

INVITED REVIEW

Glucocorticoids and the emerging importance of T cell subsets in steroid refractory diseases

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Abstract

Glucocorticoids remain the first-line treatment for a range of autoimmune and allergic diseases. However, 30% of patients fail to achieve disease control at tolerable systemic doses and continue to have an increased immune response with poor clinical outcome. This steroid refractory (SR) phenotype has previously been attributed to enhanced expression of inactive glucocorticoid receptor isoforms and cytokine-mediated suppression of glucocorticoid (GC) signaling, in particular by interleukin-2. These mechanisms are discussed, with emphasis on recent evidence for the role of the CD4⁺CD25^{int} and GC-induced T regulatory cell subsets in perpetrating SR disease.

Keywords: Human; T cells; CD25; IL-2; glucocorticoid; steroid resistance

Introduction

Glucocorticoids (GCs) are central to the normal reciprocal interaction between the neuroendocrine and immune systems, and their physiological release is under the homeostatic regulation of the hypothalamic-pituitary-adrenal (HPA) axis. Immunological, emotional, neurological and circadian stresses induce secretion of corticotropin releasing hormone (CRH) from the hypothalamus, which in turn induces release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. ACTH then acts upon the adrenal cortex to release GCs (primarily cortisol). Circulating GCs exert negative feedback on both the hypothalamus and pituitary resulting in a homeostatic control loop. Both under and overactivity of the HPA axis have been associated with enhanced susceptibility to infection, inflammation and autoimmunity.⁽¹⁾

Synthetic GC analogues were first used to treat rheumatoid arthritis (RA) in 1949.⁽²⁾ They are potent and

relatively fast acting anti-inflammatory agents which immunomodulate via induction of apoptosis and inhibition of proliferation, particularly of T-lymphocytes.⁽³⁾ Within 4–6 hours following systemic administration, monocytes, T and B cells redistribute from the blood to lymphoid compartments and circulating neutrophil numbers increase.⁽⁴⁾ Monocyte production of interleukin (IL)-10 or IL-6, and T cell IL-2 secretion, are suppressed,⁽⁵⁾ and GCs, via their interaction with nuclear factor kappa B (NFκB), impede upregulation of Intercellular Adhesion Molecule (I-CAM),^(6,7) Vascular Cell Adhesion Molecule (V-CAM),⁽⁸⁾ and E-selectins⁽⁹⁾ by vascular endothelial cells, thus blocking extravasation to sites of inflammation.

GCs remain first-line treatment for acute and chronic immune-mediated diseases, including autoimmunity and allergy, as well as forming part of the therapy for lymphoproliferative disorders. However, despite clinical success (notwithstanding GC induced side effects) there remain up to 30% of patients who fail to achieve

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disease control at tolerable systemic doses.⁽¹⁰⁾ A steroid refractory (SR) patient will therefore display increased autoimmune / inflammatory responses and potentially a severe clinical phenotype as a direct consequence of poor response to GC therapy rather than a more aggressive idiosyncratic auto-inflammatory response. As a result, despite therapy, enhanced immune responses include overexpression of cytokines, chemokines and adhesion molecules.

Overall, poor response to GC therapy and thus SR disease is the cause of considerable morbidity. Although additional immunosuppressive drugs, and more recently, specific targeted biologic therapy, are often prescribed in severe autoinflammatory disease to induce remission and offer the added potential of immunomodulation, they are also used to facilitate steroid taper and thus prevent unwanted GC-induced adverse events — particularly in patients displaying SR responses. However, these alternative immunotherapies are not always successful; either because of unfavorable side-effect profiles or treatment failure, and recurrent cycles of high dose steroids to rescue reactivating disease remain commonplace.

Glucocorticoid modulation of the immune response

To determine why patients display SR phenotypes requires dissection and interrogation of the function and structure of the GC receptor (GR), and the signaling cascades which are consequent on its activation. As such, it is important to consider that SR may be multifactorial; not solely secondary to mutations or gene polymorphisms that control the GR and potentially generate a null response, but also SR may manifest from deviant T cell responses to normal GR activation as a result of change in T cell or T cell subset behavior.

Structure of the GR

The human GR is a member of the steroid-hormone-receptor family of proteins⁽¹¹⁾ and is expressed in almost every cell in the body. It is made up of 777 amino acids and was first cloned in the mid 80s⁽¹²⁾ and sequenced in the early 90's.⁽¹³⁾ The GR gene is located on chromosome 5q31-q32 and there are 3 promoter regions which can produce several GR messenger RNAs (mRNA). Exon 9 has two isoforms, which when spliced produce GR α and GR β mRNA.⁽¹⁴⁾ These two isoforms are homologous except for their carboxyl terminus: GR β has a unique 15 amino acid sequence in place of the last 50 amino acids present in GR α .⁽¹⁵⁾ This region of variation between GR α and GR β is located in the hormone-binding domain, where it is the GR α isoform that binds ligand, DNA and

other transcription factors.⁽¹⁶⁾ GR β can bind DNA but not ligand and can, in some cell types, act as a dominant negative inhibitor of GR α .⁽¹⁷⁾

Activation of the GR

In the absence of GCs, the GR remains inactive in the cytoplasm. The inactive form is a heterodimeric complex made up of two heat shock protein (hsp) 90 molecules, a p23 subunit and one of several immunophilin-related proteins.⁽¹⁸⁾ The hsp90 molecules are important for maintaining the GR's shape and inhibiting its translocation to the nucleus. GCs are lipophilic and pass easily from the extracellular space across the cell membrane via passive diffusion. When GCs bind, the GR undergoes a conformational change and the multi-protein complex dissociates.⁽¹⁹⁾ Together, the GR-GC complex translocates into the nucleus and binds to DNA as a homodimer at glucocorticoid response elements (GREs) and GC responsive genes are transcribed (Fig.1).⁽²⁰⁾

