Important lessons learned from the study of the pharmacology of glucocorticoids in human airway smooth muscle cells: too much of a good thing may be a problem

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Abstract

Glucocorticoids (GCs) are the treatment of choice for chronic inflammatory diseases, such as asthma. Despite proven effective anti-inflammatory and immunosuppressive effects, GCs' long-term and/or systemic use can potentially induce unwanted adverse effects. Strikingly, some recent experimental evidence suggests that GCs may also exacerbate some diseases' outcomes. In this review, we will summarize evidence describing how GCs promote pro-inflammatory and remodeling features in asthma, specifically in airway structural cells, and will also cover some possible solutions to these unanticipated effects of GCs.

Keywords

Glucocorticoids, airway smooth muscle, airway remodeling, airway inflammation, asthma, adverse effects.

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Abbreviations

AR	Airway remodeling
ARDS	Acute respiratory distress syndrome
ASM	Airway smooth muscle
BAL	Broncho-alveolar lavage
cAMP	Cyclic adenosine monophosphate
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
ECM	Extra-cellular matrix
EGF	Epidermal growth factor
FoxO1	Forkhead box O1
FP	Fluticasone propionate
GCs	Glucocorticoids
G-CSF	Granulocyte colony stimulating factor
ID2	Inhibitor of DNA binding 2
MT1M	Metallothionein 1M
PGE2	Prostaglandin E2
PKA	Protein kinase A
TLRs	Toll-like receptors
TNF	Tumor necrosis factor-α

1. Introduction

Glucocorticoids (GCs) represent a cornerstone therapeutic approach in the treatment of inflammatory airways diseases, such as asthma. Despite proven effective anti-inflammatory and immunosuppressive effects, GCs' long-term and/or systemic use can potentially induce unwanted adverse effects such as osteoporosis, skin atrophy, diabetes, glaucoma, hypertension and growth retardation in children among others (Buehring, Viswanathan, Binkley, & Busse, 2013; Schacke, Docke, & Asadullah, 2002; Yamashita, et al., 2010). Importantly, a subset of patients with severe asthma appears refractory to the therapeutic actions of GCs and strikingly some of the current literature has revealed some of the "unanticipated" effects of GCs with regard to their impact on several pathological responses involved in asthma. Those include modulation of cell proliferation, or induction of some pro-inflammatory mediators and receptors which all appear to be cell- and stimuli- dependent, and as such potentially contribute to a less favorable disease state outcome. To get a better understanding of the effects of GCs in airways diseases such as asthma and specifically address whether these effects could, under certain circumstance, be ineffective and/or detrimental, we will here review evidence describing how GCs modulate airway inflammation and airway remodeling features associated with disease severity and progression. We will mainly focus on airway smooth muscle (ASM) cells, pivotal cells regulating bronchomotor tone with significant immunomodulatory functions and major contributor to the remodeling features associated with asthma ({Keglowich, 2015 #152}).

2. ASM cells phenotypic changes as a major contributor in airway remodeling in asthma.

Unequivocally, it has been established that the ASM layer in asthmatics becomes thicker through an increase in mass, particularly in more severe cases (Carroll et al, 1993, Am Rev Respir Dis 147(2); Ebina et al, 1990, Am Rev Respir Dis 141(5 pt 1); Woodruff et al, 2004, Am J Respir Crit Car Med 169(9)). The increased mass of (contractile) ASM is a typical feature of airway remodeling (AR) and is considered a major causal feature for airway hyperreactivity and

excessive narrowing that reduces airflow in asthma (Affonce 2006, J Appl Physiol 101; Wiggs 1990, J Appl Physiol 69) (Hirota, Nguyen, Schaafsma, Sharma, & Tran, 2009; Lambert, Wiggs, Kuwano, Hogg, & Pare, 1993). This is supported by evidence suggesting that targeted elimination of ASM through bronchial thermoplasty improves disease control in subjects with moderate to severe asthma (Cox et al 2007, NEJM, 356). Over the past decades, several studies have indicated that the phenotype of airway mesenchymal cells, which include ASM cells and (myo) fibroblasts, derived from asthmatic airways and propagated in cell culture is different from that of cells obtained from subjects not suffering from airway diseases, exhibiting augmented proliferative abilities (Chambers et al, 2003, AJP Lung, 285 (3); Johnson et al, 2001, AJRCCM 164(3))). These findings suggest that there is an intrinsic abnormality in the proliferation characteristics of ASM from asthmatics, and that any change in proliferation of the muscle cells over time (with increasing severity) may have robust effects on total muscle mass. Of note, it is unclear if proliferating cells are all in the same vicinity or if other mesenchymal cells migrate to the muscle bundles to contribute to the accumulating muscle mass (Henderson et al, 2007, AJP Lung, 292(4)). Paradoxically, other studies have indicated ASM cells from asthmatics are more contractile than control ASM cells (Ma et al 2002, AJP Lung 283). As suggested, ASM cells *in vivo* are subjected to a plethora of micro-environmental cues, in particular under conditions of transient (local) inflammation, and it is very conceivable that these cells express an intermediate phenotype that can be driven to either a more proliferative or contractile state, depending on the aforementioned intermittent profile of specific cues present (Hirota, et al., 2009; Lambert, et al., 1993).

