# STEROIDS AND SECOSTEROIDS IN THE MODULATION OF INFLAMMATION AND IMMUNITY

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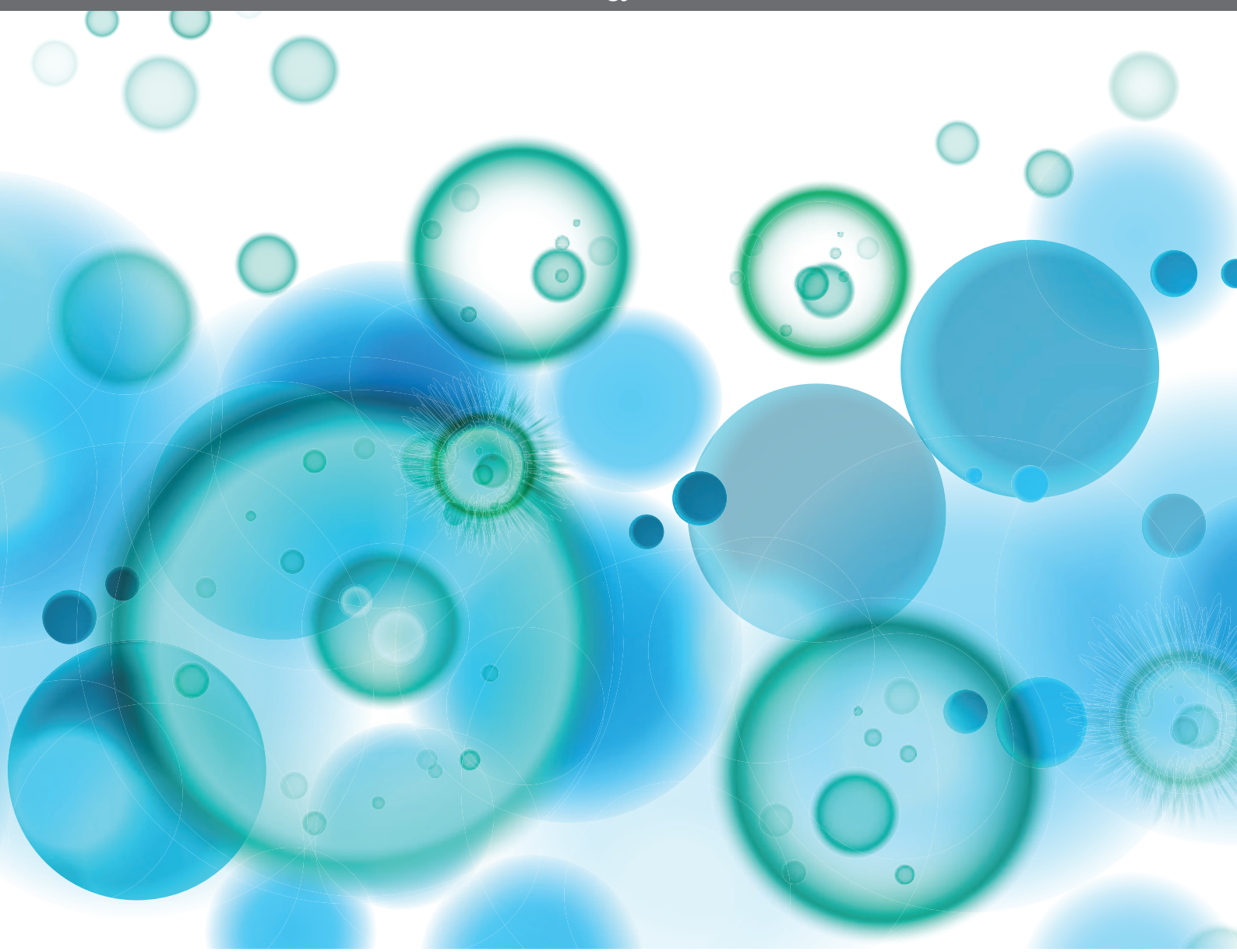

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# STEROIDS AND SECOSTEROIDS IN THE MODULATION OF INFLAMMATION AND IMMUNITY

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EDITORIAL
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# Editorial: Steroids and Secosteroids in the Modulation of Inflammation and Immunity

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**Keywords: steroids, secosteroids, steroidogenesis, secosteroidogenesis, Inflammation, immunity, vitamin D, glucocorticoids**

**Editorial on the Research Topic**

**Steroids and Secosteroids in the Modulation of Inflammation and Immunity**

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In this Research Topic of Frontiers in Immunology focused on the steroids and secosteroids in the modulation of inflammation and immunity 11 articles by experts in corresponding fields have been published (Bier et al.; Bruscoli et al.; He et al.; König et al.; Lucafò et al.; Merk et al.; Postlethwaite et al.; Quatrini et al.; Shimba et al.; Vanderhaeghen et al.; Xie et al.). These included research articles, reviews and mini-reviews papers on important aspects of steroid- and secosteroidogenesis and the role of the final or intermediate products of these pathways in regulation of inflammatory and immune activities. Mechanisms of action and of broad homeostatic activities in humans and experimental animal models have also been discussed in these expert written papers. Different aspects of biochemistry, molecular biology, cell biology, and the systems-level role of (seco) steroidogenesis in regulating physiology and pathology have been discussed.

The key role in steroidogenesis is played by an enzyme CYP11A1, a member of the cytochrome P450 family, which catalyzes the first and rate-limiting step in steroidogenesis, converting cholesterol to pregnenolone through sequential its hydroxylation at C22 and C20, with a final cleavage of the side chain (1–4). In addition to the adrenals, gonads and placenta (classical steroidogenic tissues), CYP11A1 is also expressed in the brain (6), gastrointestinal tract, immune systems (7–9), the skin (5) and other peripheral organs/tissues (10) including malignant tumors (9, 11). The roles of local steroidogenesis (i.e., extra-glandular steroidogenesis, including immune cell mediated steroidogenesis) are emerging and warrant a revisit to this important biosynthetic pathway and its functional involvement in tissue homeostasis (including immune homeostasis) and disease (7, 12, 13). Recent discoveries pointed out an important, and unexpected role for CYP11A1 in metabolism of 7-dehydrocholestrol (14), vitamin D3 (15), D2 (16), lumisterol (17, 18) and ergosterol (11) to several biologically active metabolites. Thus, CYP11A1 activity is opening several novel pathways generating Δ7 steroids, full chain and short chain lumisterol derivatives, and secosteroids (vitamin D hydroxyderivatives) (5, 19). While the biological significance of these pathways is currently being evaluated, it must be noted that CYP11A1-derived hydroxyderivatives of vitamin D (20, 21) and of lumisterol (18) are circulating in human serum and

are detectable in the epidermis. Furthermore, recent data showing that 7-Dehydrocholesterol reductase (DHCR7, which catalyzes the reduction of the C7-C8 double bond of its B-ring that is necessary for the final formation of cholesterol) did not act on 7-dehydropregnenolone and lumisterol compounds (22), enhances the importance of pathways generating Δ7 steroids and lumisterol derivatives (20, 23, 24).

As relates to secosteroids, in this Research Topic, a paper by Postlethwaite et al. reports that CYP11A1-derived 20S-hydroxyvitamin D3 [20S(OH)D3] markedly suppresses clinical signs of arthritis and joint damage in a mouse model of rheumatoid arthritis (RA). 20S(OH)D3 also changes proportion of lymphocyte subsets in peripheral blood resulting in a significant reduction in the levels of inflammatory cytokines, and decrease in complement-fixing anti-CII antibodies. The authors propose further consideration for 20S(OH)D3 in treatment of RA and other autoimmune disorders (Postlethwaite et al.).

Concerning extra-adrenal glucocorticoids synthesis, Merk et al. review a role of epithelial barriers as alternative routes for their synthesis at the local and perhaps systemic levels. Their role in the inter-organ communication through an interconnected crosstalk that counteract pro-inflammatory activities and prevent autoimmune activities are discussed (Merk et al.). These considerations are in line with similar concepts previously proposed (7, 13, 25, 26) and are consistent with communication between peripheral and central endocrine regulators (27) including hypothalamo-pituitary-adrenal axis (**Figure 1**) (7, 28, 29). Therefore, regulation of local corticosteroidogenesis may serve as a viable therapeutic alternative to using synthetic corticosteroids in the therapy of inflammatory or autoimmune disorders (Merk et al.; 7; 13). Such regulation can be achieved by using specific wavelength of ultraviolet radiation (UVR) (30).

Of similar importance are three other mini-reviews and two reviews. One on regulation of the immune system development by

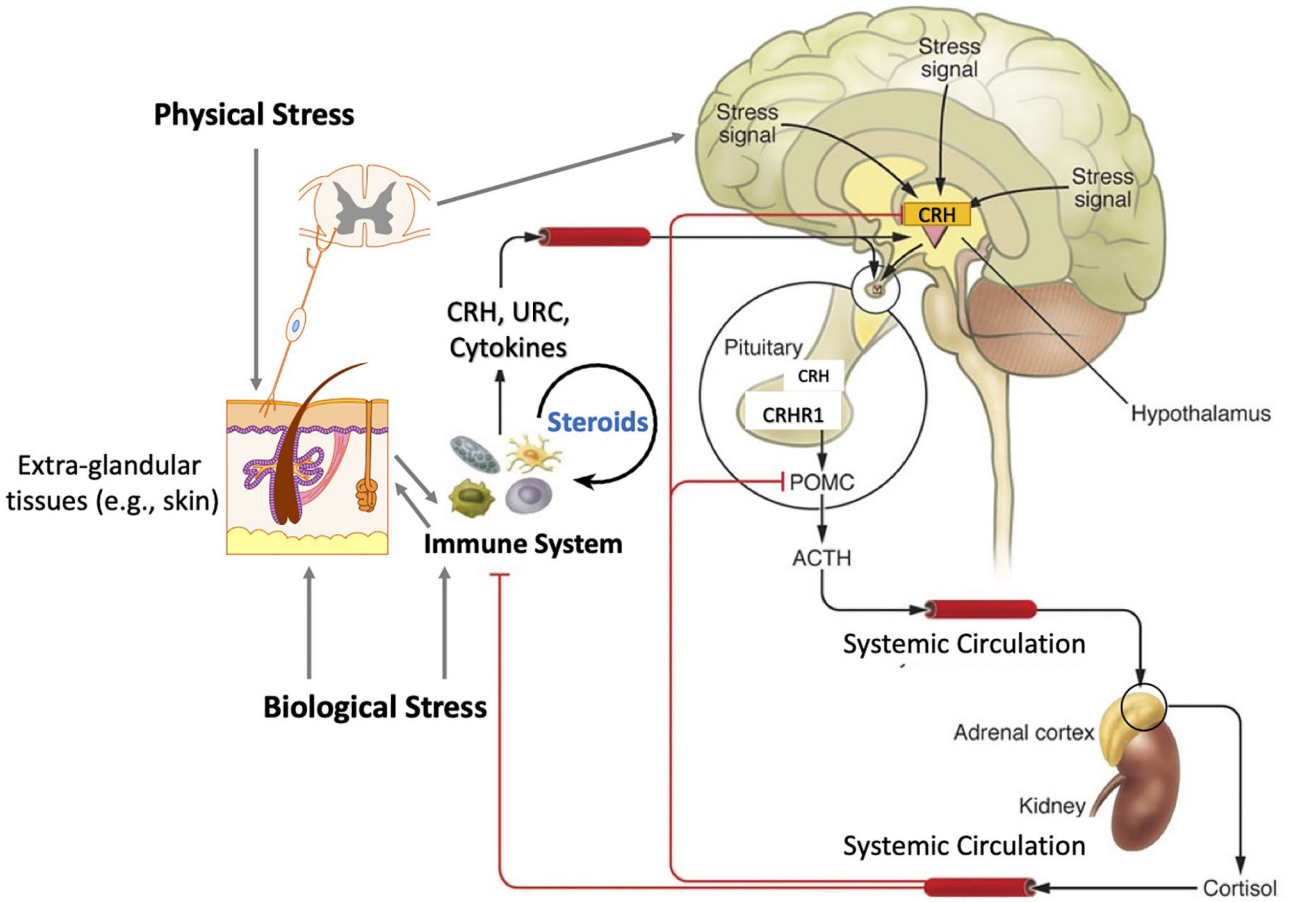


**FIGURE 1** | The functional organization of the hypothalamic-pituitary-adrenal axis with inputs from the immune system and peripheral organs. Physical and biological stress promotes the release of stress signals in both the brain, the skin, and immune cells, resulting in the hypothalamic release of corticotropin releasing hormone (CRH), which in turn stimulates the release of adrenocorticotropic hormone (ACTH) and pro-opiomelanocortin (POMC) expression and processing in the anterior pituitary. ACTH binds to the melanocortin-2 (MC2) receptor in the zona fasciculata of the adrenal cortex and stimulates the transport of cholesterol into the mitochondria and stimulates the production cortisol. Glucocorticoids not only regulate body homeostasis but also act in a negative feedback loop for CRH and POMC expression. Immune cell-mediated steroidogenesis and secosteroidogenesis are emerging as new modes of immune regulation. Reprinted with permission from the (7, 28).

glucocorticoids and sex hormones (Quatrini et al.), which also include control of the hematopoietic stem cell differentiation and subsequent maturation of immune cell subsets. Second mini-review (Bruscoli et al.) discusses glucocorticoid therapy in inflammatory bowel diseases with consideration of new glucocorticoids mediators, such as glucocorticoid-induced leucine zipper, which may have similar anti-inflammatory properties. The third mini-review (Shimba et al.) discusses pleiotropic effects of glucocorticoids on the Immune system in the context of circadian rhythm and stress. Among two reviews one (He et al.) analyses glucocorticoid-induced leucine zipper as a promising marker for monitoring and treating sepsis. The second one (Vanderhaeghen et al.) discusses bidirectional crosstalk between hypoxia inducible factors and glucocorticoid signaling in health and disease, being in line with a fascinating subject of linking immune activity with immune cells energy yielding metabolism (31).