Post-translational modifications through phosphorylation also regulate the GR. As a phosphoprotein, both ligand binding and further phosphorylation are necessary for full activation.⁽²¹⁾ There are 3 potential phosphorylation sites at serine (Ser) 203, 211, and 226. Phosphorylation at Ser 203 and 211 determines translocation and functional activation of GR. In the absence of ligand binding (presence of GC), the GR is phosphorylated at Ser 203. When GCs are present, there is an increase in the phosphorylation at Ser 211 relative to Ser 203 and it is this Ser 211-phosphorylated GR that is found in the nucleus and correlates with the amount of transcriptional activity; whilst in the absence of GC, GR phosphorylation at Ser 203 retains GC in the cytoplasm.⁽²²⁾ Mitogen activated protein kinases (MAPK) are responsible for Ser 226 phosphorylation which inhibits GR-mediated transcriptional activation by facilitating nuclear export.⁽²³⁾

Transactivation by GCs

Many inflammatory genes are controlled by GCs. Genes such as Annexin-1 (Anx-1), mitogen-activated protein kinase phosphatase-1 (MKP-1), glucocorticoid-induced leucine zipper (GILZ), and glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR) play a vital role in activation and function of immune cells⁽³⁾ as well as the production of inflammatory mediators such as prostaglandins and nitric oxide.⁽¹⁾ The GC-GR complex activates and regulates these genes via transactivation.

Anx-1, also known as lipocortin-1, was first identified as a GC-inducible protein, and has been shown to be involved with leukocyte migration, acute and chronic inflammation, ischemic damage, pain and fever.⁽²⁴⁾ It mediates GCs anti-inflammatory action on cytosolic

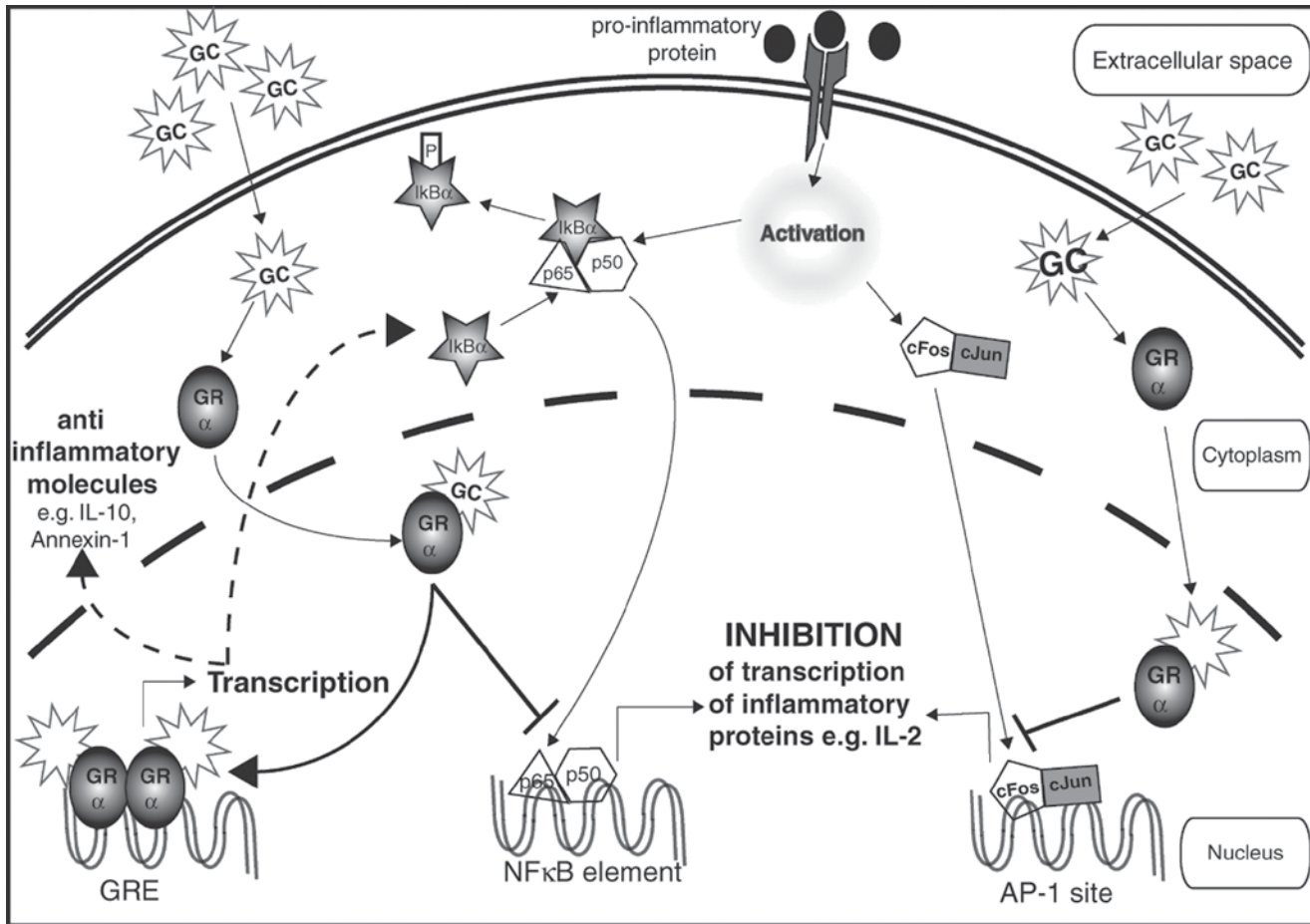


Figure 1. Mechanism of action of glucocorticoids in the inhibition of inflammation. Glucocorticoids (GCs) enter the cell and bind to the cytoplasmic GC receptor alpha (GR α). The GR α -GC complex translocates into the nucleus, binds with other activated GR α subunits and the homodimer then binds to GC response elements (GRE) on DNA. Anti-inflammatory proteins and Inhibitor of κ B alpha (I κ B α) molecules are subsequently transcribed (I κ B α causes cytosolic retention of nuclear factor- κ B, NF κ B). Activated GR α can further suppress NF κ B by inhibiting the p65 subunit. It also suppresses activator protein 1 (AP-1). Both result in transcriptional suppression of interleukin-2 (IL-2) as well as other pro-inflammatory mediators (IL-1, TNF α , IFN γ). Other abbreviations: cFos + cJun = AP-1; IL-10 (Interleukin-10); p50 (p50 subunit of NF κ B); p65 (p65 subunit of NF κ B); TNF- α (tumor necrosis factor- α); p50 + p65 = NF κ B; p = phosphorylation.