Interestingly, ASM phenotypes and functions can be altered under specific inflammatory conditions where GCs have the ability to promote/facilitate (predominantly neutrophilic) inflammation and remodeling, for instance by producing IL-8, CXCL1, G-CSF, and ECM (regulating) proteins. Furthermore, interesting data obtained from endobronchial biopsies from subjects with asthma revealed an increased expression of genes importantly involved in asthma progression and severity within the ASM bundles, including ADAM33, ADAM8 (Foley, et al.,

2007), eotaxin (Ghaffar, et al., 1999), and CCL19 (D. Kaur, et al., 2006), despite these patients being treated with high dose of GCs.

ASM cells gene expression program has been shown to be affected *in vivo* in patients with asthma after 14 days treatment with GCs. Indeed, Yick and colleagues {Yick, 2013 #121} showed that oral prednisone changed the gene expression profile of ASM layer in asthma, which was correlated with improved lung function. Notably, the gene network analysis revealed significant changes in genes associated with the network functions cellular growth, proliferation, and development, such as ERK1/2 (extracellular signal-regulated kinase 1/2), UBC (ubiquitin C), and PPP2R1B (protein phosphatase 2, regulatory subunit A, β). Additional study by Himes's group showed that an ASM-specific transcriptomic signatures associated with GC treatment {Kan, 2019 #123}. Such changes were similar in ASM cells derived from healthy donors or patients with fatal asthma. Collectively, these clinical evidence clearly highlights the role of ASM cells not only as a major contributor in the AR features in asthma but also as an *in vivo* target of GCs.

3. Mechanisms mediating the effect of GC on different ASM functions.

A number of studies using cultured human ASM cells have investigated the beneficial actions of GCs and their potential associated mechanisms. The conclusions made from different labs suggest that GCs exert a strong anti-inflammatory action on a variety of inflammatory genes induced by pro-asthmatic stimuli, although the potency/efficacy appear to be highly gene and stimuli specific. However, despite this impressive anti-inflammatory action of GC, their underlying inhibitory mechanisms have not been completely established and appear to be also complex and involve targeting both transcriptional and post-transcriptional pathways. In addition to these GC-sensitive pathways, ASM is also a unique cellular model as it displays many GC-insensitive features which could therefore be potentially altered in severe asthma and be playing a major role

in the overall GC insensitive features seen in these patients (Latifa Chachi, Adelina Gavrila, Omar Tliba, & Yassine Amrani, 2015).

3.1) Induction of pro-inflammatory genes by various pro-asthmatic stimuli is differentially regulated by GCs in ASM cells. A number of pro-inflammatory genes that have the potential to regulate various aspects of asthma pathogenesis have been reported to be inhibited by different GCs in human ASM cells. For example, dexamethasone (or fluticasone) suppressed TNF-induced production of various chemokines including CXCL8 (Oltmanns, et al., 2008; Pang & Knox, 2000), CCL5 and IL-6 (A. Ammit, et al., 2000; A. J. Ammit, et al., 2002), CCL11 (L. Pang & A. Knox, 2001), CXCL10 (Clarke, et al., 2010) and expression of ICAM-1 (Yassine Amrani, Lazaar, & Panettieri, 1999). Responses induced by IL-1β, another pro-inflammatory stimulus involved in asthma, such as MMP-12 expression/activity, production of CXCL10 and GM-CSF, an essential factor for eosinophils/neutrophils differentiation and activity, or expression of ICAM-1 were also reported to be inhibited by dexamethasone (Yassine Amrani, et al., 1999; Saunders, et al., 1997; Tran, et al., 2005; Xie, et al., 2005) or fluticasone (Seidel, et al., 2012). In addition, GCs were shown to be effective in inhibiting the production of pro-inflammatory mediators such as IL-6 or CXCL8 stimulated by GPCR agonists such as bradykinin (Huang, Tliba, Panettieri, & Amrani, 2003; Pang & Knox, 1998; Zhu, Bradbury, Pang, & Knox, 2003) or sphingosine-1 phosphate (S1P) (Rahman, et al., 2014). Ciclesonide, a GC that requires to be converted by desisobutyryl-ciclesonide by lung esterases to be clinically active, and fluticasone were equally effective in inhibiting the induction of the chemotactic mediator MCP-1 in response to TNF stimulation (Nie, Corbett, Knox, & Pang, 2005; Patel, Clifford, Deacon, & Knox, 2012). Cigarette smoke was reported to stimulate the production of CXCL8 via pathways sensitive to fluticasone but not to salmeterol (Oltmanns, et al., 2008). It is interesting to mention that GCs exert a differential suppressive effect on the expression of pro-inflammatory genes in ASM cells and that not all genes are repressed with equal potency/efficacy. Induction of some genes such

as IL-6, CCL5, CXCL10 or MCP-1 appears to be strongly inhibited by dexamethasone or fluticasone (>80-90% inhibition at 10^{-5}M), while other responses such as expression of ICAM-1, CXCL8, CCL11, or GM-CSF were found to be only partially repressed (50-60% inhibition at 10^{-5}-to-$^{-6}$M). Surprisingly, other genes such as IL-33, CX3CL1, TARC or CCL11 were found to be not affected by either dexamethasone or fluticasone (Chung, et al., 1999; Faffe, et al., 2003; Prefontaine, et al., 2009; Sukkar, et al., 2004).

These observations led to the interesting conclusions that i) different signaling pathways regulate inflammatory gene expression and ii) GC differentially modulated these genes in a stimuli-dependent manner. These results most likely reveal the differential contribution of multiple anti-inflammatory mechanisms (*transrepression* vs *transactivation*) in the therapeutic action of GCs in ASM cells (Newton, 2014).