The four original research papers discuss important experimental evidences including use of IFNγ/IL10 ratio for stratification of hydrocortisone (a synthetic but identical molecule to endogenous cortisol) therapy in patients with septic shock (König et al.), glucocorticoid-induced exacerbation of mycobacterial infection through a reduced phagocytic capacity of macrophages (Xie et al.), protection of antigen-primed effector T cells from glucocorticoid-induced apoptosis in cell culture and in a mouse model of multiple sclerosis (Bier et al.), and that gender may influence the immunosuppressive actions of prednisone (a synthetic glucocorticoids with much higher potency than cortisol) in inflammatory bowel disease (Lucafò et al.).

Thus, this Research Topic discusses several important aspects of steroidogenesis, secosteroidogenesis, and their role in cell signaling cascades in the context of physiology and pathology of inflammation and immunity. One important conclusion is that the deregulation of steroidogenic and secosteroidogenic signaling pathways may also lead to a variety of inflammatory disorders and autoimmune diseases in a gender- and context-dependent manner. Different therapeutic and preventive strategies can be deducted from the presented papers leading to practical and clinical solutions to many inflammatory and autoimmune diseases. In future, further analytical investigation is required to understand the physiological and pathological role of endogenous steroids and secosteroids.

## AUTHOR CONTRIBUTIONS

AS wrote the first-draft and others (BM, CR, OB) contributed to improve the writing and conceptual Figures. Correspondence can be made to anyone or all of these authors (AS, BM, CR, OB). All authors contributed to the article and approved the submitted version.

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REVIEW
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# Glucocorticoid-Induced Leucine Zipper: A Promising Marker for Monitoring and Treating Sepsis

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Sepsis is a clinical syndrome that resulting from a dysregulated inflammatory response to infection that leads to organ dysfunction. The dysregulated inflammatory response transitions from a hyper-inflammatory phase to a hypo-inflammatory or immunosuppressive phase. Currently, no phase-specific molecular-based therapies are available for monitoring the complex immune response and treating sepsis due to individual variations in the timing and overlap of the dysregulated immune response in most patients. Glucocorticoid-induced leucine zipper (GILZ), is broadly present in multiple tissues and circumvent glucocorticoid resistance (GCR) or unwanted side effects. Recently, the characteristics of GILZ downregulation during acute hyperinflammation and GILZ upregulation during the immunosuppressive phase in various inflammatory diseases have been well documented, and the protective effects of GILZ have gained attention in the field of sepsis. However, whether GILZ could be a promising candidate biomarker for monitoring and treating septic patients remains unknown. Here, we discuss the effect of GILZ in sepsis and sepsis-induced immunosuppression.

**Keywords: sepsis, glucocorticoid-induced leucine zipper, glucocorticoids, sepsis-induced immunosuppression, anti-inflammatory**

## INTRODUCTION

Sepsis is a complex disease that causes life-threatening organ dysfunction due to uncontrolled infection (1). The global burden of sepsis is estimated to be 30 million patient episodes, with a mortality rate approaching 30%–50% annually. Notably, most data are derived from the developed countries, and the true global burden of sepsis is much greater than suggested by these figures (2, 3). The World Health Organization (WHO) rendered sepsis a global health priority in 2017 to improve prevention, diagnosis, and management. Concomitant with early hemodynamic and respiratory support and appropriate antibiotic administration, corticosteroids have been widely used for the management of sepsis as adjuvant therapy to control the immune response to invading pathogens.

Glucocorticoids (GCs) constitute are a class of corticosteroids widely used clinically as anti-inflammatory and anti-shock drugs. The therapeutic glucocorticoids include hydrocortisone, prednisolone and dexamethasone. Several studies and meta-analyses have indicated that

low-dose hydrocortisone reduced mortality in patients with septic shock in the intensive care unit (ICU). However, the recent adjunctive corticosteroid treatment in critically ill patients with septic shock (ADRENAL) trial did not show a significantly reduced risk of mortality (4). Mortality is likely associated with the timing of treatment and multiple side effects due to the wide spectrum of effects induced by glucocorticoids (GCs) in addition to the complicated immunopathology of sepsis (5). GCs bind the GC receptor (GR), which belongs to the nuclear receptor superfamily of transcription factors to exert their broad physiological and therapeutic effects.

Glucocorticoid-induced leucine zipper (GILZ or TSC22D3), which is a GC-inducible molecule, has emerged as a GC mediator due to its anti-inflammatory effects and theoretically lacks the side effects of GCs (6). Recently, the potential effect of GILZ in sepsis has gained attention, and GILZ has been recognized as a more promising therapy for polymicrobial sepsis than GCs. Hence, notably, that GILZ may be a checkpoint or even a biomarker of innovative therapies for sepsis-mediated immune responses and is regarded as an actionable target. Here, we review the specific role of GILZ in sepsis.

## IMMUNOPATHOLOGY OF SEPSIS

Sepsis induces a complex immune response and this excessive pro-inflammatory response easily elicits the dysregulation of signaling pathways, resulting in tissue damage and organ dysfunction, and induces an immunosuppressive environment, which could increase susceptibility to secondary infections associated with poor outcomes and subsequent mortality (7), although immunosuppressive responses occur simultaneously upon the initiation of innate immune response (8–10).

Increasing evidence suggests that immune cell apoptosis, autophagy, broad metabolic defects, endotoxin tolerance, T cell exhaustion and epigenetic regulation are all contributors, and aerobic glycolysis is crucial for maintaining the function of the immune system (11). For instance, immune cells, including monocytes and T cells, undergo metabolic reprogramming and shift from oxidative phosphorylation (OXPHOS) to aerobic glycolysis when the host is infected (11). Both glycolysis and oxidative metabolism are apparently defective in leukocytes during sepsis-induced immunosuppression. The phosphorylation of extracellular signal regulated kinase (ERK) promotes B-cell death in sepsis (12). The delayed state of neutrophil apoptosis is the most significant change in innate immunity and contributes to the deficits in of bacterial eradication and ongoing continuous dysfunction (13). In addition, a deficit in the capacity to activate nuclear factor-κB (NF-κB) or deacetylation of p65 is closely related to the macrophage reprogramming (14, 15) and sepsis-induced immunosuppression (16).

Identifying treatment and diagnosis guidelines is essential for survival during sepsis. A rich profile of transcriptional shifts occurs in leukocytes within a few hours of exposure to endotoxins (17). Patients with multiple traumas exhibit an immunosuppressed state with large quantities of danger-associated molecular patterns shortly after trauma (18). Similarly, a reduction HLA-DR in monocytes occurs early and is thought to be a marker of an altered immune state (19). Multiple studies exploring sepsis-associated survival also focused on this immunopathology. For instance, studies have focused on antiapoptotic strategies that could reduce mortality in septic mice (20), and immunomodulation with thymosin α1, IFN-γ, IL-7, GM-CSF, interferon-g and the immune checkpoint inhibitor PD-1 in the clinic (11). However, the timing of treatment and side effects associated with suppressing excessive inflammation or enhancing host immunity have not been fully elucidated. Consequently, more timely, suitable and precise therapy is necessary to reduce cell and tissue damage in sepsis.

## SEPSIS AND GLUCOCORTICOIDS/ ENDOGENOUS GLUCOCORTICOIDS

In 1976, William Schumer's study showed that septic shock patients could benefit from glucocorticoid drugs, and thus, GCs have attracted much interest in the treatment of sepsis (21). Glucocorticoids represent a type of steroid hormones secreted by the renicapsule that play key roles in the regulation of reproduction, metabolism, and immunization by binding the GR. To date, more than 37 randomized clinical trials have investigated the treatment effect of steroids in sepsis. While steroid administration reverses shock in some sepsis patients, which patients could benefit from this treatment remains unclear (22, 23). The European Society of Intensive Care Medicine and the Society of Critical Care Medicine suggest that some benefit of using corticosteroids in sepsis occurs only if shock is present (24). Recently, the APPROCHSS trial involving 1241 patients evaluated the effect of hydrocortisone plus fludrocortisone therapy and revealed a lower all-cause 90-day mortality and higher vasopressor-free days in the hydrocortisone plus fludrocortisone group than the placebo group (25). However, the ADRENAL trial, which included 3800 patients, showed that septic patients who were treated with low-dose hydrocortisone did not exhibit reductions in 90-day mortality compared with the patients in the placebo group (4). Given the number of cases included, the difference of these two large-scale trials is likely associated with their broad physiological molecular mechanisms of GCs and sepsis.

Actually, sepsis also involves in neuroendocrine mechanisms (26). The neuroendocrine system is triggered once the body is infected or traumatized to restore dynamic balance and fight noxious stimulation, thus to promote tolerance (27). Cortisol, which is a product of neuroendocrine signaling and a glucocorticoid, is the executor of neuroendocrine responses. The high level of plasma cortisol concentrations may be associated with the severity of sepsis because cortisol is not only an essential response for survival but also mediates endotoxin tolerance (28, 29). Furthermore, the molecules that mediates cortisol clearance including the corticosteroid-binding globulin (CBG), cortisol carrier albumin, A-ring reductases, and 11b-hydroxysteroid dehydrogenase type 2(11β-HSD2), are

reduced during in early phase of sepsis (30–32). Notably, when was inhibited by cortisol generation with hypophysectomy, the mortality of lipopolysaccharide (LPS)-induced septic shock was significant increased (27). Thus, functioning endogenous GCs are very important for the regulation of the immune mechanism of sepsis.

However, inadequate cellular corticosteroid activity has been described as critical illness-related corticosteroid insufficiency (CIRCI) and manifests as insufficient glucocorticoid receptor (GR)-mediated downregulation of proinflammatory transcription factors (33). The following three major pathophysiological theories associated with CIRCI: dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis, altered cortisol metabolism (33) and glucocorticoid resistance (GCR), which limits the activity of endogenous GCs' and GCs' therapeutic effects (34) (**Figure 1**).

The GR is encoded by a single genetic locus, but alternative splicing of the gene product generates the following four distinct messenger RNAs (mRNAs): GRα, GRβ, GRγ, and GR-A. Each of these intracytoplasmic GRα subtypes can bind GCs and, when in the dimer form, can activate gene expression. The GRα protein normally resides in the cytoplasm, and GRα isoforms directly bind GC response elements (GREs) to induce the transcription of multiple genes, including IL-10, IL-1 receptor antagonists, and GILZ (35). GRα expression significantly decreases in patients suffering sepsis, while GRβ is upregulated and cannot bind GCs in septic patients (36). GRβ located in the nucleus could be