phospholipase A $_2$ (cPLA $_2$) controlled arachidonic acid release.^(25, 26) Attempts to interrogate the pathophysiology of Anx-1 have shown that Anx-1 null mice have upregulated cyclooxygenase-2 (COX-2 - involved in stimuli-induced prostaglandin production) and cPLA $_2$ levels, display exacerbated inflammation and are partially resistant to the synthetic GC, dexamethasone (Dex). Their leukocytes also show altered adhesion molecule expression with a reduction in total CD11b and an upregulation of CD62L on monocytes and neutrophils.⁽²⁴⁾

Another important anti-inflammatory protein transcribed by GCs is MKP-1. Transcription of inflammatory genes is inhibited when MKP-1 inhibits c-Jun mediated transcription as well as the MAPK family of proteins. Consequently, phosphorylation of cPLA $_2$ by MAPKs and MAPK-interacting kinase is also blocked⁽¹¹⁾. Upregulation of MKP-1 and Anx-1 by GCs inhibits

MAP kinases, p38 and c-Jun-n-terminal Kinase (JNK) pathways, leading to a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines.⁽²⁷⁾ Similarly, GCs inhibit NF κ B via upregulation of the NF κ B inhibitor, I κ B α (see transrepression below), and the anti-inflammatory cytokine, IL-10, is upregulated.⁽²⁸⁾

Transrepression by GCs

GRE sequences lack genes that encode proteins for inhibition of pro-inflammatory molecules and it has been shown that the transcription factors: activator protein 1 (AP-1), NF κ B, nuclear factor of activated T cells (NFAT), cyclic adenosine monophosphate response element binding (CREB), and members of the signal transducers and activators of transcription (STAT) family, can be inhibited ("transrepressed") by

the GR, without the GR binding to the GRE.⁽¹⁰⁾ The first described GR protein-protein interaction was with AP-1, the result of which is mutual repression of both proteins. Initially, this effect was thought to be mediated by reduced binding to their respective recognition sites in DNA. However, the GR interacts with the AP-1 components, Jun and Fos⁽²⁹⁻³¹⁾ and antagonizes AP-1 activity without altering AP-1 site occupancy *in vivo* (Figure 1).⁽³²⁾ This is corroborated by studies using GR^{dim/dim} mice. Unlike GR deficient mice, in which the mutation is lethal, viable GR^{dim/dim} mice have a point mutation in the GR gene DNA binding domain which prevents dimerization and DNA binding.⁽³³⁾ Results convincingly show that although unable to mediate transcription from the GRE, the GR is still able to modulate transcription of AP-1.⁽³⁴⁾

A major anti-inflammatory mechanism results from the GR-NFkB complex. NFkB is ubiquitous and key to the induction of many cytokines, chemokines and adhesion molecules. It consists of a p65 (RelA) and a p50 subunit, is found in the cytoplasm in its inactive form and is tightly controlled by IkB subunits. Activation signals, such as TNF- α , IL-1 β , lipopolysaccharide (LPS), and stress and viral agents lead to phosphorylation and degradation of IkB and activation of NFkB.⁽³⁵⁾ In a negative feedback loop, IkB α , which plays a central role in NFkB regulation, is quickly resynthesized and terminates NFkB activity. Indirectly, GCs are able to affect NFkB activity by transactivating upregulation of the IKBA gene, leading to increased IkB α protein synthesis and subsequent inhibition of NFkB (Fig. 1).⁽³⁶⁾

However, this is not the only mechanism of GR-mediated inhibition of NFkB activity as GR^{dim/dim} mice still show GC dependent inhibition of NFkB⁽³⁴⁾ which, like AP-1, is mediated by direct interaction between the GR and endogenous NFkB, preventing NFkB from binding to DNA. Other studies have shown that cytokine-induced nuclear translocation of NFkB is also blocked via the interaction of activated GR with its p50 and c-rel subunits.⁽³⁷⁾

Transrepression of the IL-2 gene is another significant immunosuppressive action of GCs. Dex inhibits nuclear transcription of the IL-2 gene in human T cells, although not through direct binding to the gene as it lacks a GRE.⁽³⁸⁾ How GCs mediate negative transcription of the IL-2 gene has been explored extensively (although somewhat less in human lymphocytes), revealing that GCs are able to interfere with the binding sites of NFAT, NFkB, and AP-1 for the IL-2 promoter.^(38,39)

Non-genomic effects of GCs

Apart from the previously discussed classical genomic effects of GCs which are mediated through the GR,

there are also rapidly acting, non-genomic effects. These mechanisms occur too quickly to be explained by up or down regulation of protein synthesis and therefore are unlikely to involve gene expression.^(11,40) The best described involves activation of endothelial nitric oxide synthase (eNOS), in which the GR-GC complex stimulates phosphatidylinositol-3-hydroxykinase (PI3K) in a GR-dependent but transcription-independent manner. Once activated, PI3K phosphorylates a succession of downstream molecules, leading to the eventual activation of eNOS and nitric oxide (NO)-dependent vasorelaxation.⁽⁴¹⁾ These non-genomic effects are especially pertinent to the treatment of acute conditions, such as anaphylaxis and spinal cord injury,⁽⁴²⁾ but are unlikely to be as significant as gene transrepression and transactivation in the context of GC therapy for chronic disease.