3.2) Differential regulation of pro-inflammatory signaling pathways by GCs in ASM cells. The mechanisms by which GCs exert their anti-inflammatory action in ASM cells have not been extensively investigated. The findings that a number of genes including CXCL8 (Rahman, et al., 2014), MCP-1 (Patel et al. 2012), GM-CSF (Tran et al. 2005) were inhibited at the mRNA levels by GCs strongly suggest the involvement of transcriptional mechanisms. Several studies using selective inhibitors and gene promoter constructs have then attempted to dissect the signaling pathways driving the expression of inflammatory genes in ASM cells. Reports found that various transcription factors STAT1/2, NF-κB, AP-1, IRF-1 and signaling pathways such as MAPKs (JNK, p38 MAPK, ERK1/2), often acting in concert, were involved in the transcription of pro-asthmatic genes in human ASM cells (Alrashdan, et al., 2012; A. Ammit, et al., 2000; Yassine Amrani, et al., 1999; Clarke, et al., 2010; Hardaker, et al., 2003; Rahman, et al., 2014; Robins, et al., 2011; Sukkar, et al., 2004; Tirumurugaan, et al., 2008; O. Tliba, et al., 2008; Omar Tliba, et al., 2003; J. Zhang, et al., 2015). The study of whether GCs suppress these signaling pathways has led to some very interesting conclusions regarding the unique anti-inflammatory strategies

used by GCs in ASM cells. In contrast to the popular belief that NF-kB is a main target of GCs (Newton, 2014), studies conducted in ASM cells have revealed that the impact of GCs on NF-κB function was highly complex and highly dependent on the type of activating stimuli. Indeed, dexamethasone was found to be less effective in inhibiting NF-κB pathways (assessed using reporter constructs) when activated by TNF or IL-1β (Yassine Amrani, et al., 1999; Moore, et al., 1999). In contrast, NF-κB activation in response to either thrombin, IL-1β (Tran, et al., 2005) or even bradykinin (Zhu, et al., 2003) was found to be strongly inhibited by dexamethasone. Gerber's lab has shown that the transcriptional cooperation between GR and NF-κB as the main mechanism explaining the augmentation of TNF-induced A20 expression by dexamethasone (Sasse, et al., 2016). The impact of GCs on the function of MAPKs has been investigated and found to be variable and stimuli specific. This is an important observation as immunohistochemistry and PCR assays demonstrated that p38 MAPK was activated *in vivo* in ASM bundles of severe asthmatic patients taking either oral or inhaled GCs (Robins, et al., 2011). The authors showed that in cultured ASM cells, activation of p38 MAPK by either IL-1β or activation by FGF-1 (and FGF-2) was sensitive to dexamethasone or fluticasone (Fernandes, et al., 1999; Tran, et al., 2005; Willems-Widyastuti, et al., 2013) while $ERK_{1/2}$ activation by TNF was found to be insensitive to GCs (Fernandes, et al., 1999; Robins, et al., 2011). In our recent study, we showed that $ERK_{1/2}$ was required for dexamethasone to induce pentraxin-3, a multifunctional protein regulating both innate and adaptive immunity (J. Zhang, et al., 2019). The overall message is that the therapeutic action of GCs in ASM cells is still poorly understood and additional studies are required to determine how GCs interfere with various signaling pathways, knowing that their anti-inflammatory actions will vary according to the nature of the stimulus and the presence of other therapeutic drugs such as β2-agonists.

3.3) Importance of transactivation in GC beneficial effects in ASM cells. Different studies have performed ASM transcriptomics to determine the profile of anti-inflammatory genes

induced by GCs in human cells in health and diseases (Himes, et al., 2014; Kan, et al., 2019; Masuno, et al., 2011; Misior, et al., 2009; Yick, et al., 2013). From these studies, it has emerged that budesonide or dexamethasone can stimulate the expression of a variety of induced genes that possess anti-inflammatory activities in ASM cells. Among these genes, *CRISPLD2* (Cysteine-rich secretory protein LCCL domain-containing 2), which has been associated with lung development and response to endotoxin, was reported to be up regulated by dexamethasone. siRNA assays showed that knockdown of CRISPLD2 protein enhanced the expression of IL-6 and IL-8 induced by IL-1β and reduced the inhibitory action of dexamethasone (Himes, et al., 2014). Another GC responsive gene found in ASM cells is called Kruppel Like Factor 15 (*KLF15*), which belongs to a KLF family of zinc finger transcriptional regulators that play a critical role in development, differentiation, and organ homeostasis. Masuno and colleagues found that *KLF15* expression was increased by dexamethasone at 4 and 24 hr. Knockdown experiments showed that KLF15 regulates *in vitro* apoptosis and proliferation in ASM cells and *in vivo* airway hyper-responsiveness in a murine model of allergic asthma (Masuno, et al., 2011). The same group recently identified phospholipase C delta 1 as a KLF15-regulated gene that inhibits ASM cell proliferation (Sasse, et al., 2017). Similar to KLF15, induction of A20 (i.e., TNFAIP3) by GCs in human ASM cells has been later reported to act as a negative feedback mechanism to inflammatory cytokines. A20 was shown to be essential for the anti-inflammatory action of dexamethasone in repressing the expression of a number of genes (i.e., IL-1A, IL-6, CXCL8, CCL2, TNF). The mechanisms of action of A20 is likely due to its strong inhibitory action on NF-κB pathways (Sasse, et al., 2017). However, one of the most studied GC inducible genes in ASM cells is MKP-1 (DUPS1), a dual phosphatase that plays a pivotal role in the inhibition of p38 MAPK and JNK pathways. Studies from Ammit's group and others have provided strong evidence supporting the implication of MKP-1 in the repression of different pro-asthmatic genes (CD38, GRO-alpha and IL-6) induced by a variety of stimuli including IL-1β, TNF and S1P (Che, et al.,