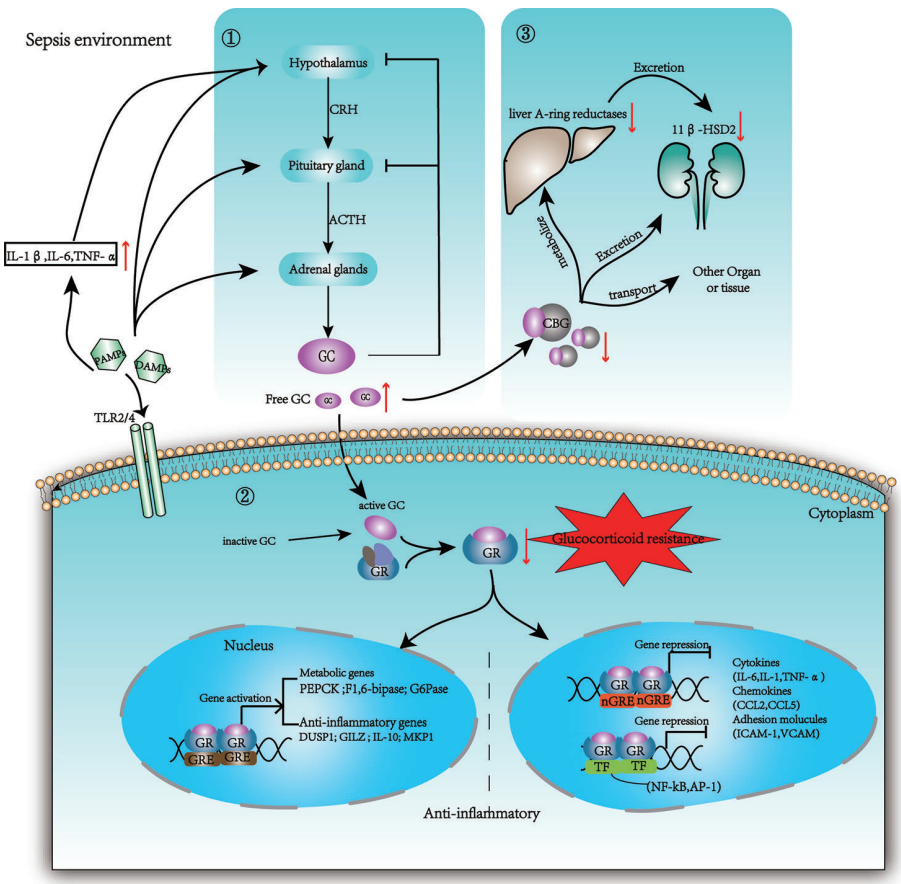


**FIGURE 1** | Changes in glucocorticoid production, metabolism, and regulation in sepsis. ① The HPA axis is activated by stress (both physically and mentally), tissue damage, and infection. The paraventricular nucleus of the hypothalamus secretes CRH, and the anterior pituitary gland secretes ACTH or corticotropin; then GC is secreted by the adrenal cortex. Cytokines such as IL-1β, TNF-α, and IL-6 can be projected in the hypothalamus through neuroafferent projections. DAMPs and PAMPs can also directly stimulate adrenocortical cells with toll receptors (TLR), leading to the synthesis of ACTH dependent cortisol. Circulating inflammatory mediators break the brain barrier and act on the hypothalamus. GC exerts negative feedback on both CRH and ACTH production when GC exceeds the threshold. ② Then 5% GC that is free and activated binds the GR and enters the nucleus to influence gene expression. The inactive GC could be reactivated by 11b-HSD1. GR binds promoters to promote metabolic genes and anti-inflammation genes. Additionally, GR binds negative GRE elements (nGRE) on DNA or some transcription factors (TF) to inhibit the expression of target genes. ③75% GC bound to CBG is transported to various tissue. During sepsis, the host protein production is reduced and the CBG in the inflammatory site is cleaved by neutrophil elastases. Cortisol is cleared mainly through A-ring reductases in the liver and through 11β-HSD2 in the kidneys. These enzymes are limited in sepsis resulting in reduced clearance of GC. PAMPs: pathogen-associated molecular patterns; DAMPs: damage-associated molecular patterns; HPA: hypothalamic–pituitary–adrenal axis; CRH: corticotropin-releasing hormone; ACTH: adrenocorticotropic hormone; CBG: corticosteroid-binding globulin; 11β-HSD2: 11b-hydroxysteroid dehydrogenase type 2; 11b-HSD1: 11b-hydroxysteroid dehydrogenase type 1.

induced by several proinflammatory cytokines, such as IL-2, IL-4, IL-17A, IL-17F, IL-23, and TNF-α (37, 38),and is thought to be a negative regulator of GC activity. The overexpression of GRβ inhibits GRα-mediated gene transcription (39, 40). Thus, clearly, high GRβ levels seem to be associated with GCR in sepsis.

There are other forms of GRs, leading to different variants of GR proteins in addition to GRα and GRβ. GR isoforms and associated subtypes within organs may explain sepsis-induced alterations in GC responses, revealing different clinical responses in septic patients (41). Investigators have identified 27 splice variants of the GR gene and hundreds of single nucleotide polymorphisms (SNPs), insertions and deletions, which could lead to different variants of GR proteins (42). Recently, ANP32E has been shown to correlate with GCR. This protein has been linked to the exchange of H2A. z histone and promotes GR-induced transcription (43). The mechanisms of GC-mediated hypo-responsiveness are heterogeneous due to the various cell types and cytokines involved, and further studies of GR biology may be an important step in promoting GC-based therapies. Hence, supplementing active GCs and activating downstream target molecules are critical for this treatment. Consequently, the identification of a potential treatment that can replace GCs and mediate effective anti-inflammatory effects and endotoxin tolerance without causing GC-associated adverse effects has become the main focus of current studies.

## ANTI-INFLAMMATORY/ IMMUNOSUPPRESSIVE EFFECTS OF GLUCOCORTICOIDS AND GILZ

Glucocorticoids function at physiological and pharmacological levels and are mediated by the GR. Following glucocorticoid exposure, the GC/GR complex translocates into the nucleus and directly binds to glucocorticoid response elements (GREs) to promote downstream transcription factors, including GILZ (44). In turn, GILZ binds NF-κB and prevents its nuclear translocation to elicit anti-inflammatory effects. GILZ can also directly bind c-Jun and c-Fos, which are two constituents of AP-1, to inhibit their transcriptional activity and gene expression of proinflammatory molecules (**Figure 2**). Additionally, GILZ interacts directly with Ras and Raf, thereby inhibiting the downstream activation of mitogen-activated protein kinase 1 (MAPK1), to mediate antiproliferative effects (45, 46).

GILZ, which is widely expressed in various human and mouse organs and is most highly expressed in the lungs (47, 48), was

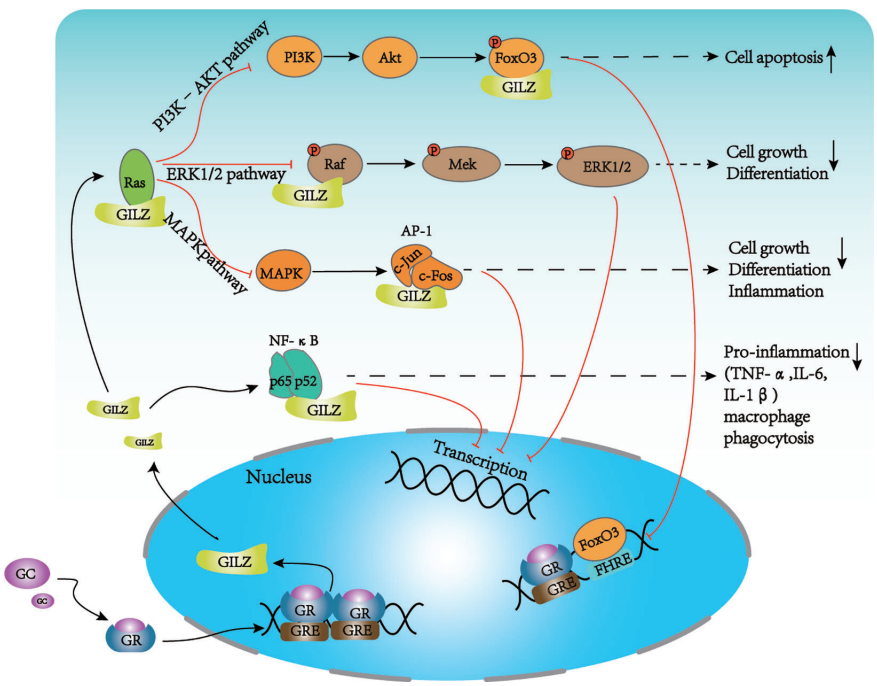


**FIGURE 2** | Major signaling pathways of GILZ. GILZ can regulate cell activation, apoptosis, proliferation, and inflammation mainly through several signaling pathways. GILZ directly binds p65 and p52 to inhibit NF-κB signaling to reduce the production of proinflammatory factors and macrophage phagocytosis. GILZ can directly bind Ras and inhibit downstream pathways. (1) inhibits the PI3K–Akt pathway to regulate apoptosis and cell survival; GILZ inhibits FOXO3A-mediated transcription, such as the pro-apoptotic protein, Bim. (2) The direct binding of GILZ to Ras and Raf leads to reduced activation of MEK, ERK, and MAPK, which inhibits cell growth and proliferation. (3) GILZ can directly bind c-Fos and c-Jun to inhibit AP-1 signaling, which prevents cell growth, cell differentiation, and inflammation. NF-κB, nuclear factor κB; AP-1, activator protein 1; FoxO3, forkhead box protein O3; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; AKT: Protein kinase B (PKB), also known as Akt.

originally identified in 1997 (49). GILZ contains the following three main regions: an N-terminal domain (1–75 amino acids) with a tuberous sclerosis complex (TSC) domain, a leucine zipper (76–97 amino acids) that largely mediates the homodimerization of GILZ, and a proline-rich C-terminal domain (98–137 amino acids) and at least three typical GREs (50) at transcriptional initiation site. GILZ mediates the endogenous and exogenous anti-inflammatory and immunosuppressive effects of GCs in various types of cells, including lymphoid cells, in which it may regulate the activation and apoptosis of cells, which is an important pathophysiological characteristic of the immunopathology of sepsis (6, 51).

GILZ inhibits T lymphocyte activation, apoptosis, and cell proliferation either directly or through antigen-presenting cells (APCs) (52, 53), and inhibit IL-2 withdrawal-induced apoptosis by inhibiting Forkhead Box O3 (FoxO3) transcriptional activity and the proapoptotic gene Bim (54). GILZ also preventsTh-1 and promotes Th-2 differentiation by inhibiting NF-κB activation and nuclear translocation. Transgenic GILZ-overexpressing (GILZ-Tg) mice are less susceptible to Th-1-mediated experimental dinitrobenzene sulfonic acid- (DNBS-) colitis and spinal cord injury. Regulatory T Cells (Tregs) constitute are a subpopulation of T cells that accumulates in the bone marrow and can modulate the immune system, maintain tolerance to self-antigens and control autoimmune disorders. GILZ expression in bone marrow-derived mesenchymal stem cells (BMSCs) and DCs exerts anti-inflammatory effects depending on IL-10-producing Treg and activity. Additionally, GILZ promotes Treg differentiation by activating TGF-β signaling, which is a typical anti-inflammatory factor (55). However, GILZ-KO mice did not have worse collagen-induced arthritis (CIA) compared with WT mice, although the GILZ-KO mice displayed higher T cell proliferation. Furthermore, replenishing GILZ reduces inflammation but does not affect T cell proliferation. Consequently, as a key modulator of Tregs, GILZ plays an anti-inflammatory role in most experimental settings, but the actual role of GILZ in T cells is complex and requires more exploration.

GILZ exerts suppressive effects on B cells by inhibiting cell activation, proliferation, differentiation, apoptosis and IgG production (56). The accumulation of B cell precursors in the bone marrow and peripheral lymphoid organs with elevated Bcl-2 and NF-κB activity has been shown in GILZ-deficient mice (57). The deletion of GILZ increased IFN-γ and AP-1 activity in B cells in a colitis mouse model (58). Furthermore, the above effects were all reversed by the compensation of the GILZ protein, demonstrating the potential therapeutic role of GILZ in regulating B cell-dependent diseases (58).

GILZ not only plays an important role in adaptive immunity, but also inhibits the activity of the innate immune system. In DCs, GILZ and GCs are critical for balancing the anti-inflammatory response and tolerance phenotypes (59, 60), which are closely related to sepsis-induced immunosuppression. GILZ could mediate the downregulation of MHC class II molecules, costimulatory factors, and Treg cell generation (61). Some pro-inflammatory factors including IL-1α, IL-1β, IL-6, and IL-23, were strongly increased in GILZ-KO bone marrow-derived DCs (BMDCs) following upon TLR4 and TLR7 stimulation (62, 63). GILZ prevents DCs from activating the antigen-specific T lymphocyte response. $GILZ^{-/-}$ DCs increased IFN-γ and IL-17 secretion in $CD4^+$ T cells and $CD8^+$ T cells (64, 65). Moreover, GILZ could regulate antigen capture and cross-presentation by DCs and limits antigen internalization in DCs from $GILZ^{-/-}$ mice (62, 66). Dexamethasone enhanced antigen capture by DCs (67, 68), which does not different from the roles of GILZ. Additionally, GILZ can inhibit DC maturation, which could increase the production of IL-10 and promote the development of tolerant DC phenotypes (65, 69).

The GILZ protein and mRNA levels were obviously decreased in alveolar macrophages (AMs) and THP-1 cells exposed to LPS (6, 46, 70). GILZ could decrease macrophage sensitivity to LPS and proinflammatory cytokines expression (71). GILZ deficiency enhances NF-κB pathway-mediated macrophage phagocytosis. Consistent with this result, GILZ-KO macrophages were observed to have increased NO production (45). Interestingly, GC exerts the opposite effect to promote macrophage phagocytosis (72).

GILZ also inhibits neutrophil migration to inflammatory sites *via* annexin A1 (73) and alleviates the proinflammatory response by inhibiting reactive oxygen species (ROS) generation and the accumulation of leukocytes at the site of inflammation or inducing neutrophil apoptosis (57, 74). Neutrophils derived from GILZ-KO mice showed a stronger capacity to clear pathogens in a candida albicans intraperitoneal infection model. Although the role of GILZ is extensive, it has not been deeply studied in sepsis patients. Furthermore, few studies investigated about the immune response associated with GILZ in myeloid-derived suppressor cells, which perform potent immunosuppressive functions and act on both innate and adaptive immunity.

## GILZ IN SEPSIS

GILZ has been studied in septic patients and CLP models (**Table 1**). During the hyperinflammatory stage of sepsis, GILZ regulation is contrary to inflammatory release. GILZ mRNA was reduced by 50% in peripheral polymorphonuclear cells from critically ill patients (75, 80). In an experimental model of CLP, the GILZ expression level was also downregulated in both blood cells and the liver after two hours of the CLP procedure (75). Human alveolar macrophages (AMs) were treated with LPS, upon TLR4 activation, both the mRNA and protein levels of GILZ rapidly decreased, while the TNF and IL-6 mRNA levels increased (70, 77).