GCs and T cell subsets

It is well established that Type 2 helper T cell (Th2) responses, which are characterized by the production of anti-inflammatory cytokines such as IL-4, IL-5, IL-10, and IL-13 and an antibody-mediated defense against foreign antigens, are promoted by GCs.⁽⁴³⁾ GCs exert this switch from Th1 (IFN γ , IL-2) to Th2 mainly by down regulation of macrophage and dendritic cell (DC) derived IL-12.^(44,45) Although suppression of IL-2 and IFN- γ ⁽³⁴⁾ (and secretion of IL-4 and IL-10 by macrophages, DCs, and mast cells) is induced in presence of GCs, a study on peripheral blood mononuclear cells (PBMCs) demonstrated that GCs differentially regulate IL-10 and IL-12 expression as Dex was still able to inhibit IL-12 production⁽⁴⁶⁾ when IL-10 was neutralized. These changes all contribute to the Th2 shift and ultimately DC, macrophage and T cell inactivation.

As more mechanisms of GC action are elucidated, more detailed effects on T cell subsets are being revealed which have the potential to provide novel approaches to the treatment of autoimmune diseases and allograft rejection. Of particular interest are T regulatory cells (Tregs), which are responsible for inhibiting immune responses and maintaining self-tolerance. Naturally occurring Treg populations (nTregs) contain a member of the Forkhead/winged-helix transcriptional regulators, FoxP3. In humans, these predominantly reside in the 2.5% of CD4⁺ cells which most highly express CD25.⁽⁴⁷⁾ However, a distinct subset of CD4⁺ Tregs (called Tr1 cells), that secrete IL-10 and are FoxP3 negative, can be induced by GCs.^(48,49) This may be a hitherto overlooked, but an important mechanism of GC induced immunosuppression, which is supported by the observation that Dex driven Tr1 cells inhibit autologous T cell proliferation in an IL-10 dependent manner.^(50,51)

In animal studies, BALB/c mice injected with Dex have increased numbers of FoxP3⁺ CD4⁺CD25⁺ Tregs, and the ratio of CD4⁺CD25⁺ to CD4⁺CD25⁻ cells is enhanced in lymphoid organs.⁽⁵²⁾ Furthermore, *in vitro*, CD4⁺CD25⁺ cells, show increased levels of GR, greater resistance to dexamethasone-induced apoptosis and persistent inhibition of proliferation in responder cells.⁽⁵²⁾ This is of translational relevance as asthma patients receiving GC treatment upregulate FoxP3 and IL-10 mRNA, adding weight to the suggestion that an important mode of GC action is through the induction of Tregs.⁽⁵³⁾

What drives steroid refractory disease?

The SR phenotype reportedly affects up to a third of patients with ulcerative colitis (UC),⁽⁵⁴⁾ RA,⁽⁵⁵⁾ systemic lupus erythematosus (SLE),⁽⁵⁶⁾ asthma,⁽⁵⁷⁾ uveitis,⁽⁵⁸⁾ allograft rejection,⁽⁵⁹⁾ and childhood acute lymphoblastic leukemia (ALL).⁽⁶⁰⁾ Hence, from a cross-specialty perspective, the burden of morbidity and mortality attributable to SR disease is considerable. One approach to elucidate its cause is to investigate GC modulation of effector responses, namely T cell function. With this in mind, approximately 30% of healthy volunteers' PBMCs also fail to respond to GCs *in vitro*, in that they maintain proliferation to PHA in the presence of Dex.⁽⁶¹⁾ Such findings allude to a concept of a common underlying phenotype that is intrinsic to an individual and not attributable to disease type, or due to prolonged or severe inflammation.⁽⁶¹⁾

Despite the clinical significance of SR disease, its molecular basis is poorly understood. Given that GCs and the GR have such a wide range of effects on signal transduction, there are a number of potential mechanisms that could be affected (as discussed above). These include different GR isoforms and their interaction, GR gene mutations and polymorphisms, differences in GR-transcription factor "cross-talk", and the relationship between GCs and cytokines - both pro-inflammatory, such as IL-2, and anti-inflammatory, such as IL-10.

GR isoforms

GC effects are mediated through GR α while the GR β isoform does not bind ligand or activate GC-sensitive genes. Predominantly found in the nucleus (independent of hormone), GR β has an intact DNA binding domain and can bind GREs.^(17,62,63) Overall, GR β accounts for 0.2–1% of total GR expression.⁽⁶⁴⁾ However, the actual level of expression throughout tissue types is not homogeneous; for example, the ratio of GR α /GR β in PBMCs is 600:1⁽⁶⁵⁾ and in the pituitary is 40:1.⁽⁶⁶⁾

Equally, GR β is overexpressed by PBMCs from SR asthmatics,^(67–69) SR RA,^(64,70) SR UC⁽⁶⁵⁾ and SR Crohn's disease⁽⁷¹⁾ patients when compared to their steroid sensitive (SS) counterparts.

Additionally, low GR α expression with normal to high expression of GR β was found in leukemic lymphoblasts from ALL patients in comparison to controls.⁽⁷²⁾ The increased or altered GR β levels relative to GR α is hypothesized to drive SR phenotype but the mechanism of action of GR β has not been fully elucidated. It is thought to be a dominant negative regulator of GR α , as GR β is able to inhibit activated GR α in a dose dependent manner, by forming a heterodimer with GR α and rendering it transcriptionally inactive.⁽⁷³⁾ However, in a GC-responsive reporter system, GR α -GC affinity was not altered by GR β and its inhibitory effect was thought to be due to competition for GRE binding sites on DNA.⁽¹⁷⁾

Much of this work has been carried out in transfected systems and there is contradictory evidence to suggest that this dominant negative role of GR β is cell specific and not likely to solely account for SR.^(74,75) In a recent study of healthy volunteers' PBMCs, the GR α /GR β ratio showed a normal distribution with a higher expression of GR α and inter-individual variability between GR α and GR β . No correlation between the GR α /GR β ratio and the dose of Dex required to suppress plasma cortisol was found. Together the data suggest that variability of GC sensitivity in healthy individuals is unlikely to be secondary to changes in GR α and GR β isoform status,⁽⁷⁶⁾ notwithstanding that GR β expression may have pathophysiological and therapeutic implications.

