKD, protein kinase D

PKR, protein kinase RNA-activated

PR progesterone receptor

PRRs, pattern recognition receptors

PTMs, post-translational modifications

RANTES, regulated on activation, normal T cell expressed and secreted

RDRP, RNA-dependent RNA polymerase

RIG-1, retinoic acid-inducible gene 1

RLRs, RIG-like receptors

RNaseL, latent ribonuclease L

ROs, replication organelles

RSAD2, radical S-adenosyl methionine domain-containing 2

RSV, respiratory syncytial virus

RV, Rhinovirus

SABA, short acting β2-agonists

SEGRAs, selective glucocorticoid receptor agonists

SEGRMs, selective glucocorticoid receptor modulators

SH2, STAT Src-homology 2

SHP2, SH2 domain-containing protein-tyrosine phosphatase 2

SIN3A, SIN3 transcription regulator homologue A

siRNA, short interfering RNA

SLPI, secretory leukoprotease inhibitor

SMRT, silencing mediator of retinoic acid and thyroid hormone receptor

SOCS, suppressor of cytokine signalling

SRC-2, steroid receptor coactivator-2

ssRNA, single-stranded RNA

STAT1, signal transducer and activator of transcription 1

SUMO, small ubiquitin-like modifier

SWI/SNF, switching/sucrose non-fermenting

TAD, transcriptional activation domain

TAFIIs, TBP-associated factors

TANK, TRAF family member-associated NF-kB activator

TBK1, TANK-binding kinase1

TBP, TATA-binding protein

TC-PTP, T cell protein tyrosine phosphatase

TGF-β, transforming growth factor-β

Th_2, T helper 2

TIF2, transcriptional mediators/intermediary factor 2

TIR, Toll/interleukin-1 receptor

TLRs, toll-like receptors

TNF-α, tumor necrosis factor alpha

TRAF6, TNF receptor-associated factor 6

TRIF, TIR domain-containing adaptor protein inducing IFN-β

TRIM, tripartite motif

TSLP, thymic stromal limphopoietin

TTP, tristetrapolin

TYK, tyrosine kinase

USP18, ubiquitin carboxy-terminal hydrolase 18

UTR, untranslated region

VP, viral protein

WHO, World Health Organization

β2ARs, β2-adrenergic receptors

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