The inbred mouse strain SPRET/Ei has been shown to exhibit marked resistance to LPS that depending at least partially on the GR levels and GILZ mRNA was higher than that in C57BL/6 mice (78, 81). In addition, the TAT-GILZ fusion protein, which is a synthetic fusion construct containing the TAT peptide followed by the GILZ cDNA sequence from C57BL/6 and SPRET/Ei, has been shown to reduce mortality [58]. The

**TABLE 1** | A Summary of Preclinical Evidence that revealing the Involvement of glucocorticoid-induced leucine zipper (GILZ) in the pathogenesis of sepsis.

| Reference | Mouse Model of sepsis | GILZ-dependent Effects | Treatment |
|---|---|---|---|
| Ballegeer et al. (75) | C57BL/6 and GILZ-Tg mice; CLP induced septic peritonitis. | GILZ-Tg peritoneal leukocytes (CD45+) displayed a significantly higher phagocytic capacity than GILZ-WT cells | **N/A** |
| Ellouze et al. (76) | C57BL/6 and GILZ-Tg mice; CLP induced septic peritonitis. | Monocytes and macrophages from GILZ-Tg mice showed greater phagocytic capacity and faster bacterial clearance. | **N/A** |
| Hoppstadter et al. (77) | C57BL/6J and myeloid-specific GILZ knockout (KO) mice; LPS induced endotoxin tolerance. | GILZ-deficient macrophages displayed increased TNF-a and IL-1β expression due to the activation of ERK signaling pathways. | **N/A** |
| Pinheiro et al. (78) | C57BL/6 and SPRET/Ei mice; LPS-induced lethal inflammation. | Macrophages transfected with TAT-GILZ, containing either the C57BL/6 or SPRET/Ei sequence, showed reduced cytokine production. | TAT-GILZ administration reduced mortality and IL6 serum concentrations |
| Hang et al. (79) | C57BL/6 mice; LPS-induced septic shock. | Suppression of NF-κB signaling pathways | Short-Chain Alcohols Protect Mice from LPS-induced Septic Shock |

recombinant GILZ protein was first demonstrated to attenuate colon inflammation in DNBS-induced colitis in 2009 (82). The administration of the TAT-GILZ fusion protein could reduce tissue edema and increase perfusion of the ischemic/reperfused kidney in mice with acute kidney injury (AKI), and improve the disruption of mitochondrial membrane potential and cell death *in vitro* (83, 84). GILZ-Tg mice had a lower mortality and blood bacterial load than GILZ-WT mice in a CLP model (75). Peritoneal leukocytes in GILZ-Tg mice are more likely have a higher phagocytic capacity, which may be mediated in inflammatory resolution due to neutrophils apoptosis or M2 –type macrophages polarization (74, 85, 86) In addition, mice with GILZ overexpress mononuclear macrophages (M/M) exhibited an increased survival rate and reduced levels of plasma inflammatory cytokines and blood bacterial load (76). It is likely that overexpression of GILZ elicits an increase in macrophages phagocytosis. A recent study found that the management of short-chain alcohols protects mice from LPS septic shock by increasing peripheral blood GILZ in a dose-dependent manner and suppressing IκB phosphorylation, which is a mechanisms that promoting immune tolerance (16, 79).

Although the upregulation of GILZ can alleviate inflammatory storms and improve survival during the early stage of sepsis, but not endotoxin tolerance (77), the downregulation of GILZ expression independent of GR activation abrogated LPS tolerance and increased responsiveness to LPS by enhancing ERK activity and rescuing MAPK signals, which was independent of GR activation (77). Meanwhile, Wang et al. (87) found that the elevated level of GCs or GILZ is related to late stage inflammation in sepsis, and artesunate can inhibit the upregulation of GILZ mRNA and increase bacterial clearance of hydrocortisone-induced immunosuppression peritoneal macrophages.

Immune phenotypes of infection, especially in sepsis, have many similarities with those in cancer (88). GILZ is a crucial immunosuppressive molecule mediated by GCs in the immunosuppressive tumor environment. Recently, GILZ was shown to be highly expressed in the immunosuppressive tumor microenvironment, which was sufficient to abolish the therapeutic control of tumors (89). DC-specific GILZ deletion or GR antagonists could reverse these negative effects. An online survival analysis suggested that the GILZ level was negatively correlated with prognosis in lung cancer patients (90, 91). These seemingly conflicting data may indicate the cell-specific nature and different stages of the regulation of GILZ in sepsis, which is similar to GCs. As mentioned above, GILZ may play a pivotal role in the immune tolerance of sepsis, although only a few related researches. Of note is, GCs are responsible for osteoporosis though osteoblast formation after long-term GC treatment. In contrast, GILZ increases osteogenic differentiation and inhibits adipocyte formation in mesenchymal stem cells (MSCs) (92). Consistently, cell-specific GILZ overexpression in osteoblasts induced high bone mass and increased bone formation and the osteoblast number (93). Whether GILZ exerts the opposite effect on other GC side effects remains unclear, although the latest published data indicate that GILZ is a critical mediator leading to statin-induced myopathy (90).

Collectively, the potential significance of GILZ in the potential diagnosis, treatment, and prediction of sepsis is worth discussing. To date, few studies investigated the mechanism and therapeutic applications of GILZ in inflammatory diseases and sepsis, and more studies are needed to determine its efficacy and safety.

## PROSPECTS OF GILZ IN SEPSIS

Sepsis and septic shock are both severe diseases that should be treated and resuscitated immediately. Evidence increasingly suggests that the early treatment of sepsis increases the chances of survival, and no study has shown that treatment works better when applied later (94). Although the immune system changes as sepsis progresses, GILZ expression is stable during the late stage of inflammation, especially during immunosuppression, which is probably due to GILZ acting as a downstream molecule and regulation by endogenous cortisol *in vivo*. Hence, GILZ might become a potential monitoring molecule under the regulation of endogenous glucocorticoids or guide treatment with glucocorticoids. Currently, studies are exploring the key role of GILZ in sepsis and whether it can be a critical marker, such as the Rapid Recognition of Corticosteroid Resistant or Sensitive Sepsis (RECORDS) trial (NCT04280497) (95).

According to the current study, GILZ overexpression mice or using GILZ peptides all simulated the effect of early anti-inflammatory agents in acute sepsis. Recombinant GILZ, which

couples GILZ to membrane-penetrating sequences, is an attractive prospect, although further studies are needed to demonstrate the feasibility and safety of GILZ in clinical setting. Alternatively, the targeted activation of GILZ expression using the CRISPR/dCas9 activator complex specifically in macrophages could be used (96). It should be noted that a peptide targeting the C-terminal region of GILZ 115-137 aa in the mouse GILZ sequence has demonstrated a potential therapeutic effect in experimental autoimmune encephalitis (EAE) (97), which suggesting that diverse regions of GILZ can bind different partner proteins. It may be necessary to further determine the optional GILZ peptide that binds specific targets.

## SUMMARY

Sepsis is a syndrome associated with an immune system disorder in response to infection. Currently, the mechanism underlying sepsis development is unclear, further limiting treatment. Clinical studies have shown that the earlier sepsis is resolved, the more likely the patient is to survive. GILZ, which is downstream of the GR, has powerful anti-inflammatory effects similar to GCs and might circumvent GCR in sepsis. Proof-of-concept studies have shown that GILZ has significant therapeutic effects in CLP-induced septic models. Additionally, cell-specific targeting of GILZ may circumvent side effects, which could be of particular interest in sepsis models or other experimental models. Cortisol is essential for inflammatory responses, and endogenous and exogenous GCs induce GILZ to change dynamically. Detection of GILZ expression over time can guide the use of GCs and evaluate the timeline of sepsis, which is of great importance for the treatment of sepsis. As a potential alternative treatment to GCs, further research concerning the role of GILZ in sepsis is needed to identify its powerful regulatory role and molecular mechanisms.

## AUTHOR CONTRIBUTIONS

Y-JH, J-QX, and YS designed the review. Y-JH and J-QX wrote the manuscript with supervision of YS. All authors contributed to the article and approved the submitted version.

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# Use of IFNγ/IL10 Ratio for Stratification of Hydrocortisone Therapy in Patients With Septic Shock

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Large clinical trials testing hydrocortisone therapy in septic shock have produced conflicting results. Subgroups may benefit of hydrocortisone treatment depending on their individual immune response. We performed an exploratory analysis of the database from the international randomized controlled clinical trial Corticosteroid Therapy of Septic Shock (CORTICUS) employing machine learning to a panel of 137 variables collected from the Berlin subcohort comprising 83 patients including demographic and clinical measures, organ failure scores, leukocyte counts and levels of circulating cytokines. The identified theranostic marker was validated against data from a cohort of the Hellenic Sepsis Study Group (HSSG) ($n = 246$), patients enrolled in the clinical trial of Sodium Selenite and Procalcitonin Guided Antimicrobial Therapy in Severe Sepsis (SISPCT, $n = 118$), and another, smaller clinical trial (Crossover study, $n = 20$). In addition, *in vitro* blood culture experiments and *in vivo* experiments in mouse models were performed to assess biological plausibility. A low serum IFNγ/IL10 ratio predicted increased survival in the hydrocortisone group whereas a high ratio predicted better survival in the placebo group. Using this marker for a decision rule, we applied it to three validation sets and observed the same trend. Experimental studies *in vitro* revealed that IFNγ/IL10 was

negatively associated with the load of (heat inactivated) pathogens in spiked human blood and in septic mouse models. Accordingly, an *in silico* analysis of published IFNγ and IL10 values in bacteremic and non-bacteremic patients with the Systemic Inflammatory Response Syndrome supported this association between the ratio and pathogen burden. We propose IFNγ/IL10 as a molecular marker supporting the decision to administer hydrocortisone to patients in septic shock. Prospective clinical studies are necessary and standard operating procedures need to be implemented, particularly to define a generic threshold. If confirmed, IFNγ/IL10 may become a suitable theranostic marker for an urging clinical need.

**Keywords: sepsis, hydrocortisone, immune therapy, machine learning, theranostics, steroids, IFNgamma/IL10**

## INTRODUCTION

Though prospective, randomized, controlled multicenter trials have consistently reported faster shock resolution (1, 2), the utility of "low-dose" hydrocortisone (HC) in patients with septic shock remains controversial. Whereas, two French studies reported outcome benefit from a combination of hydrocortisone plus oral fludrocortisone (3, 4), the pan-European CORTICUS trial and the 5-country ADRENAL trial found no survival effect from hydrocortisone alone (2, 5). Possible explanations for this disparity included differences in mortality risk in the populations with a two-fold higher risk of mortality in the French control group [of ref. (1)] compared to CORTICUS (61 vs. 31%, respectively), and an increase in superinfections, variations in other aspects of clinical management, and genetic variations. Of note, a subset analysis of the ADRENAL trial indicated survival benefit from hydrocortisone in Australasian patients, no effect in British and Danish patients, and a trend to harm in patients enrolled in Saudi Arabia (2). It is increasingly recognized that patients presenting in septic shock are hyper-inflamed yet at the same time immunosuppressed (6–8). Corticosteroids are traditionally considered to induce immune suppression *via* the glucocorticoid receptor (GR) and its repressive effect on pro-inflammatory transcription factors such as AP-1 and NFκB (9). Thus, patients in an overall state of immunosuppression may be potentially compromised by administration of an immunosuppressive drug. This argument is, however, complicated by increasing evidence implicating corticosteroids and GRs in immune-reconstitutive processes (10, 11).

In human monocytes corticosteroid treatment induced expression of innate immune-related genes, such as Toll-like Receptors and anti-inflammatory genes (10, 11). In macrophages and derived cell lines glucocorticoids induced a central component of the inflammasome (NLRP3) and, upon stimulation with endotoxin (lipopolysaccharide, LPS), induced secretion of pro-inflammatory cytokines such as Tumor Necrosis Factor alpha (TNFα) (11). Furthermore, glucocorticoid-dependent NLRP3 induction resulted in sensitization of innate immune cells to extracellular ATP and, thus, in an enhanced ATP-mediated secretion of pro-inflammatory cytokines following endotoxin stimulation (12). This immune-activating role of corticosteroids has been described as a response to acute stress enhancing the peripheral immune response, whereas chronic corticosteroid exposure leads to immune suppression (13, 14). These diverging effects of corticosteroids support the need for biomarkers to guide their application.

We applied machine learning to physiological and laboratory data from patients enrolled within a sub-population of the CORTICUS study to search for a potential theranostic marker for hydrocortisone treatment. Using the ratio of serum interferon gamma (IFNγ) to interleukin 10 (IL10), we were able to identify specific sub-cohorts with increased and decreased survival upon treatment. Furthermore, we explored the predictive utility of this biomarker in further datasets of septic shock patients and performed experimental studies regarding this marker as a function of pathogen challenge.