GR polymorphisms and potential abnormalities in transcription factors

Evidence for polymorphic differences within the GR being the cause of SR disease is inconclusive. For example, a polymorphism at codon 363 in the GR has been shown to increase GC sensitivity but has also been shown not to correlate with the SR phenotype.⁽⁷⁷⁾ In addition, there is a rare hereditary condition called familial GC resistance, which is characterized by GR mutation and diminished cortisol action, with compensatory hyperactivity of the HPA axis.^(78,79)

Release of pro-inflammatory cytokines triggers numerous signaling molecules and, as described above, the activation of NF κ B, AP-1, Anx-1, MKP-1, and the STAT family are all known to be modulated by GCs and therefore may counter-regulate GC/GR interactions. Thus, the SR phenotype could feasibly be perpetuated by a mutation within a transcription factor and/or any deficiency in the GR's interaction with it. Consistent with this, CD4⁺ T cells from SR nephrotic syndrome children, show a

decrease in the NF κ B p65 subunit as well as increased IL-2 mRNA when compared to SS nephrotic syndrome patients.⁽⁸⁰⁾ Nevertheless, the precise mechanistic implications of these observations remain unclear.

Cytokine-mediated suppression of GC signaling

Whether the SR phenotype is generated from a deviant T cell response in the face of GCs has been mooted since 1979, following reports that *in vitro* human T cells given IL-2, proliferate in response to mitogenic stimulation despite the presence of GCs.⁽⁸¹⁾ There is much support for this concept. First, IL-2, the canonical cytokine for lymphocyte proliferation, growth and survival is transcriptionally regulated by GCs.⁽⁸²⁾ Second, IL-2 (in combination with IL-4) decreases PBMC GR-binding affinity, similar to that seen in SR patients.⁽⁸³⁾ Third, Dex inhibits IL-2 production in cells from SS asthmatic patients but given to SR asthmatic patients' cells, is not able to inhibit IL-2 production and

consequently a higher dose of GC is required to inhibit T cell proliferation.^(84,85) The amount of IL-2 present in PBMC cultures from SR individuals is also greater than in SS PBMC cultures.⁽⁸⁶⁾

Could this decreased IL-2 responsive to Dex explain why some patients are SR? It is not known why SR patients have a decreased response to IL-2 (Fig. 2). However, there is evidence to suggest that STAT5 plays a central role.^(87,88) IL-2 receptor engagement rapidly phosphorylates STAT5 and the GR reciprocally acts as a transcriptional co-activator for STAT5 at the gene encoding β -casein.⁽⁸⁷⁾ So, GR and STAT5 bind together, and enhance STAT5 dependent transcription independent of a GRE.

Conversely, IL-2 mediated STAT5 activation can diminish the induction of GC-responsive genes leading to a reduced GC response of a GRE-containing promoter.^(19,87) Furthermore, in an IL-2 dependent murine line, culturing with IL-2 rendered cells SR and the GR did not translocate to the nucleus. Additionally, splenocytes from STAT5 knockout mice were not SR when cultured with IL-2. Taking