2014; Issa, et al., 2007; Kang, Jude, Panettieri, Walseth, & Kannan, 2008; Prabhala, Bunge, Ge, & Ammit, 2016; Quante, et al., 2008). The specific contribution of each of these different GC-inducible genes in the overall potency and efficacy of GCs reported in ASM cells (see previous section) remain to be further explored. A nice study by Newton and colleagues reported that many GC-inducible genes including MKP-1 and GC-induced leucine zipper (GILZ) were enhanced by GC/β2-agonist combination, providing at least one mechanisms supporting the superior clinical benefit of the combination therapies (M. Kaur, Chivers, Giembycz, & Newton, 2008). We and others have previously reported that GILZ was a GC responsive gene in ASM both *in vitro* in cultured cells treated with fluticasone and *in vivo* in lung biopsies from patients treated with inhaled budesonide (Chachi, et al., 2017; Chachi, et al., 2013; Kelly, et al., 2012).

4. Impaired and unanticipated effects of GC in ASM in severe asthma.

4.1) Clinical evidence of impairment of GC actions in ASM cells. Elegant studies from Martin's lab and others have provided strong evidence that some ASM abnormalities associated with severe asthma (i.e., increased ASM mass) despite patients being treated with oral and inhaled GC therapy (Benayoun, Druilhe, Dombret, Aubier, & Pretolani, 2003; Hassan, et al., 2010; Ichikawa, et al., 2019; Pepe, et al., 2005; Ramos-Barbon, et al., 2010). Additionally, the wall thickening of the central airways of patients with asthma has been shown to be only partially responsive to inhaled corticosteroids ({Niimi, 2004 #119}). These studies have raised the possibility that severe asthma is associated with an impaired therapeutic response to GC in the lungs including in the ASM. Different studies including from our lab comparing the therapeutic action of GCs in ASM cells have indeed supported this hypothesis by showing that GC sensitivity was blunted in cells from severe asthmatics when compared to cells derived from healthy subjects (Chachi, et al., 2017; Chang, Bhavsar, Michaeloudes, Khorasani, & Chung, 2012; Chang, et al., 2015; J. H. Liu, Li, Zhang, & Zhang, 2020; Perry, Baker, Gibeon, Adcock, & Chung, 2014; Roth, et al., 2004). These studies revealed that the anti-inflammatory (ability to inhibit chemokine

secretion) and anti-remodeling (ability to inhibit cell proliferation) actions were significantly reduced in ASM cells from severe asthmatics. The underlying mechanisms appear to be very complex involving multiple mechanisms affecting mostly GC receptor (GR) function including a decreased receptor expression, receptor nuclear translocation, receptor phosphorylation at serine211 or transactivation of genes (GILZ). These studies have highlighted the mechanistic complexity of GC insensitivity seen in ASM tissues in severe asthma which could reflect the heterogeneity of clinical and/or inflammatory profiles seen in these patients. Nonetheless, we have identified the protein phosphatase PP5 while others found microRNA (mir-21) as the main pathways blunting GC sensitivity in ASM cells of severe asthmatics {Chachi, 2017 #1605;Liu, 2020 #1750;Bouazza, 2012 #100}. These are important findings with clinical implications as high expression of several pro-asthmatic mediators including cytokines or chemokines has been shown in the ASM bundles of asthma patients despite treatment with either oral or high doses of inhaled GCs (reviewed in (Latifa Chachi, et al., 2015)). The GC insensitive mediators produced by ASM *in vivo* have the capacity to regulate various aspects of asthma pathogenesis including airway remodeling, airway hyper-responsiveness and airway inflammation (reviewed in (Chachi, et al., 2017)). One study, however, failed to detect any significant difference in GC response in ASM cells between fatal asthma and healthy when assessing GC transcriptome, although the small sample size, cells isolated from tracheal tissues, experimental design and lack of clinical data may have influenced the significance of the study (Kan, et al., 2019).

4.2) Unanticipated effects of GCs on airway inflammatory features. While GCs exhibit anti-inflammatory actions, such as suppressing the secretion of cytokines and chemokines in cells such as ASM cells, under certain inflammatory conditions they not only lose their anti-inflammatory properties but can enhance the expression of inflammatory genes (L. Chachi, A. Gavrila, O. Tliba, & Y. Amrani, 2015; Sukkar, et al., 2004).

a) CX3CL1: Levels of CX3CL1, a chemokine implicated in cell adhesion, chemoattraction of various inflammatory cells associated with asthma, such as $CD4_+$ T cells (Mionnet, et al., 2010) or mast cells (L. Chachi, et al., 2015), are increased in broncho-alveolar lavage (BAL) of patients with asthma (Rimaniol, et al., 2003). Further, CX3CL1 appears to mediate asthma exacerbations associated with respiratory virus infection and allergen exposure (Loxham, et al., 2018). Mechanistic studies showed that the enhancing effect of GC on CX3CL1 production in the presence of cytokines was unassociated with mRNA stability, but was due to an increased transcriptional activity (Sukkar, et al., 2004). The potentiation of CX3CL1 secretion by GC in the presence of TNF/IFNγ might be unique to ASM, since CX3CL1 induction by these cytokines was suppressed by GC in human bronchial epithelial cells (Bhavsar, Sukkar, Khorasani, Lee, & Chung, 2008).