## MATERIALS AND METHODS

### Included Cohorts and Studies

The discovery dataset based on the Berlin subcohort of the CORTICUS trial. The CORTICUS study was approved by local Ethics Committees (No: 153/2001). Written informed consent was obtained from patients, proxies or their legal representatives. Eligible patients were enrolled if they met the following inclusion criteria: clinical evidence of infection, evidence of a systemic response to infection, the onset of shock within the previous 72 h and hypoperfusion or organ dysfunction attributable to sepsis. Notable exclusion criteria included an underlying disease process with a poor prognosis, life expectancy <24 h, long-term immunosuppression and treatment with long-term corticosteroids within the past 6 months or with short-term corticosteroids within the past 4 weeks. Detailed eligibility criteria are given in **Supplementary Table 1** and in the original study (5). Patients were randomized to receive either *placebo* or 200 mg hydrocortisone (HC) daily for 5

**Abbreviations:** ACTH, Adrenocorticotropic hormone; ADRENAL, Adjunctive corticosteroid treatment in critically ill patients with septic shock; CI, Confidence interval; CORTICUS, Corticosteroid therapy of septic shock; HC, Hydrocortisone; HSSG, Hellenic sepsis study group; NE, Norepinephrine; PSM, Propensity score matching; OR, Odds ratio; SISPCT, Sodium selenite and procalcitonin guided antimicrobial therapy in severe sepsis; SOFA, Sequential organ failure assessment; VASST, Vasopressin and septic shock trial.

days, followed by a tapering dose until day 11. Demographic, baseline and progression characteristics were extracted from the CORTICUS database.

In addition to the standard protocol, the Berlin sub-group of CORTICUS (consisting of 13 of the 52 study centers) sampled blood for subsequent measurement of cytokines and other circulating inflammatory mediators from all 84 patients. Seventy-nine out of these patients received norepinephrine at baseline while none received epinephrine. Blood samples were taken directly before an ACTH stimulation test and administration of the study medication. **Supplementary Table 2** shows the timing of blood sampling relative to the onset of shock. The average time period between onset of septic shock and blood sampling was 29.4 h (standard deviation: 16.6 h). Blood samples were collected on day 0, on day 2, on the morning of day 5 (end of full dose HC application), on day 12 (day after HC cessation), day 17 and day 27. The soluble mediators interleukin (IL) 6, 8, 10, 12p70, IFNγ, TNFα, soluble TNF-receptor-I (sTNF-RI), soluble FAS (all OptEIA™ Set Human, BD Biosciences, New Jersey, USA), and E-selectin (R&D, Minnesota, USA) were measured in serum, plasma, or culture supernatant by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instructions. This included calculating calibration and standard curves. All measurements were performed in duplicate. One patient was removed due to lack of cytokine data. Of the remaining 83 patients, serum lactate values (pre-treatment) and serial values were available in 53 and 41 patients, respectively. For 10 patients either IFNγ or IL10 were below the detection limit. Excluding these patients did not change the overall results. For details, see **Supplementary Text 1**.

The first validation set was obtained by the Hellenic Sepsis Study Group from septic shock patients with community-acquired pneumonia or intraabdominal infection. This study included a prospective collection of clinical data and biosamples from patients admitted to 45 study sites in Greece. Patients were enrolled after written informed consent provided by themselves or their legal representatives. Eligibility criteria are given in **Supplementary Table 1**. All enrolled patients had been reclassified into infection and sepsis using the Sepsis-3 classification criteria (15, 16). In HC-treated patients 200 mg/day HC had been administered for 7 days followed by gradual tapering. Start of administration of hydrocortisone and sampling of blood was later than 3 h after onset of septic shock. After discarding data from patients dying or being discharged on the day of admission, a total of 342 shock patients were selected. Secreted cytokines were measured using the LEGENDplex Human Inflammation Panel (13-plex, BioLegend, San Diego, USA) according to the manufacturer's instructions with half of the reagents volume and sample incubation at 4°C overnight. After removing all specimens with <8 of 13 successful cytokine measurements or without IFNγ and IL10 measurements, a total of 246 eligible shock patients (HC treatment: $n = 93$, No HC treatment: $n = 153$) were selected. If only one of the cytokines (IFNγ or IL10) was below the detection limit, the respective value was set as the detection limit. Excluding these samples from the analysis did not alter the findings (**Supplementary Text 7**). Propensity score matching (PSM) was not necessary as the available dataset did not consist of unbiased baseline variables. For more details and the patient characteristics see **Supplementary Text 2** and **Supplementary Table 3**. As a further validation cohort, we investigated serum IFNγ/IL10 of patients in the *placebo* arm of the randomized and *placebo*-controlled trial of Sodium Selenite and Procalcitonin-guided antimicrobial therapy in Severe Sepsis (SISPCT) (17). SISPCT was a multicenter, randomized, clinical, 2 × 2 factorial trial performed in 33 intensive care units in Germany. It was conducted from November 6, 2009, to June 6, 2013, including a 90-day follow-up period. In this study patients were randomly assigned to receive sodium selenite or placebo. In addition, patients were randomized to receive anti-infectious therapy guided by a procalcitonin algorithm or without procalcitonin guidance. Using the same protocol as for HSSG blood samples, secreted cytokines were measured for our study using the LEGENDplex Human Inflammation Panel (13-plex, BioLegend, San Diego, USA) according to the manufacturer's instructions with half of the reagents volume and sample incubation at 4°C overnight. We selected septic shock patients from the SISPCT trial randomized to the placebo (non-selenite) treated arm with or without procalcitonin guidance. Here, administration of HC was at the discretion of the treating physician. Patients were included for our study only, if they were either not treated with HC (arm not treated with HC), or treated with at least 50 mg daily hydrocortisone for at least the first 3 days (HC treated arm). Patients were excluded if IFNγ and IL10 both were below the detection limits. If only one of these cytokines (IFNγ, IL10) was below the detection limit, the respective value was set at the detection limit. After removing all specimens with <8 out of 13 successful cytokine measurements or without IFNγ and IL10 measurements, a total of 254 eligible shock patients (HC treatment: $n = 77$, No HC treatment: $n = 177$) were selected. We observed considerable differences in their clinical variables (**Supplementary Table 4a**), hence we needed to select a balanced cohort employing propensity score matching (see below, **Supplementary Table 4b**). In addition, we analyzed the serum IFNγ/IL10 ratio of patients from an earlier small crossover study of patients in septic shock (18). Details about this study and the crossover scheme is given in **Table 2g**, **Supplementary Text 3**. In this study, the early arm got a comparable HC application as the/verum arm of CORTICUS, and was also used for validating our marker. The cytokine values of the crossover study were taken from the original publication.

## Propensity Score Matching

To account for the non-random application of hydrocortisone within the HSSG and SISPCT studies, we performed a PSM procedure using the 24 and 182 available baseline variables of these studies. According to (19), features are unbalanced if they show an absolute standardized mean difference $d > 0.1$ between HC-treated and untreated patients, and are thus candidates for PSM. Furthermore, to identify true confounding variables, we applied the method described by Austin (20) and determined features which were significantly correlated with treatment (HC) and outcome (28-days survival). Within HSSG, no variables fulfilled these criteria, and thus patient balancing using PSM

was not applied. Within SISPCT, 11 out of 182 variables were significantly correlated with treatment and outcome, and showed $d > 0.1$. These variables were regarded as true confounding and comprised the variables minimal value of thrombocytes (<24 h before inclusion), distorted metabolism of glucose (yes/no), deregulated body temperature (yes/no), known location of infection (yes/no), sodium minima (<24 h before inclusion), gram positive infections (yes/no), SOFA subscore of renal function, $paO_2/FIO_2$ ratio, amount of urine, potassium maxima (<24 h before inclusion and arterial hypotension (yes/no)). This set of true confounding features was used for PSM at both leaves of IFNγ/IL10 ratio high and low using the R package matchIt (method "nearest," caliper value of 0.2). We repeated this PSM analysis 99 times (with different random seeds) and calculated a robust significance value by calculating the median *p*-value of the corresponding Fisher's exact-tests. By counting the re-occurrence of patient numbers within 100,000 Propensity Score Matching runs, we determined the consensus cohort comprising patients with the maximal number of selections ($n = 86{,}273$). By this, a balanced cohort of $n = 118$ patients (HC treatment: $n = 42$, No HC treatment: $n = 76$) was determined and used for the following analysis. **Supplementary Tables 7a**, **7b** show the patients' characteristics before and after balancing.

## *In vitro* Whole Blood Culture Experiments and *in vivo* Studies on Septic Mouse Models

To assess biological plausibility of the data-driven biomarker IFNγ/IL10, we performed *in vitro* whole blood culture experiments in which blood of healthy donors was challenged with bacterial lysates or LPS (endotoxin of *E. coli B4:O111,* Sigma Aldrich) to simulate pathogen burden. Two hundred microliter of diluted (HBSS, 1:1, V/V) heparinized whole blood obtained from healthy volunteers (male, 20–25 years, $N = 2$–5) was stimulated with serial dilutions either of LPS, lysates from two *E. coli*, two *S. aureus*, one *E. faecalis* or one *E. faecium* isolates obtained from septic patients. In addition, lysates from lab strain *S. aureus USA300* were obtained. Bacterial lysates were created by heat inactivation, following sonification and a serial dilution of the obtained fragment stock. Following incubation (37°C, 18 h, gentle agitation at 2 rpm) plasma supernatant was prepared by centrifugation (2,500 g at room temperature for 10 min). Secreted cytokine levels were measured using the LEGENDplex Human Inflammation Panel (13-plex, BioLegend), as described above. Comparison was made to vehicle controls. The data of the *in vivo* septic mouse model [cecal ligation and puncture (CLP)] for IFNγ and IL10 was provided from Dahlke et al. (21) and the log2-ratio of IFNγ and IL10 was calculated. Additionally, a second group of mice were infected by peritoneal contamination and infection (PCI). For the study, male C57BL/6J mice had been used (untreated controls: $n = 7$, sham: $n = 3$, CLP septic mice: $n = 5$, PCI septic mice: $n = 6$). Throughout all experiments, animals were kept under standardized conditions with access to food and water *ad libitum*. Cecal ligation and puncture and PCI were performed as described elsewhere (22). Whole blood was taken from CLP, PCI, sham, and control mice, for CLP, PCI, and sham mice 6 h post-intervention. For plasma preparation whole blood was centrifuged at 2,000 g for 10 min. Afterwards samples were frozen and stored at −80°C for further analysis. Plasma cytokine levels were measured using the CBA assay (Cytometric Bead Array, CBA; Mouse Inflammation Kit, Becton Dickinson, East Rutherford, USA) by FACS according to product manuals.

## Strategy for Data Analysis

We used data consisting of 137 features (patient variables, potential predictors) from 83 patients of a subcohort of the CORTICUS study population to perform an exploratory data analysis. Only data which was available at baseline was used for propensity matching and machine learning. This table also included measured cytokine levels and ratios of all possible cytokine combinations. We aimed to find a theranostic marker distinguishing HC responders (i.e., survivors) from non-responders (non-survivors). The complete list of these features is shown in **Supplementary Table 5** and the workflow is depicted in **Supplementary Figure 1**. From this data, we sought the best predictor of 28-day survival for the placebo arm using one-level decision trees (stumps) calculated by a leave-one-out cross-validation scheme. The best stumps were chosen by enumeration of all available variables. To apply the marker to other datasets than the CORTICUS subset, the threshold was newly calculated by selecting the one with best significance of improved survival (employing a Fisher's exact-test). All analyses were carried out in R (www.r-project.org) using custom scripts.

The results from the CORTICUS, the HSSG, the SISPCT and the Crossover study cohorts were integrated by using a Mantel-Haenszel test implementing the R stats function mantelhaen.test.

# RESULTS

## IFNγ/IL10 Stratifies CORTICUS Patients

The CORTICUS study included 499 patients (5). The Berlin sub-study (Immune sub-study) comprised of 84 patients, and only for these cytokine measurements were available (see section Materials and Methods). Hence, our analyses focused on this sub-cohort. Its patients' characteristics are summarized in **Table 1**. Median plasma concentrations of soluble mediators and leukocytes were not significantly different between the arms at baseline (**Table 1**). Age differed but was not a confounder (see **Supplementary Text 4**). Analysis of baseline characteristics was performed on 137 variables including demographic and clinical variables, Sepsis-related Organ Failure Assessment (SOFA) scores, lymphocyte counts, plasma protein concentrations of cytokines and patient blood stimulation experiments. We performed a leave-one-out cross-validation with one-level decision trees (using only one predictor at a time) to the *placebo* arm to discriminate between 28-day-survivors and non-survivors. In 95% of the cross-validation runs, the serum IFNγ/IL10 ratio (referred to as IFNγ/IL10 or ratio in the following) was selected as the best predictor with a stable threshold of 0.95. Patients in which IFNγ/IL10 was below 0.95 were denoted "low-ratio patients," the others "high-ratio patients." Untreated high-ratio patients showed a higher survival rate than such with a low ratio (95 vs. 45%, **Table 2a**).