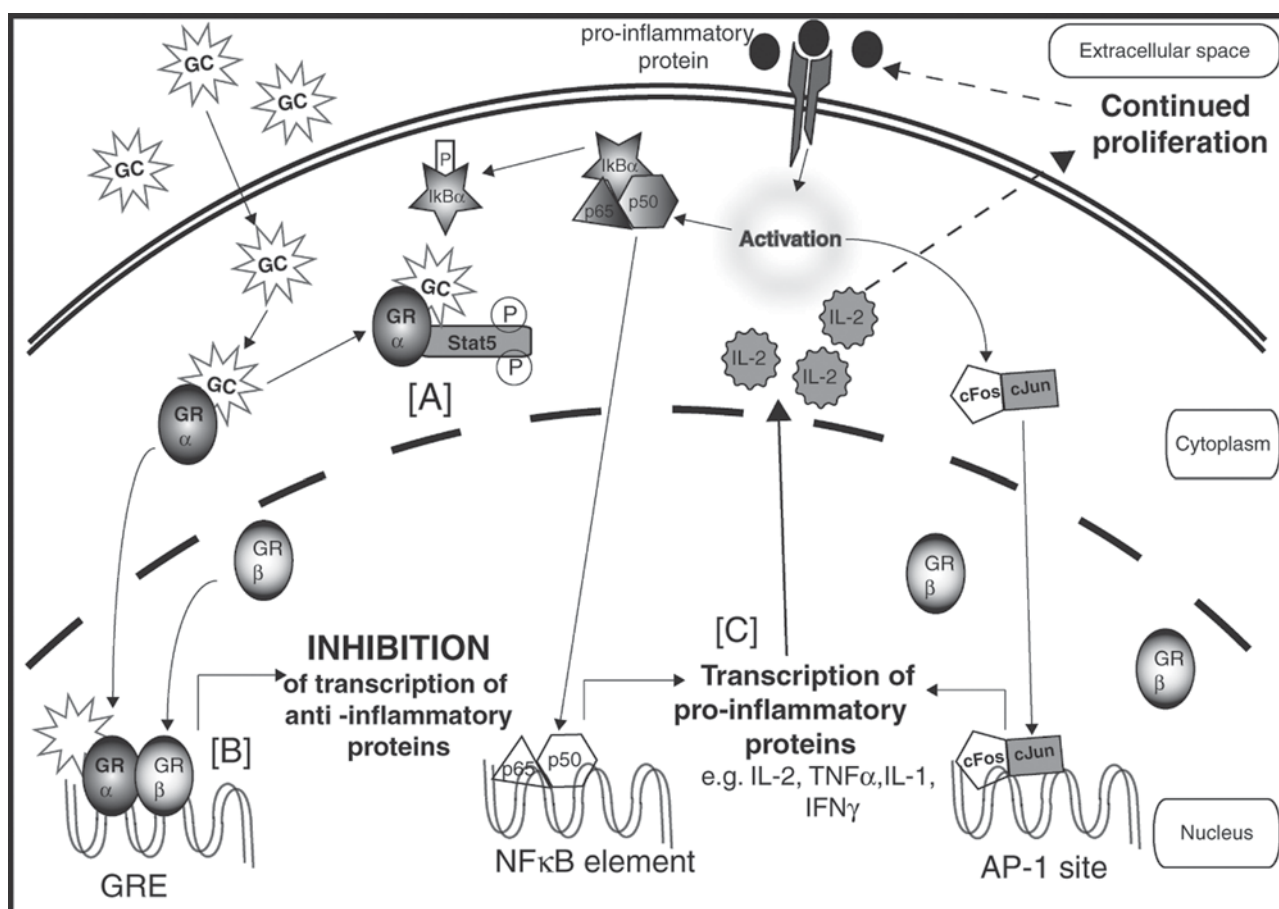


Figure 2. Inhibition of glucocorticoid action in steroid refractory disease. [A] Interleukin-2 (IL-2) induced activation of Signal Transducer and Activator of Transcription-5 (STAT5) inhibits nuclear translocation of ligand-bound glucocorticoid receptor alpha (GR α), thus preventing [B] transcription of glucocorticoid (GC) induced anti-inflammatory proteins and [C] inhibition of pro-inflammatory protein transcription. Increased amounts of GR β further inhibit GC action by forming inactive heterodimers with GR α at GC response elements (GRE) in DNA. Other abbreviations: cFos + cJun = AP-1; IL-10 (Interleukin-10); p50 (p50 subunit of NF κ B); p65 (p65 subunit of NF κ B); TNF- α (tumor necrosis factor- α); p50 + p65 = NF κ B; p = phosphorylation; Nuclear factor- κ B (NF κ B), activator protein 1 (cFos/cJun); I κ B α (Inhibitor of κ B alpha).