b) G-CSF: GCs can also increase the plasma levels of Granulocyte Colony stimulating factor (G-CSF) in healthy individuals (Jilma, et al., 1998), likely through its production and release from mononuclear cells (Witek-Janusek & Mathews, 1999). Interestingly, others investigated the impact of GC-induced G-CSF on neutrophilic lung inflammation using murine model of lung injury (Banuelos, et al., 2017). In this model, LPS challenge increased the number of BAL neutrophils, which was then further enhanced by dexamethasone exposure. Dexamethasone also maintained LPS-induced airway G-CSF while suppressing TNF and IL-6. Interestingly, *in situ* hybridization revealed that epithelial cells, ASM cells, and infiltrating leukocytes were the source of G-CSF in the lungs. When BEAS-2B bronchial epithelial cells, A549 lung epithelial cells, human monocyte-derived macrophages, and human neutrophils were used, dexamethasone and pro-inflammatory stimuli (IL-1β or TNF) synergistically induced G-CSF (Banuelos, et al., 2017; Files, et al., 2015). These observations clearly show that GCs enhance the production of some pro-asthmatic mediators with a potential to regulate neutrophilic asthma, one of the important granulocyte-based inflammatory phenotypes in severe asthma (O. Tliba & Panettieri, 2019).

c) CCL20: CCL20, another pro-inflammatory mediator induced by GCs, is increased in human bronchial epithelial cells (Zijlstra, et al., 2014). The clinical relevance of CCL20 in asthma is supported by the strong correlation observed between the levels of CCL20 found in the sputum, sputum neutrophil counts (Zijlstra, et al., 2014), mucus hypersecretion (Faiz, et al., 2018) and the dose of inhaled GC (budesonide) used. This is not entirely surprising as CCL20 is a neutrophil and Th17-cell chemoattractant and Th17-mediated neutrophilic airway inflammation has been associated with asthma severity including poor response to GC therapy. Interestingly, ASM cells derived from subjects with moderate asthma produce more CCL20 than cells derived from subjects with mild asthma suggesting that ASM as a potential source of CCL20 in asthma (Faiz, et al., 2018). However, whether CCL20 directly affects the therapeutic response to GC remains to be further explored. Additional mechanistic studies revealed that budesonide increased TNF-induced release of CCL20 by primary bronchial epithelial cells, while suppressing CXCL8 secretion, suggesting that the effects of GCs on the expression of chemokines are gene-specific (Zijlstra, et al., 2014). Although TNF-induced CCL20 secretion requires the activation of signaling pathways such as ERK, p38 and STAT3, none of these pathways were affected by budesonide. Furthermore, this GC action was only inhibited when GR was inhibited (Zijlstra, et al., 2014), suggesting the involvement of GR dependent mechanisms. It would be interesting to examine the common mechanisms by which GCs drive the expression of CCL20 and G-CSF.

d) TLRs: GCs also have the capacity to modulate the innate immune response by affecting the expression of Toll-like receptors (TLRs). For instance, dexamethasone enhanced the expression of TLR2 induced by TNF and IFNγ in ASM cells (Sukkar, et al., 2006), while in alveolar macrophages budesonide enhanced the expression of TLR2 induced by TLR ligands (Ji, et al., 2016). These observations strongly suggest that the modulation of TLR2 by GC could amplify the inflammatory responses in the airways (Manetsch, et al., 2012). In contrast, it is worth to mention that in primary human airway epithelial cells, dexamethasone decreased the expression of TLR2

induced by cytokines (Winder, et al., 2009). In human lung epithelial A549 cells, TNF and GCs were shown to cooperatively regulate components of innate immunity such as TLR2 (Hermoso 2004, Mol Cell Biol, 24(11)). Indeed, while dexamethasone repressed IL8 mRNA, it enhanced TLR2 mRNA expression in TNF-treated cells. Further mechanistic studies showed that TNF and dexamethasone activated unique intracellular mechanisms promoting the transcription of the TLR2 gene. Although dexamethasone alone did not appear to induce TLR2 promoter activity, may be due to the presence of only one single GRE site in the promoter region of TLR2 gene, such single binding site was required for the synergistic induction of TLR2-dependent gene expression to occur between TNF and GC (Hermoso 2004, Mol Cell Biol, 24(11)). Collectively, these studies show that the modulation of TLRs by GCs is highly dependent on the cell type and nature of the stimulus used.