**TABLE 1 |** Patient characteristics, initial cytokine and blood counts of the studied CORTICUS sub-cohort.

| | HC (*N* = 42, 49.4%) | Placebo (*N* = 41, 50.6%) |
|---|---|---|
| Sex (female) | 13 (31%) | 11 (27%) |
| Age (years) | 59 ± 15* | 69 ± 11* |
| **Admission category** | | |
| - Medical | 1 (2%) | 0 (0%) |
| - Emergency surgery | 39 (93%) | 37 (90%) |
| - Elective surgery | 2 (5%) | 4 (10%) |
| Temperature (°C) | 38.5 ± 1.2 | 38.6 ± 0.8 |
| Heart rate (bpm) | 121 ± 18 | 123 ± 24 |
| Systolic blood pressure (mmHg) | 83 ± 17 | 90 ± 22 |
| SOFA score at inclusion | 10.4 ± 3.2 | 9.8 ± 2.7 |
| SAPS II score | 43.7 ± 15.6 | 48.0 ± 14.9 |
| Leukocytes (thousands/mm$^2$) | 13.8 ± 7.3 | 15.1 ± 8.8 |
| Platelets (thousands/mm$^2$) | 187 ± 121 | 165 ± 98 |
| Arterial lactate (mmol/L) | 3.0 ± 2.3 | 3.9 ± 4.0 |
| **Survival** | | |
| - Day 28 (survivors) | 31 (74%) | 29 (71%) |
| - ICU (survivors/all) | 27/42 (64%) | 28/41 (68%) |
| - Hospital (survivors/all) | 25/42 (60%) | 26/41 (63%) |
| - One year (survivors/all) | 20/41 (49%) | 17/40 (42%) |
| ICU-stay (days) all patients | 28 ± 27 | 24 ± 19 |
| Hospital stay (days) all patients | 51 ± 42 | 46 ± 31 |
| Cytokines** | Median (Q1–Q3) | Median (Q1–Q3) |
| IFNγ (pg/ml) | 35.05 (15.79–152.13) | 30.16 (17.93–57.80) |
| IL10 (pg/ml) | 31.21 (13.30–76.22) | 31.66 (15.68–54.76) |
| IL12 (pg/ml) | 10.03 (3.90–43.60) | 13.00 (3.90–43.27) |
| IL6 (pg/ml) | 388.02 (177.80–487.27) | 377.21 (237.17–509.01) |
| IL8 (pg/ml) | 130.95 (56.63–226.28) | 86.81 (53.81–244.44) |
| sFas (pg/ml) | 2137.17 (1544.84–3335.26) | 2155.38 (1551.38–3196.85) |
| sTNF-R1 (pg/ml) | 25,806 (13,322–40,599) | 18,809 (12,009–28,885) |
| **White blood cell counts**** | | |
| Lymphocytes (/nl) | 0.71 (0.34–0.93) | 0.75 (0.41–1.05) |
| Monocytes (/nl) | 0.53 (0.36–0.80) | 0.56 (0.33–0.92) |
| NK cells (/nl) | 0.04 (0.02–0.07) | 0.05 (0.03–0.12) |
| PMNs (/nl) | 11.69 (7.17–14.36) | 11.06 (7.75–15.21) |
| B-lymphocytes (/nl) | 0.09 (0.04–0.20) | 0.08 (0.05–0.13) |
| T-Helper lymphocytes (/nl) | 0.30 (0.13–0.43) | 0.26 (0.14–0.50) |
| T-Suppressor lymphocytes (/nl) | 0.10 (0.05–0.14) | 0.07 (0.04–0.17) |
| Thrombocytes (/nl) | 170.00 (99.50–250.00) | 146.50 (92.00–203.25) |

*Values are given in means ± SD for all continuous variables of the patient characteristics.
**All cytokine and blood count values did not significantly differ between verum and placebo treated patients.

**TABLE 2 |** Survival rates according to high and low IFNγ/IL10.

| | Non-survivors | Survivors | % Survivors |
|---|---|---|---|
| **(a) CORTICUS patients treated with *placebo*** | | | |
| IFNγ/IL10 high | 1 | 20 | **95%**☑* |
| IFNγ/IL10 low | 11 | 9 | **45%**☒ |
| **(b) CORTICUS patients treated with verum (HC, hydrocortisone)** | | | |
| IFNγ/IL10 high | 9 | 20 | **69%**☒ |
| IFNγ/IL10 low | 2 | 11 | **85%**☑ |
| **(c) HSSG patients, not treated with HC** | | | |
| IFNγ/IL10 high | 43 | 36 | **46%**☑ |
| IFNγ/IL10 low | 53 | 21 | **28%**☒ |
| **(d) HSSG patients, treated with HC** | | | |
| IFNγ/IL10 high | 31 | 14 | **31%**☒ |
| IFNγ/IL10 low | 29 | 19 | **40%**☑ |
| **(e) SISPCT patients, not treated with HC** | | | |
| IFNγ/IL10 high | 0 | 15 | **100%**☑ |
| IFNγ/IL10 low | 13 | 48 | **79%**☒ |
| **(f) SISPCT patients, treated with HC** | | | |
| IFNγ/IL10 high | 2 | 7 | **78%**☒ |
| IFNγ/IL10 low | 5 | 28 | **85%**☑ |
| **(g) Patients from the early arm of the crossover study (treated with HC)** | | | |
| IFNγ/IL10 high | 5 | 6 | **55%**☒ |
| IFNγ/IL10 low | 1 | 8 | **89%**☑ |

*☑ *Compliant to the rule;* ☒ *Non-compliant to the rule.*

Upon applying this predictor to HC-treated patients, the reverse behavior was observed i.e., a low IFNγ/IL10 indicated a high likelihood of survival (85 vs. 69%, **Table 2b**).

Performing a logistic regression with HC treatment, IFNγ/IL10 ratio and their interaction as independent variables and 28-day survival as the dependent variable, we observed a significant effect of the interaction term ($p = 0.042$, **Supplementary Text 5** and **Supplementary Figure 2A**). These observations suggested a new decision rule, that is, no HC treatment if the ratio is high, and HC treatment if it is low. This decision rule yielded an odds ratio of survival of OR= 6.97 (95% Cl: 1.79–40.50), $p = 0.0013$ (**Figure 1**). Notably, the identified rule was valid also when regarding an earlier or later onset of septic shock before inclusion (0–14, 24–48, and 48–72 h, see **Supplementary Figure 6F**). Regarding white blood cell (WBC) counts of different leukocytes did not reveal a clear separation of IFNγ and IL10 producers. The IFNγ/IL10 ratio did not significantly correlate with any of the investigated leukocyte subtypes. Notably, IL-10 negatively correlated with most of the leukocyte subtypes (**Supplementary Table 6**). However, neither IFNγ, IL10 nor any WBC count alone could be used for a treatment rule.

## Validation Based on Patients From the Hellenic Sepsis Study Group, a Published Crossover Study and the "Trial of Sodium Selenite and Procalcitonin-Guided Antimicrobial Therapy in Severe Sepsis"

**Supplementary Table 3** summarizes the demographics of the HSSG validation cohort. Applying IFNγ/IL10 to HSSG patients, we observed a similar pattern as for the CORTICUS subgroup (**Tables 2c**, **d**). High IFNγ/IL10 indicated distinct higher survival

| Study | Number of patients | Odds Ratio | P | CI (lower, upper) | |
|---|---|---|---|---|---|
| CORTICUS | 83 | 6.97 | 1.3e-03 | 1.79 | 40.50 |
| HSSG | 246 | 1.83 | 2.5e-02 | 1.05 | 3.22 |
| SISPCT | 118 | 2.36 | 1.4e-01 | 0.74 | 8.99 |
| Crossover | 20 | 6.07 | 1.6e-01 | 0.49 | 353.00 |
| Pooled | 467 | 2.42 | 6.9e-05 | 1.58 | 3.72 |

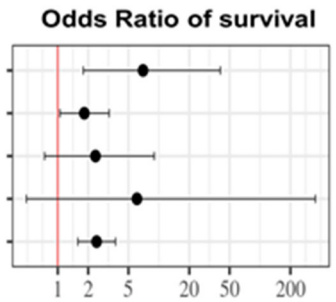


**FIGURE 1 |** Forest plot showing the outcome in the investigated cohorts depending on hydrocortisone administration according to the theranostic marker. Odds ratios including confidence intervals and thresholds between low and high ratios are shown regarding the IFNγ/IL10 prediction rule for the sub-cohorts of CORTICUS (discovery set), HSSG (validation set), SISPCT (validation set) and the crossover study (validation set).

of the HC non-treated patients (46 vs. 28%). In contrast, in the HC treated group we observed the opposite behavior (31 vs. 40%), yielding an odds ratio of 1.83 (95% CI: 1.05–3.22), $p = 0.025$. As the means to measure the cytokines differed, we had to adjust the IFNγ/IL10 ratio threshold for the HSSG (threshold = 1.01). For the HSSG study, days of survival were available. Thus, we assembled survival curves for patients treated according and against our rule (**Supplementary Figure 3**). During the first 10 days, patients with a high IFNγ/IL10 ratio showed a higher rate of survival, suggesting an initial better condition of patients with higher ratios (this is evidenced also by their lactate levels, see next section). After 10 days, the HC treatment (during the first 7 days following by gradual tapering) seems to impact the outcome, as the survival rate of the low ratio patients with HC-treatment (treatment in compliance with our rule) crossed the high ratio patients with HC-treatment (treatment not in compliance with our rule). The HSSG cohort consisted of patients in sepsis either caused by community acquired pneumonia (CAP) or intra-abdominal infection. To note, our rule performed better for the subcohort of patients with intra-abdominal infection [$n = 98$ patients, OR = 2.55 (95% CI: 1.03–6.49), $p = 0.038$], compared to patients with CAP [$n = 148$ patients, OR = 1.73 (95% CI: 0.82–3.73), not significant].

Next, we analyzed serum IFNγ/IL10 of patients from an earlier, smaller crossover study (18) in which the early arm got a comparable HC application as the HC arm of CORTICUS (details about this study and the crossover scheme is given in **Supplementary Text 3**). In line to the results from CORTICUS and HSSG, low IFNγ/IL10 indicated better survival (89% survivors), whereas high IFN/IL10 was an indicator for considerably worse outcome (55% survivors), yielding an odds ratio of 6.07 (95% CI: 0.49-353), $p = 0.16$. Due to the small sample size the result from the crossover study failed to achieve significance.

Furthermore, we investigated serum IFNγ/IL10 of propensity score matched patients from the *placebo* arm of the randomized *placebo*-controlled trial of Sodium Selenite and Procalcitonin-guided antimicrobial therapy in Severe Sepsis (SISPCT) (17). For details about the study and the selection of patients, see Methods and **Supplementary Text 6**. In contrast to the former patient populations, the 28-day-survival rate of SISPCT patients receiving or not receiving HC differed considerably (68 vs. 84%, respectively, $p = 0.0041$, Fisher's exact-test, see **Supplementary Figure 4A**). We evaluated if there was a variable which may elucidate such an uneven distribution and identified the time of septic shock before inclusion (and HC treatment) to be strongly correlated with the survival rate of the HC-treated patients. This variable was not correlated with the survival of the non-HC-treated patients (**Supplementary Figure 4B**). Thus, we excluded patients with shorter time intervals between shock and study inclusion (<7 h), and balanced the remaining patients after separation into IFNγ/IL10 ratio low and high. The cohort comprised of n=118 patients and showed comparable survival rates of HC- and non-HC-treated patients (83 vs. 84%).

In summary, and comparable results when applying our treatment rule (**Tables 2e,f**). the investigated patients from all investigated validation studies evidenced IFNγ/IL10 as a potential theranostic marker for HC application in septic shock. **Figure 1** shows the forest plot including all investigated studies.

## CORTICUS Time Courses of Serum Lactate and Norepinephrine Requirement Reflect Hemodynamic Stabilization in Patients Treated in Compliance With the Decision Rule

High serum lactate levels have been demonstrated to indicate severity of metabolic derangements and increased mortality in sepsis (15). In all studied subpopulations where it was available, median initial lactate was distinctly lower in patients with high compared to patients with low IFNγ/IL10 ratio (CORTICUS: 1.90 vs. 2.89 mmol/L, SISPCT: 2.20 vs. 3.00 mmol/L, Crossover study: 1.49 vs. 2.24 mmol/L). Together, lactate values were significantly different between low ratio and high ratio patients ($p = 0.0029$, $n = 330$, Yuen's bootstrap-test), suggesting that IFNγ/IL10 associated with disease severity. Of note, although the lactate levels correlate inversely with IFNγ/IL10 at baseline, serum lactate itself performed worse as a theranostic marker. Notably, we identified a significantly stronger decrease of the lactate values of patients which were treated in compliance

with the decision rule in comparison to patients which were treated against the rule (**Figure 2A**). Hemodynamic stabilization was also supported by a visible stronger trend of reduction of norepinephrine (NE) requirement specifically in the group of patients in compliance with the decision rule (**Figure 2B**). In summary, time series of serum lactate and NE requirement reflect better recovery of septic shock in patients treated in compliance with the proposed theranostic marker.

## IFNγ/IL10 as an Indicator of Pathogen Challenge

In light of published evidence associating IFNγ and IL10 with the severity of parasitic infection and tuberculosis (23, 24), we investigated whether IFNγ/IL10 reflects the pathogen burden of immune cells when challenged with typical pathogens found in sepsis. We spiked blood from healthy donors with fragments of clinical isolates (*Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium*) or endotoxin (LPS) across a wide range of concentrations. As expected, the higher the pathogen challenge, the higher the immune response and hence the concentration of IFNγ and IL10 in the supernatant. Remarkably, we observed the inverse behavior for the ratio, i.e., a high IFNγ/IL10 ratio for low pathogen concentrations and *vice versa*. **Figure 3** shows the results exemplarily for one *E. coli* isolate. Results for the second *E. coli* isolate, *S. aureus, E. faecalis* and *E. faecium* isolates and LPS are shown in the **Supplementary Material** (**Supplementary Figure 5**). To note, testing also a lab strain of *S. aureus* (USA 300) showed not this tendency (**Supplementary Figure 5**, see section Discussion). To validate the obtained results in an *in vivo* sepsis model we challenged mice by cecal ligation and puncture (CLP sepsis model) as well as peritoneal contamination and infection (PCI), and compared them to untreated controls and sham treated mice (sham-operated mice underwent an identical operation except for the actual cecal ligation and puncture). Indeed, we observed the same behavior: the ratio of IFNγ/IL10 was distinctively higher in the controls (negative and sham treated mice) and lower in septic mice (**Supplementary Figure 6**).