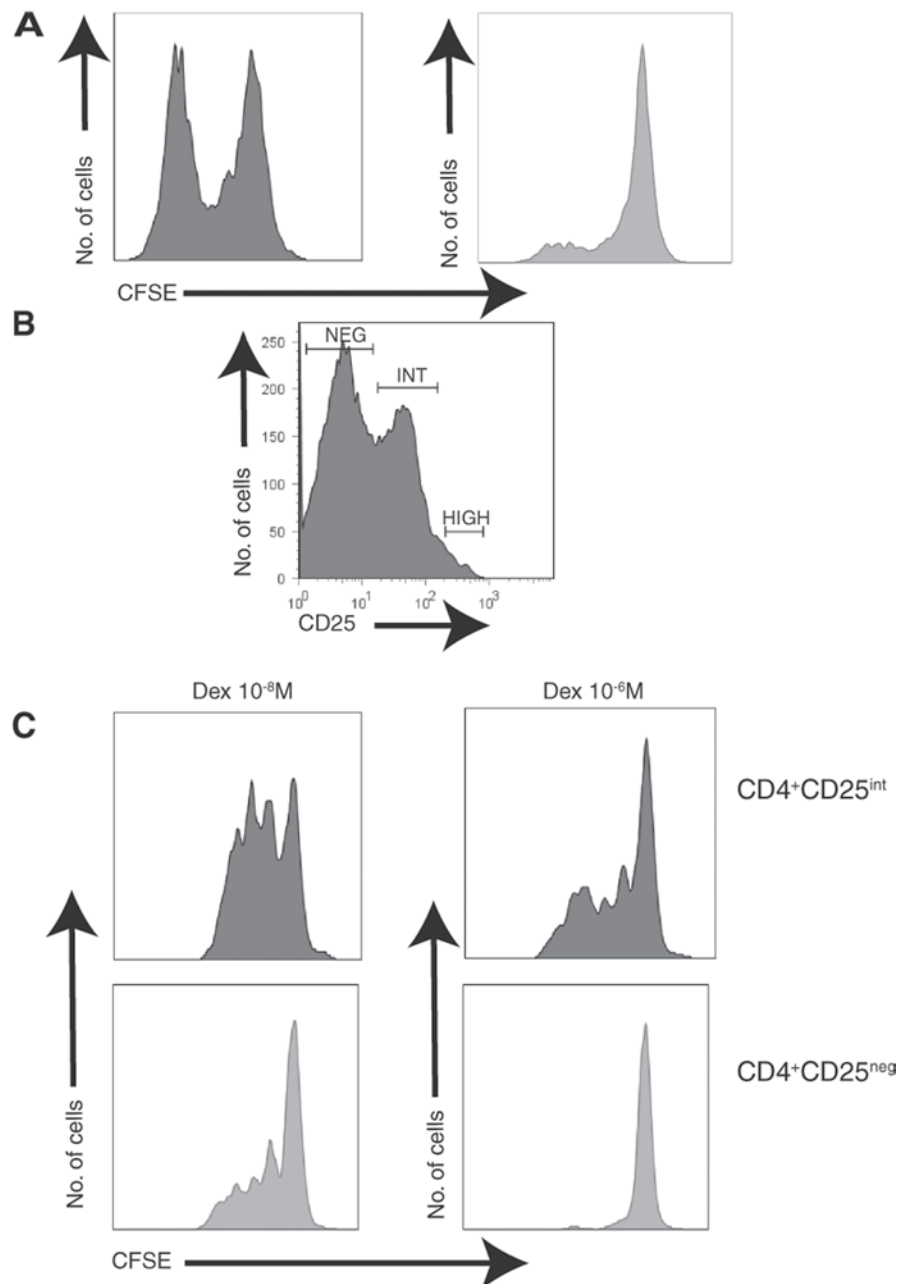


Figure 3. Characteristics of CD4⁺CD25^{neg} and CD4⁺CD25^{int} cells. A, Carboxyfluorescein succinimidyl ester (CFSE) dilution in CD4⁺ T cells from a patient with steroid refractory (SR) ulcerative colitis (left graph) and a steroid sensitive (SS) normal volunteer (right graph) after *in vitro* culture in the presence of high dose glucocorticoids (GCs). The SR patient has a distinct subpopulation of T cells which continue to divide despite the presence of GCs (left peak). B, Expression of CD25 in human peripheral blood CD4⁺ T cells. Neg = no expression (approx 60% of cells). Int = intermediate expression (approx 37.5% of cells). Hi = maximal expression (approx 2.5% of cells, almost all of which co-express the T regulatory cell marker FoxP3). C, Representative CFSE proliferation profiles of CD4⁺CD25^{int} (top row) and CD4⁺CD25^{neg} (bottom row) cells after *in vitro* culture with dexamethasone (Dex). CD4⁺CD25^{neg} cells are exquisitely SS, whereas CD4⁺CD25^{int} cells are relatively SR, even in the presence of high dose (10⁻⁶M) Dex.

these data together, when STAT5 is already activated (e.g., by IL-2), it binds the ubiquitous GR, preventing its translocation and subsequent suppression of gene transcription and offers a mechanism by which IL-2 induces SR in lymphocytes.⁽⁸⁸⁾ As such, inhibiting IL-2 mediated STAT5 activation has attractive therapeutic potential to sensitize patients to GC treatment (see below).

What is the role of T cell subsets in steroid refractory disease?

IL-10 secreting Tr1 cells

In severe asthma, IL-10 secretion from alveolar macrophages and circulating monocytes is abnormally

suppressed.⁽⁸⁹⁾ Additionally, CD4⁺ T cells from SR asthmatics do not generate IL-10 when cultured in the presence of Dex,⁽⁹⁰⁾ leading to a postulate linking the SR phenotype with failure to induce IL-10 secreting Tr1 cells (see above). Accordingly, interventions which restore Dex-induced IL-10 secretion in SR patients' CD4⁺ cells have therapeutic potential. The most promising candidate to date is vitamin D3, which has been shown to enhance IL-10 production in T cells from SR asthmatics to levels seen in SS controls.⁽⁹¹⁾ The potential applicability of this strategy to the clinic has been further demonstrated in a proof-of-concept pilot study in which oral administration of vitamin D3 increased Dex-induced IL-10 secretion by CD4⁺ T cells from SR patients.⁽⁹¹⁾ However, as yet there are no reports relating this observation to improved clinical outcomes.

CD4⁺CD25^{int} cells

As the disparities in GC-induced Tr1 cells between SS and SR asthmatics demonstrate, there is still much to be

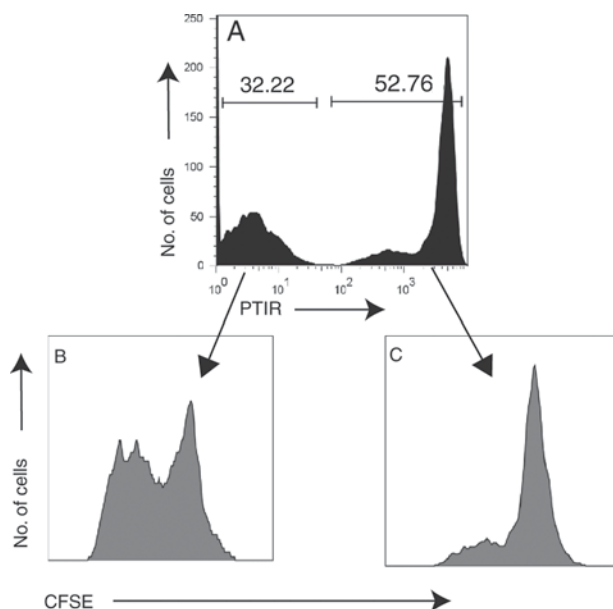


Figure 4. CD4⁺CD25^{int} cell proliferation in mixed CD4⁺ cell cultures. CD4⁺CD25^{neg} and CD4⁺CD25^{int} cells were cell sorted (FACS Vantage), separately stained with CFSE and then the CD4⁺CD25^{neg} cells were additionally stained with a membrane dye, PTIR. Both subsets were remixed in the proportion originally found in peripheral blood mononuclear cells (PBMCs), set up in culture with/without dex and stimulated with CD3/28 beads for 5 days. **A**, Representative PTIR profile ($n = 4$) of cells cultured with 10^{-6} M Dex. Clearly defined PTIR^{pos} and PTIR^{neg} populations are marked. **B**, CFSE profile of CD4⁺CD25^{int} (PTIR^{neg}) cells, demonstrating that in mixed CD4⁺ cell cultures it is this subpopulation which continues to vigorously proliferate in the presence of high dose GCs. **C**, CFSE profile of CD4⁺CD25^{neg} (PTIR^{pos}) cells, demonstrating that high dose GCs suppress their proliferation. In cultures with no dex (data not shown), both the PTIR^{pos} and PTIR^{neg} populations had equivalent CFSE proliferation, indicating that the lack of proliferation seen in C is not due to a toxic effect of the combined PTIR and CFSE stains. Other abbreviations: CFSE (carboxyfluorescein succinimidyl ester); FACS (Fluorescence-activated cell sorting).

learned from honing in on more specific cell subpopulations with genuine potential for bench-to-bedside translation. This is further illustrated by evidence for the presence of a characteristic SR CD4⁺ T cell subpopulation in patients with SR UC (Fig. 3A),⁽⁹²⁾ which corroborates the hypothesis that the SR phenotype is post-GR-receptor controlled and is not a feature of all immune cells. We have also recently shown that the same SR CD4⁺ T cells found in SR UC patients are present in SR uveitis patients, thus substantiating the concept of the SR phenotype as a discrete entity, independent of disease type.⁽⁹³⁾