e) MAPKs: Several studies have shown that treatment with GCs results in a loss of MAPK activity (ERK, p38, JNK) in a variety of cells, including mast cells (Kassel et al EMBO 2001 20)), HeLa cells (Lasa 2002 Mol Cell Biol 22), and human pulmonary epithelial A549 cells (Shah 2014 JBC 289). Although sustained stimulation (several hours) with GC does not activate MAPK signaling pathway, short-term acute GC treatment has been shown to activate such inflammatory pathways in some other cell types (reviewed in Panettieri & Tliba, 2019 {Panettieri, 2019 #114}). For instance, in PC12 cells (cell line derived from rat adrenal gland), corticosterone induced rapid activation (within 15 min) of ERK1/2, p38, and JNK in a PKC-dependent manner (Li, et al., 2001; Qiu, et al., 2001). The activation of MAPK pathways following GC treatment appears to be mediated by the putative membrane GR, since corticosterone-BSA can rapidly (within 15 min) activate all MAPKs (Li, et al., 2001; Qiu, et al., 2001). Similarly, in rat vascular smooth muscle cells, dexamethasone either alone or in combination with norepinephrine, rapidly (within 10 min) induces ERK1/2 and p38 MAPK activities (T. Zhang, et al., 2013). Interestingly, we recently showed that the stimulation of human ASM with dexamethasone increased mRNA and protein levels of pentraxin-3 (PTX3), a soluble pattern receptor involved in both innate and adaptive

immunity, which was markedly reduced by inhibition of p42/44 ERK (but not p38 or JNK) and GR blockade (ZHANG 2019 PloS One 14(8) suggesting the involvement of MAPK in PTX3 induction by GC. PTX3 expression has been shown to be increased in bronchial biopsies and BAL of severe asthmatics, and was shown to potently inhibit ASM migration induced by fibroblast growth factor-2 (FGF-2) and to augment CCL11/eotaxin-1 release (ZHANG 2012, PloS One, 7(4)). In addition, PTX3 deficient mice exhibit enhanced inflammation, AHR and mucus production following ovalbumin sensitization and challenge (Balhara 2017, Clin Immunol 139(3)). These findings implicate a possible dual role for PTX3 in asthma, and suggest GCs can modulate PTX3 levels in a GR and ERK dependent fashion. Since airway inflammation has generally been associated with the activation of MAPK signaling pathways (Y. Amrani, Ammit, & Panettieri, 2001; Baraldo, et al., 2003; Hallsworth, Moir, Lai, & Hirst, 2001), future investigations are warranted to explore the rapid effects of GCs on MAPK signaling in different airway structural cells derived from patients with various stages of asthma severity and to determine whether such non-genomic acute effects of GC affect asthma pathogenesis (reviewed in {Panettieri, 2019 #114}).

f) Additional examples from non-ASM cells. Interestingly, further evidence from immune cells and non-ASM cells demonstrated that GCs also upregulate certain inflammatory molecules such as inflammasome and Serpin A3.

Under certain conditions, GCs have been shown to exacerbate inflammatory response, by upregulating the expression of inflammasome regulators such as nucleotide-binding domain and leucine-rich repeat protein-3 (NLRP3). NLRP3 is a member of NOD-like receptors (NLRs), which activates an inflammasome complex in response to elevated levels of various molecules released in disease states, including extracellular ATP. For instance, treatment with either dexamethasone or cortisol rapidly enhanced NLRP3 mRNA and protein expression in THP-1 cells. Interestingly, such increase enhanced cell sensitivity to extracellular ATP and augmented the production of pro-inflammatory cytokines (2). In HMEC-1 cells, dexamethasone increased the

mRNA expression of IL-6, via a GR-dependent mechanism. Such increase was due to the upregulation of purinergic P2Y2 receptor (P2Y2R) expression, a Gq protein-coupled receptor, activated by ATP and UTP, with a particularly high affinity for ATP. Interestingly, pre-incubation with dexamethasone enhanced ATP-induced transcription of adhesion molecules ICAM-1, VCAM-1, and SELE, and the release of IL-8. These results suggest that exogenous GCs may enhance pro-inflammatory responses induced by ATP binding to the P2Y2R receptor (3). Interestingly, in human ASM cells, studies from Ammit's group ({Hirota, 2013 #116} {Prabhala, 2015 #117}) showed that TNF induced IL6 secretion in an inflammasome-independent manner and that TLR-2 treatment of ASM cells does not activate the inflammasome. Further studies are still needed to characterize the role inflammasome in ASM cells and importanty whether GCs potentiate inflammasome regulators as seen in immune cells.

An additional gene that was shown to be co-regulated by GCs and inflammatory cytokines is Serpin A3 (α-1 antichymotrypsin), an acute phase protein released during inflammatory processes. Indeed, Cidlowski group showed, using microarray analysis in A549 lung cells, that dexamethasone and TNF coregulate, rather than antagonistically regulate many genes involved in inflammatory disease such as S*erpinA3*. Such finding was confirmed *in vivo*, when treatment of C57BL/6 mice with dexamethasone and TNF led to an additive increase in SerpinA3 mRNA levels in the liver and the lung, although to a lesser extent than in the cell culture model. Furthermore, ChIP analysis suggested that GR binding at the serpinA3 transcriptional start site increased slightly when A549 cells were treated with either dexamethasone or TNF alone, but was markedely enhanced by their combination (4).

Collectively, these data suggest that the unanticipated effects of GCs in inflammatory features described above, may participate in the pathogenesis of severe asthma where GCs actions are impaired.

4.3) Unanticipated effects of GCs on ASM proliferation and airway remodeling.

a) GC, cAMP/PKA signaling, and ASM proliferation. Inhaled β-agonists constitute the most effective therapy for reversing acute bronchoconstriction associated with an asthma attack. Protein kinase A (PKA) has been identified as the primary cyclic adenosine monophosphate (cAMP) effector molecule in β2-agonist-mediated relaxation of ASM (Morgan et al 2014, JBC,289(33)).