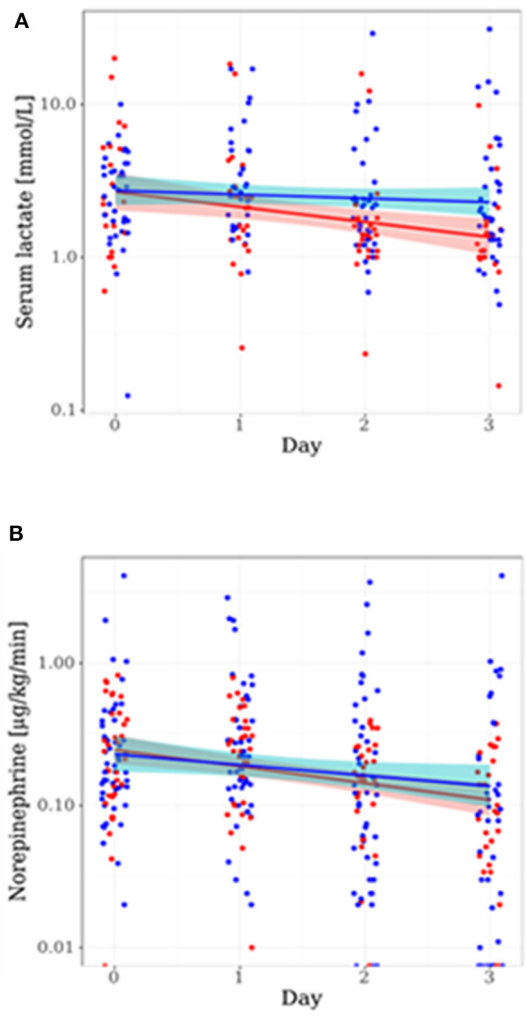


**FIGURE 2 |** Time courses of serum lactate and norepinephrine requirement are depending on the proposed theranostic rule. Shown are patient values and linear models including color-shaded confidence intervals (95%) for the sub-populations of CORTICUS treated in compliance (red) and not in compliance (blue) to our rule. The time course is given in days after start of the study, **(A)** shows the course of serum lactate, and **(B)** shows the course of the norepinephrine requirement.

Furthermore, we associated IFNγ/IL10 to the bacterial load in critical ill patients, and compared publically available data of patients with and without bacteremia. Matera et al. (25) investigated 52 patients (39 survivors and 13 non-survivors) with signs of SIRS at hospital admission. Only 4% of them were in septic shock. Twenty-eight of 52 were diagnosed with bacteremia. In line with our experimental observations, a distinctively higher ratio (1.6 ± 0.77) for IFN/IL10 in SIRS patients was observed compared to patients with bacteremia (0.8 ± 0.88, details: **Supplementary Text 7**). Healthy volunteers showed the highest IFNγ/IL10 ratio (2.80 ± 1.22) of all groups investigated by Matera et al.

In summary, IFNγ/IL10 inversely correlates with the immunological load of infection in an *in vitro* system, in septic mice and when comparing bacteremic and non-bacteremic critically ill patients.

# DISCUSSION

Starting from a larger panel of 137 predictors derived from septic shock patients ($n = 83$) in CORTICUS, we identified the ratio of serum IFNγ and IL10 as a promising biomarker to guide the treatment decision for or against HC. We validated our results by applying the marker to three other datasets.

Metabolically, high serum lactate indicates severity of cellular disturbances (15) and is associated with poor outcome (26). Consistent with this, patients with low IFNγ/IL10 showed high serum lactate. Furthermore, stratifying patients in compliance with the proposed decision rule, we observed a considerable decrease over time in serum lactate specifically in the group of

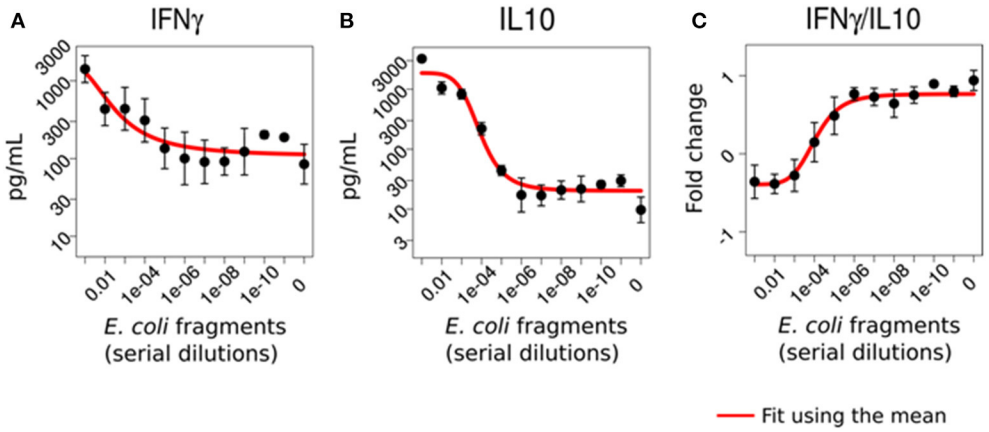


**FIGURE 3 |** IFNγ/IL10 reflects the immunological load of immune cells *in vitro*. Whole blood from five healthy donors was challenged with varying dilutions of *E. coli* fragments mimicking the bacterial burden on the immune system in sepsis. IFNγ **(A)** and IL10 **(B)** were elevated with increasing bacterial challenge while their ratio **(C)** showed the opposite behavior, i.e., a high stimulus was associated with a lower ratio (given in log-two-fold-changes).

patients, which were treated in compliance with the theranostic marker. This further extends the concept that HC is generally able to stabilize cardiovascular function in septic shock and suggests that this effect might also be restricted to patients with a high burden of the disease, and compares to the different beneficial effects of steroids in septic shock observed in the trials mentioned in the introduction (French studies with higher mortality showed a beneficial effect compared to no beneficial effect in the studies ADRENAL and CORTICUS).

IFNγ and IL10 have been suggested as biomarkers for parasitic (23) and other infections, such as tuberculosis (24). E.g. McIlleron et al. reported the time course of IFNγ indicating response to treatment in pulmonary tuberculosis (27). However, for diagnosis of pulmonary tuberculosis, IFNγ alone has limitations relating to sensitivity (28). In turn, high IL10 was observed with mycobacterial persistence (29) suggesting that in particular the ratio of these cytokines might reflect the immunological burden of infection. In line with this, it has been shown that the ratio of IFNγ and IL10 correlates with disease severity of tuberculosis and may differentiate pulmonary from extra-pulmonary forms (30). To elucidate the functional implications of low and high IFNγ/IL10, we performed blood culture assays challenging the immune cells with fragments of bacterial isolates from septic patients. Indeed, we observed the ratio to be negatively correlated with the load of pathogen fragments. Similarly, we observed a significant lower ratio in untreated and sham controls compared to septic mice. This was supported by clinical data from Matera et al. (25) when regarding critically ill patients with bacteraemia (higher bacterial load) and without bacteraemia (no or non-detectable bacterial load). Thus, IFNγ/IL10 is supposedly (negatively) associated with the pathogen load.

IFNγ has been consistently documented to activate cells of adaptive immunity (31), and IL10 as a suppressor of innate immune responses and inflammation (32, 33). Hence, high IL-10 may be a response to a high load to prevent and limit over-whelming immune reactions and, in consequence, tissue damage, whereas low IFNγ reflects a low adaptive immune response.

Interestingly, Sommerfeld et al. observed recently that mouse mutants lacking the receptor of complement factor C5a showed increased survival in mild septic mouse models, and, IFNγ/IL10 was increased compared to wildtype mice under the same conditions (34). The mutants also showed a similar survival benefit when mutant and wildtype mice were treated with antibiotics. Sommerfeld et al. explained the effect by an increased adaptive immune response in the mutants. It is reasonable that a high pathogen load causes repression or even disruption of the adaptive immune system or/and induces an overwhelming innate immune response monitored by the ratio of IFNγ/IL10. Nevertheless, this needs exploration in future studies. We observed *in vitro, in vivo* and in patients that blood challenged with bacteria/bacterial fragments displayed lower IFNγ/IL10 at higher loads, and *vice versa*. Transferring these observations to septic shock patients with low IFNγ/IL10, we speculate that HC treatment yielded a better outcome in these HC-responders because HC may allow the highly loaded immune system more pace for recovery. This is evidenced by the reduction of serum lactate and norepinephrine requirements as measures of recovery particularly in the subgroup of very sick patients.

To mention, we also challenged the blood culture with the lab strain *S. aureus* USA300, for which this behavior was not observed. We speculate that such a pathogen causing rather skin and soft tissue inflammation than bacteraemia and sepsis may evoke other cytokine responses and suggest follow up analyses on this aspect in more detail.

The concern about side effects of corticosteroids such as infections in patients with less severe septic shock stipulated more restrictive recommendations by the Surviving Sepsis

Campaign. HC application is currently recommended only for patients not responding to adequate fluid resuscitation and vasopressor therapy (35, 36). Considering an immune biomarker, such as IFNγ/IL10 reflecting the status of the patients' immune system status may intuitively be a promising path to improve the treatment decision. Consistent with this concept, Bentzer et al. studied data from patients of the "VAsopressin vs. norepinephrine in Septic Shock Trial" (VASST) investigating corticosteroid treated vs. non-treated patients (only vasopressin or catecholamine vasopressors). They identified a signature of three cytokines (IL3, IL6, CCL4) suggesting response to corticosteroid treatment (37). However, these results based on a study, which was not randomized, blinded or protocolized according to corticosteroid treatment, and Bentzer et al. did not elaborate how these cytokines interact with corticosteroid treatment.

In a very recent randomized controlled trial, patients who were hospitalized with Covid-19 were randomly assigned to receive dexamethasone (RECOVERY) or placebo. This trial elucidated that dexamethasone treatment is beneficial for patients receiving either invasive mechanical ventilation or oxygen alone at randomization but not among those receiving no respiratory support (38). Here the supportive measurement of the affected organ was sufficient for predictive enrichment of steroids treatment. In our case (septic shock), predictive enrichment basing solely on the supportive measurement (vasopressor requirement) was not sufficient and we needed to monitor the immune status (by the cytokines IFNγ and IL10). As an intriguing project for the future, we propose developing a generic rule distinguishing different disease/syndrome entities employing such categories.

### Limitations and Strengths

We excluded patients from the very early window of septic shock of SISPCT (<7 h) to balance the survival between HC-treated and HC-untreated patients. This difference was not surprising, as the HC treatment was applied to patients in more severe conditions, as suggested by guidelines. This is consistent with the CORTICUS data where inclusion criteria were broader (up to 72 h) and the onset of sepsis in CORTICUS occurred on average 29 h before inclusion. In the light of the current recommendations to use HC restrictively, it might indicate, that HC treatment could be supported by a theragnostic biomarker. In addition, we were not able to outbalance every variable in SISPCT. Another limitation of our study is the variability of thresholds to distinguish between high and low IFNγ/IL10 ratio. For CORTICUS it was 0.95, for HSSG 1.01, for SISPCT 0.48, and for the crossover study it was 0.81. DeJager et al. (33) reported that timing of sampling, sample storage, the choice of the platform and the blood collection tubes are crucial issues when measuring cytokines from blood. Cytokines can be stored below −80°C for a longer time, but even at −80°C degradation was observed, in particular of IL10. Most of these parameters varied between the different studies that we included in our analysis making it difficult to compare the ratios between the studies. Our cytokine measurements seemed to depend on the assay and sample handling described by DeJager et al. The discrepancies of the optimal threshold between the studies may also be due to the rather older time point when Corticus and the Crosscover study were performed. Hence, when implementing the rule into the clinics, well defined standard operating procedures need to be developed to obtain an appropriate, center independent threshold. A limitation of the *in vitro* blood culture study is that we used only blood of younger male volunteers.

A strength of our study is that our rule seems to be valid across studies of very heterogenous survival rates (CORTICUS: 72%, Crossover: 70%, HSSG: 37%, SISPCT: 84%). Applying our decision rule successfully across a broad range of these studies suggests the marker to be generic. The analyzed Berlin cohort of CORTICUS contained a high number of surgical patients and a rather low number of medical patients. Also the averaged ICU and hospital stay was longer as usual for septic shock patients [when compared e.g. to the prospective INSEP study (39)]. The validation cohorts didn't show this notable discrepancy suggesting a good generalizability of our rule. A randomized controlled trial also better representing these variables is, thus, in every case inevitable to confirm the identified rule. Furthermore, strengths of our study relates to the use of (i) well-phenotyped cohorts, (ii) that our marker showed remarkably similar results across all studies, and (iii) that we provide clinical, *in vivo* and *in vitro* data relating it to the pathogen challenge of the immune system.