CD4⁺ T cells expressing intermediate levels of CD25 (CD4⁺CD25^{int}) (Fig. 3B) are primed to proliferate, and as such they are obvious candidates for involvement with, or indeed perpetration, of SR disease. As CD25 is the alpha subunit of the IL-2 receptor, it is by implication central to the modulation of *in vitro* and *in vivo* steroid responsiveness by IL-2, as discussed above. Hence, it is logical that CD4⁺CD25⁺ cells have been shown to be more SR than CD4⁺CD25⁻ cells (Fig. 3C);⁽⁹²⁾ a finding that has recently been confirmed using cell-tracking dyes in mixed CD4⁺ cell cultures (Fig. 4), and is also consistent with Phase II clinical trial evidence that anti-CD25 monoclonal antibody (mAb) therapies rescue SR UC⁽⁹⁴⁾ and SR uveitis.⁽⁹⁵⁾

So could the SR phenotype be due to greater CD4⁺CD25^{int} cell activation? Preliminary evidence suggests not. CD4⁺CD25^{int} cells account for 30% of all circulating CD3⁺ cells and less than 25% are activated by CD69, HLA-DR, CD62L and CD44 expression. Furthermore, CD4⁺CD25⁻ cells activated to express intermediate levels of CD25 expression before culture do not become SR.⁽⁹²⁾ There is also no difference in the proportion of CD4⁺CD25^{int} cells in the peripheral blood of SR UC patients and normal volunteers (NVs).⁽⁹²⁾ Hence, it is likely that activation/CD25^{int} expression alone does not account for the SR phenotype, and SR CD4⁺ T cells may therefore represent only a fraction of the CD25^{int} cell subset.

Conclusion

As a whole, current evidence suggests that cytokine-mediated suppression of GC signaling (in particular IL-2), and disparities in GC-induced IL-10 responses, are likely to make a greater contribution to the SR phenotype than GR polymorphisms or isoforms. This has recently been refined to implicate specific IL-2 responsive (CD25^{int}), and IL-10 secreting (Tr1), CD4⁺ T-cell subsets in the perpetration of SR disease. Hence, the field is now moving toward immunotherapies which specifically target these T cell subsets to combat the cross-specialty burden of SR disease. Of these, anti-CD25 mAbs are the only treatments to be tested in clinical trials (although only

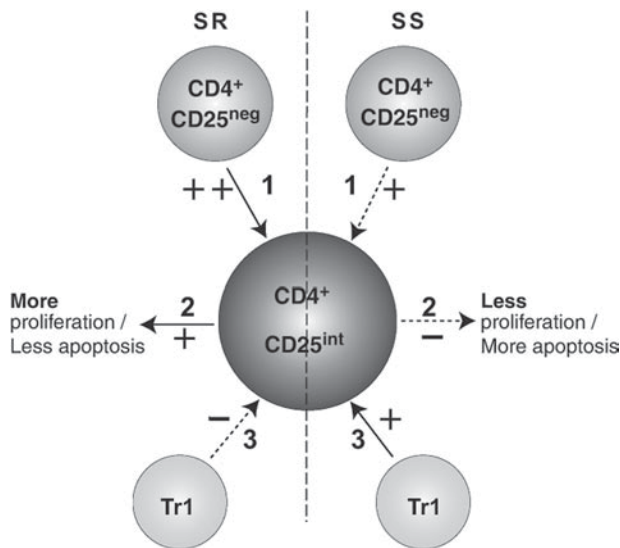


Figure 5. Potential mechanisms by which $CD4^+CD25^{int}$ cells perpetrate SR disease. (1) $CD4^+CD25^{int}$ cells are generated more readily and rapidly in steroid refractory (SR) than steroid sensitive (SS) individuals. (2) SR $CD4^+CD25^{int}$ cells are more resistant to glucocorticoids (GCs) than their SS counterparts; either due to enhanced proliferation or resistance to GC-induced apoptosis. (3) $CD4^+CD25^{int}$ cells may continue to proliferate in SR patients because of impaired induction of Tr1 cells, which are a distinct subset of GC-induced, interleukin (IL)-10 secreting T regulatory cells.

currently involving Phase II studies), with promising results.

We therefore postulate a new paradigm for the SR phenotype with $CD4^+CD25^{int}$ cells being central. We have showed that GCs positively select SR $CD4^+CD25^{int}$ cells which may in turn generate a drug refractory subpopulation, thus perpetuating on-going inflammation. The potential mechanism by which $CD4^+CD25^{int}$ cells perpetrate the SR phenotype is still unknown. However, we propose that the generation, response and regulation of the $CD4^+CD25^{int}$ cells all play a role (Fig. 5).

These ideas raise the following questions: do $CD4^+CD25^{int}$ cells from SR patients proliferate more in the presence of GCs when compared to SS individuals, and is GR function or GC induced apoptosis altered in $CD4^+CD25^{int}$ cells from SR patients? Finally, what is the relationship between SR $CD4^+CD25^{int}$ cells and Tregs, and given that the induction of Tr1 cells is defective in SR asthma patients,⁽⁹¹⁾ what is the contribution of a blunted GC-Treg drive to the SR phenotype as a whole, i.e. is Tr1 suppression also impaired in other SR diseases?

The benefits of answering these questions extend beyond identifying novel therapies, as identification of SR disease-associated T cell subsets also potentially holds the key to diagnosis of the SR phenotype. Such an advance would enable patients' GC requirement to be determined *a priori*, thus circumventing the current empirical approach to therapy, which frequently results in the delayed introduction of

additional immunotherapies. To this end, work is ongoing toward development of an application to predict SR disease and facilitate tailored, individualized immunosuppression.

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