The impact of GCs versus cAMP/ PKA stimulating agents on ASM proliferative function has been well documented and provided key information regarding the sensitivity of ASM proliferation responses to current anti-asthma drugs. For instance, PKA stimulating agents generally suppress mitogen-induced growth of ASM cells in culture (Bonacci & Stewart, 2006; Stewart, et al., 1999). In contrast, the effects of GCs on ASM cell mitogenesis are far less consistent and appear dependent on the type of mitogenic stimulus, with inhibitory effects on growth triggered by GPCR agonists, e.g. thrombin and leukotriene D4, and little effect on proliferation induced by growth factors such as epidermal growth factor (EGF) (Schramm, Omlor, Quinn, & Noveral, 1996; Stewart, Fernandes, & Tomlinson, 1995).

Interestingly, GCs could shift cytokine function from inhibitors to enhancers of mitogen-induced ASM growth (Misior, et al., 2008). Cytokines such as IL-1β and TNF activate cyclooxygenase-2 (COX-2) dependent production of prostaglandin E2 (PGE2) while inhibiting EGF-induced [3H]-thymidine incorporation in ASM cells. Since exogenous PGE2 inhibits ASM growth, likely in a cAMP/PKA dependent manner, COX-2 dependent PGE2 production emerged as a likely candidate to mediate inhibition of ASM proliferation by IL-1β and TNF. Interestingly, cell pretreatment with GC (fluticasone propionate (FP)) inhibited the induction of COX2/PGE2/PKA signaling cascade and markedly promoted EGF-induced cell growth (Misior, et al., 2008). Direct inhibition of PKA via heterologous expression of PKA inhibitors PKI or RevAB, also similarly augmented mitogen-induced ASM growth in the presence of cytokines, suggesting

a role for PKA in mediating the anti-mitogenic effects of cytokines. Thus, GCs potentially enhance ASM proliferation in the presence of some inflammatory stimuli via the inhibition of COX-2-dependent PKA pathways.

Elegant transcriptomic studies have provided some mechanistic insight into the deleterious effects of GCs in ASM proliferation. These studies showed that the treatment of human ASM cells with cytokines (e.g. IL-1β), mitogens (e.g. EGF), and GC (e.g. FP) (i) significantly increased transcripts encoding for zinc finger-containing proteins (e.g. ZBTB16, ZNF22, and PFH17), (ii) modulated the transcripts of several proteins known to regulate transcription factor activity such as metallothionein 1M (MT1M), forkhead box O1 (FoxO1), and inhibitor of DNA binding 2 (ID2), and (iii) markedly increased the expression of several putative regulators of mitogenesis such as C10orf10, Fam107A, and Wisp1 (Misior, et al., 2009). Other studies, however, revealed that the "direct" pro-mitogenic effect of FP is limited. Indeed, when considering proliferation, the only pro-mitogenic genes regulated directly by FP were C13ORF15, CYR61, and ID2 (Misior, et al., 2009; Misior, et al., 2008) while most of the pro-mitogenic effects of FP were "indirect" through PKA inhibition. These findings are of clinical relevance, since GC activation of genes that promote pro-mitogenic ASM phenotype could be counteracted by cAMP/PKA stimulating agents such as inhaled β2-agonists explaining thereby the therapeutic benefit of GC/β2-agonist combination therapy in some patients (Miller-Larsson & Selroos, 2006).

b) GC, ECM, and ASM proliferation. Other evidence suggests that the extra-cellular matrix (ECM) modulates ASM phenotype (Dekkers, Schaafsma, Nelemans, Zaagsma, & Meurs, 2007). Indeed, ECM proteins surrounding ASM cells have been shown to affect the proliferative (Bonacci, Harris, & Stewart, 2003; Dekkers, Bos, Halayko, Zaagsma, & Meurs, 2010) and contractile responses (Dekkers, et al., 2007) of ASM cells. When ASM cells were treated with EGF and IL-1β, FP enhanced the expression of genes closely associated with cell-ECM interactions such as MMP19, vinculin, integrins α5 and α10, collagen IVα1, providing an

additional mechanism by which GCs potentially promote ASM proliferation through the modulation of cell-ECM dynamics (Misior, et al., 2009). Interestingly, in a collagen-rich environment, ASM appears to be insensitive to the anti-proliferative action of GCs (Bonacci, Harris, Wilson, & Stewart, 2003; Bonacci, Schuliga, Harris, & Stewart, 2006). Together, these studies suggest that the nature of the ECM environment not only modulate a number of ASM remodeling responses seen in asthma, including proliferation and contraction, but also determine the therapeutic responses to GCs (Parameswaran, et al., 2006).

Collectively, from these different studies it is conceivable that depending on the inflammatory and ECM micro-environment in asthma, GCs might in fact promote and/or fail to inhibit abnormal ASM proliferation, a critical component of AR associated with disease progression and severity (Prakash, et al., 2017). This GC effect may be even more pronounced under conditions where β_2 adrenergic receptor/Gs/AC/cAMP/PKA pathway is impaired due to β2-receptor desensitization, a feature that may develop in patients with severe asthma (Chachi, et al., 2018) or after long-term treatment with β2-agonists (Y. Amrani & Bradding, 2017). Although GCs prevent β2-receptor desensitization in various model systems, including precision-cut lung slices (Cooper & Panettieri, 2008), the clinical relevance of such *in vitro* observations remains to be confirmed since poor responses to β2-receptor agonists is a key feature of patients with severe asthma despite being treated with high doses of inhaled or oral corticosteroids (Chachi, et al., 2018).

5. Alternative strategies to modulate the therapeutic actions of GCs in ASM cells. As discussed previously, the potency/efficacy of GCs in suppressing the expression of pro-inflammatory genes is greatly influenced by the type of stimulus and associated signaling pathways. Investigators have looked at ways to enhance the anti-inflammatory actions of GCs or to treat the GC-insensitive features. Several studies in human ASM cells have demonstrated the

therapeutic value of combining GCs with β2-agonists in the regulation of hyaluronan metabolism (Papakonstantinou, et al., 2012), and suppression of various inflammatory genes such as CCL5 (A. J. Ammit, et al., 2002), CXCL8 (Pang & Knox, 2000), or CCL11 (L. Pang & A. J. Knox, 2001). Interestingly, expression of other genes such as MCP-1 (Patel, et al., 2012) or IL-16 (A. J. Ammit, et al., 2002) were not affected by drug combination. Features of ASM remodeling are also synergistically repressed by GCs/β2-agonists combination (Dekkers, et al., 2012; Roth, et al., 2002). Combination therapy has also been reported to prevent some of the deleterious effects of β2-agonist monotherapy such as the increased expression of M3 muscarinic receptor/signaling (Y. H. Liu, Wu, Wang, Huang, & Liu, 2015), or receptor desensitization and hyper-responsiveness (Nino, Hu, Grunstein, & Grunstein, 2010). The mechanisms underlying the superior therapeutic effect of GCs/β2-agonists combination in ASM cells has been attributed to epigenetic changes at target gene promoters (Nie, Knox, & Pang, 2005), increased and/or restoration of GC-dependent transactivation (M. Kaur, et al., 2008; Rider, King, Holden, Giembycz, & Newton, 2011) including MKP-1 (Manetsch, et al., 2013) or A20 (Altonsy, Mostafa, Gerber, & Newton, 2017), decreased cellular uptake of β2-agonists via GC-induced inhibition of the organic cation transporter (OCT3) (Horvath, et al., 2007). Inhibition of NF-κB using selective IKK inhibitors has also been proposed as an alternative strategy to inhibit GC sensitive and insensitive genes in ASM cells (Catley, et al., 2006). Targeting the transcription factor IRF-1 or the protein phosphatase PP5 could also represent novel therapeutic option in ASM cells to suppress GC insensitive features (Latifa Chachi, et al., 2015).

6. Conclusion and Future Perspectives.

Overall, a better understanding of potentially deleterious effects of GCs may provide novel insights into the design of GCs with more specific anti-inflammatory actions capable of treating patients with severe asthma without engendering any unwanted effects.

Conflicts of Interest Statement

The authors declare that there are no conflicts of interest.

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Figure Legends

Figure 1. Schematic representation of the effects of GCs on ASM proliferation and airway inflammation. Under normal conditions, GCs exhibit powerful anti-proliferative and anti-inflammatory action in the lungs. However, under certain conditions, such as the presence of pro-inflammatory cytokines, growth factors, or ECM proteins, treatment with GCs may activate a range of mechanisms, leading to paradoxical pro-mitogenic and pro-inflammatory responses. Abbreviations: ASM, airway smooth muscle; CCL20, C-C Motif Chemokine Ligand 20; COX2, cyclooxygenase-2; CX3CL1, C-X3-C Motif Chemokine Ligand 1; ECM, extra-cellular matrix; EGF, Epidermal growth factor; GCs, Glucocorticoids; G-CSF, Granulocyte-colony stimulating factor; IL-1β, Interleukin 1 beta; IFNγ, Interferon gamma; PGE2, Prostaglandin E2; PKA, protein kinase A; TLR, Toll-like receptors; TNF, Tumor necrosis factor alpha. *Figure was created with images adapted from Servier Medical Art (smart.servier.com/)*.

528 **Table 1. Mechanisms mediating the unanticipated effects of GC on asthma features**

GC	Conditions	Paradoxical effect	Proposed mechanism	References
Fluticasone propionate	IL-1β and TNF plus EGF	ASM proliferation	Inhibition of COX2/PGE_2/PKA pathways	Misior, 2008
	IL-1β plus EGF	ASM proliferation (potential)	Pro-mitogenic effect PKA inhibition	Misior, 2008 Misior,2009
	IL-1β plus EGF	ASM proliferation	Modulation of cell-ECM dynamics	Misior, 2009
Dexamethasone	Collagen-rich environment	ASM proliferation	Failure to reduce cyclin D1 levels	Bonacci, 2003 Bonacci, 2006
	TNF and IFN-γ	Increase of pro-inflammatory mediators	Potentiation of CX3CL1 secretion by ASM	Sukkar, 2004
	TNF and IFN-γ	Pro-inflammatory	Increase of TLR2 in ASM	Sukkar, 2006
	TNF and IFN-γ	Pro-inflammatory	Negative regulator of functional TLR2 expression in airway epithelial cells	Winder, 2009
Dexamethasone **Budesonide**		Neutrophil activity and survival	Increase of anti-apoptotic protein Mcl-1L Increase of G-CSF levels Increase of CCL20	Sivertson, 2007 Banuelos, 2017 Faiz, 2018
Budesonide	TNF	Increase of CCL20 levels in airway epithelium	GR dependent mechanisms	Zijlstra, 2014
	TLR ligands	Pro-inflammatory	Enhanced expression of TLR2 in alveolar macrophages	Ji, 2016
Corticosterone		MAPK pathway activation	Activation of putative membrane GR in epithelial cells	Li, 2001; Qiu, 2001

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