## CONCLUSIONS

We identified the ratio of serum cytokines IFNγ and IL10 as a potential theranostic marker for hydrocortisone treatment in septic shock. An accompanying study into the mechanism suggests that this ratio monitors the immune response challenged across distinct pathogen loads. The development of a standard operating procedure for obtaining the cytokine concentrations in serum followed by a clinical trial is necessary confirming our rule before implementing it into the clinical routine.

## DATA AVAILABILITY STATEMENT

The original contributions generated in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The CORTICUS trial was a multicenter study, the protocol was approved by the Ethics Committee at each of the 52 participating intensive care units. In addition to the standard CORTICUS protocol, the Berlin study group sampled blood for subsequent measurement of cytokines and other circulating inflammatory mediators. This was approved by the Local Ethics Committee (No: 153/2001). Written informed consent was obtained from patients, proxies or their legal representatives. The

HSSG cohort represents a prospective collection of clinical data and biosamples in 45 study sites in Greece. The study protocol was approved from the Ethics Committees of all participating hospitals. Patients were enrolled after written informed consent provided by themselves or by first-degree relatives if patients were unable to consent. The Placebo-Controlled Trial of Sodium Selenite and Procalcitonin Guided Antimicrobial Therapy in Severe Sepsis (SISPCT) was a multicenter clinical trial. It was conducted in 33 multidisciplinary intensive care units across Germany. The study protocol was approved by the ethics board of Jena University Hospital. Written informed consent was obtained from all patients or their legal representatives. The study protocol for the crossover study was approved by the institutional (Charite, Berlin) Ethics Committee. All animal experiments were in accordance with the German legislation on protection of animals and with permission of the regional animal welfare committee.

## AUTHOR CONTRIBUTIONS

RK, AK, MOS, DK, and MB conceptualized and designed the study. MOS, RK, AK, and VK developed the methodology. AK, VK, MB, MS, JB, DK, DR, and RK analyzed and interpreted the data. Animal experiments were performed by OS. Administrative, technical, or material support was given by OA, DR, GD, IT, EA, HB, ML, CS, MS, FB, MOP, HG, RC, SC, and JB. The study was supervised by RK, DK, and MB, and they are the garantor of the article. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.607217/full#supplementary-material

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REVIEW
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# Regulation of Tissue Immune Responses by Local Glucocorticoids at Epithelial Barriers and Their Impact on Interorgan Crosstalk

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The anti-inflammatory role of extra-adrenal glucocorticoid (GC) synthesis at epithelial barriers is of increasing interest with regard to the search for alternatives to synthetic corticosteroids in the therapy of inflammatory disorders. Despite being very effective in many situations the use of synthetic corticosteroids is often controversial, as exemplified in the treatment of influenza patients and only recently in the current COVID-19 pandemic. Exploring the regulatory capacity of locally produced GCs in balancing immune responses in barrier tissues and in pathogenic disorders that lead to symptoms in multiple organs, could provide new perspectives for drug development. Intestine, skin and lung represent the first contact zones between potentially harmful pathogens or substances and the body, and are therefore important sites of immunoregulatory mechanisms. Here, we review the role of locally produced GCs in the regulation of type 2 immune responses, like asthma, atopic dermatitis and ulcerative colitis, as well as type 1 and type 3 infectious, inflammatory and autoimmune diseases, like influenza infection, psoriasis and Crohn's disease. In particular, we focus on the role of locally produced GCs in the interorgan communication, referred to as gut-skin axis, gut-lung axis or lung-skin axis, all of which are interconnected in the pathogenic crosstalk atopic march.

**Keywords: extra-adrenal glucocorticoids, immune regulation, inflammation, interorgan crosstalk, gut-lung axis, atopic march, gut-skin axis**

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## EXTRA-ADRENAL GLUCOCORTICOID SYNTHESIS

Glucocorticoids (GCs) and their anti-inflammatory action are known since decades and are still of great importance in the treatment of inflammatory disorders. Although GCs are mainly produced in the *zona fasciculata* of the adrenal glands, regulated *via* the hypothalamus-pituitary-adrenal axis (HPA), extra-adrenal sources of GCs have received increasing attention in recent years. Since adrenal and extra-adrenal GC synthesis has been extensively reviewed in the past (1–3), we only provide a short overview on extra-adrenal GC synthesis in gut, skin and lung in this introduction. The epithelia of the gut, skin and lung serve as connection point between the body and the outside world. Local GC synthesis potentially contributes to local immune cell regulation and thereby to the prevention of immunopathologies due to excessive inflammatory processes. Despite some

similarities in their basic functions, each epithelium meets a variety of tissue-specific functions and therefore differs in its cellular composition.

Gut epithelial GCs derive from the crypt region of the small and large intestine. The nuclear receptor liver receptor homolog-1 (LRH-1/NR5A2) in the intestinal crypts regulates the expression of steroidogenic enzymes and thereby *de novo* intestinal GC synthesis (4). Moreover, under inflammatory conditions the pro-inflammatory cytokine tumor necrosis factor (TNF) seems to have a dual role in the promotion and regulation of intestinal inflammation (5). Noti et al. reported that besides pro-inflammatory activities, TNF also induces intestinal GC synthesis, thereby limiting the pathogenesis of acute intestinal inflammation (5).

While the role of the intestinal epithelium is to efficiently absorb nutrients, the main function of the skin is to provide a protective surface barrier. The different layers of the epidermis are mainly composed of keratinocytes in different stages of differentiation (6). Local GCs in the skin are produced by keratinocytes and their synthesis is regulated *via* a "cutaneous HPA-axis", UV irradiation and cytokines (e.g. IL-1β, TNF) (7–10). Hannen et al. further linked deficiency of skin GC synthesis with inflammatory disorders of the skin, like psoriasis or atopic dermatitis (7). Active GCs can be either synthesized *de novo* from cholesterol with the final conversion catalyzed by 11β-hydroxylase-1 (CYP11B1, Cyp11b1) or *via* reactivation from inactive cortisone, respectively 11-dehydrocorticosterone catalyzed by 11-β hydroxysteroid dehydrogenase 1 (HSD11B1, Hsb11b1). Mouse models of keratinocyte-specific *Hsd11b1* or *Cyp11b1* deficiency, resulting in abrogation of skin GC reactivation or *de novo* synthesis, revealed that the absence of either enzyme results in an imbalance of skin immune homeostasis and aggravation of inflammatory skin diseases (11, 12).

While local production and regulation of intestinal and skin-derived GC synthesis were described previously, the underlying molecular and biochemical basis of lung GC synthesis remains to be elucidated. Nevertheless, systemic immune cell activation *via* anti-CD3 antibody, lipopolysaccharide (LPS) or TNF is able to induce local GC synthesis in the lung (13). Although all steroidogenic enzymes are expressed in the lung, analysis of adrenalectomized mice revealed a dominant role of GC reactivation, rather than *de novo* synthesis (13). Evidence for local *de novo* GC synthesis in the pathogenesis of human disease was found in a study in patients with rhinosinusitis (14). The upper respiratory tract seems to be capable of producing active GCs either *de novo* or *via* reactivation and both systems are activated in patients with chronic rhinosinusitis (14).

The immunomodulatory and anti-inflammatory actions of GCs can be explained by their GC receptor (GR)-mediated gene regulation, GC-induced cell death and other regulatory mechanisms that have been extensively reviewed before (15). Classically, GC suppression of T helper ($T_H$) 2 cell differentiation by inhibiting p38 activation, consequent GATA3 inhibition and type 2 cytokine expression appears to be only moderate compared to the potent inhibition of type 1 immune responses (16–21). Thus, GC-mediated immune regulation appears to shift immune responses towards humoral $T_H2$ responses. Whether this dogma, mostly derived from *in vitro* studies involving cytokine cocktails, is correct and reflects the dynamics *in vivo* environment is still unclear and up to debate. *In vivo* studies in the lung and the intestine did only describe minor induction of local GC synthesis in response to type 2 immune responses, like ovalbumin-induced airway hyperresponsiveness and oxazolone-induced colitis (5, 13). However, recent evidence indicates a non-negligible role of endogenous GCs in controlling $T_H2$ type responses, although their production is not strongly induced during these processes. Differently, atopic dermatitis (AD)-like skin inflammation as induced by the vitamin D3 analog MC903 was sufficient to promote local GC synthesis in keratinocytes, but the abrogation of local GC synthesis did not exacerbate AD-like skin inflammation (12). Although past research revealed the regulatory potential of extra-adrenal GCs in local immune responses at barrier tissues, current detailed insight into the respective processes is still limited. In the following, we discuss recently described situations where a potential contribution of local GCs in the regulation of interorgan crosstalk and disease pathogenesis has been suggested.

## TISSUE-IMMUNE REGULATION AND INTERORGAN CROSSTALK

In biological research, organs are often considered in isolation, and while this simplifies research and may be justified in many respects, the reality is nevertheless more complicated and research on interorgan crosstalk is becoming increasingly important. Especially with regard to GCs, their simultaneous and differential effects on the entire body cannot be ignored. It is known for decades that starting from the adrenal cortex, steroid hormones are systemically distributed throughout the body *via* the blood circulatory system with different effects on the various organs. It is therefore striking that three steroidogenic organs (i.e., lung, skin, intestine) are linked *via* an interorgan crosstalk, raising the possibility that local steroidogenesis may play a role in the action and regulation of infections and allergies. This interorgan crosstalk is called the gut-lung axis, gut-skin axis and skin-lung axis, which in turn interact and cooperate in the atopic march.

### Regulation of Type 2 Responses by GCs Along the Gut-Lung Axis

The gut-lung axis describes the influence of gut microbiota on the lung immune system and *vice versa* the influence of lung immunity on gastrointestinal homeostasis (22). Dysbiosis in the gut is not only associated with inflammation in the gastrointestinal tract, e.g. inflammatory bowel diseases (IBD), but also with inflammatory airway diseases, like asthma and chronic obstructive pulmonary disease (COPD) (23). Likewise, viral infections of the lung can cause gastrointestinal symptoms, like diarrhea, and also influence the microbiota of the gut *via* activities of the immune system (24, 25). Although underlying

pathways and mechanisms of the gut-lung axis remain to be elucidated, short chain fatty acids (SCFA) have been identified as important immunomodulatory metabolites that derive from the intestinal microbiome (22, 26). In the following, we discuss the role of GCs in the regulation of type 2 immune responses along the gut-lung axis.

Asthma is a common chronic disease characterized by symptoms, such as coughing, chest tightness and shortness of breath. Chronic airway inflammation and the infiltration of inflammatory cells lead to a narrowing of the airway lumen, which massively reduces the quality of life of affected patients. GCs in combination with bronchodilators are successfully applied to treat asthma patients. The anti-inflammatory action of GCs dampens the acute overreaction of the immune system and helps to overcome asthma attacks. The application of synthetic GCs, like budesonide or ciclesonide, inhibits type 2 cytokine synthesis and dampens the inflammation during the allergic reaction (27, 28). Through the activity of GRα, GCs inhibit the expression of type 2 cytokines by antagonizing the activity of other transcription factors, like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein-1 (AP-1) and CCAAT/enhancer binding protein β (C/EBPβ) (29). In addition to that, GCs were found to inhibit the transcriptional activity of GATA3 in $T_H2$ cells by preventing its p38 mitogen-activated protein kinase-mediated phosphorylation, thereby limiting the effect of this major type 2 response regulator (16). Experimental allergic airway inflammation induced by i.p. ovalbumin-alum sensitization and ovalbumin aerosol challenge revealed only minor induction of local GC synthesis, especially when compared to the effect of type 1-inducing LPS or anti-CD3 application (13). Since GCs are constitutively present in the lung and there is evidence that lack of GCs exacerbates the allergic response, it is reasonable to assume that GCs not only suppress type 1 responses, but also control type 2 responses without being massively induced by it. Classically, asthma is associated with eosinophilic infiltration and increased serum immunoglobulin E (IgE) levels, as well as typical $T_H2$ cytokines (IL-4, IL-5, IL-13), produced not only by $T_H2$ cells but also by basophils, mast cells and type 2 innate lymphoid cells (ILC2) (30). Traditional allergy models, as for example ovalbumin-induced airway hypersensitivity, have contributed much to the study of type 2 responses. However, the characteristics of human asthma are often more complicated and are now classified into different categories from "Type 2-low" to "Type 2-high" asthma depending on the respective immune responses (30). In order to better reflect the diversity of human asthma in experiments, there has been an increased use of natural allergens, such as house dust mite (HDM) or cockroach extracts. In this context, the question naturally arises which role lung-derived GCs play in balancing type 1 and type 2 responses in asthma models of natural allergens. However, this remains to be investigated and is subject of ongoing research.

With regard to the interorgan crosstalk in the control of asthma it has been reported that environmental factors and diet influence the susceptibility to allergic asthma (31, 32). For example, a farming environment protects children from allergy and asthma. Toll-like receptor (TLR) 4 activation in experimental HDM allergy has been shown to be crucial for allergic sensitization and dendritic cell (DC) activation (33). However, chronic exposure to low dose endotoxin or farm dust protects mice from HDM allergy by the induction of A20 (ubiquitin-modifying enzyme) resulting in reduced cytokine production (33, 34). This suggests that this intervention in the epithelial-immune interaction may contribute to the protective effect of rural environments. Interestingly, the "farm effect" as well as diet also contribute to the microbiome composition in the gut (31, 32). As mentioned above, it is well known that dietary fiber and gut-derived SCFA contribute to airway homeostasis and exert a protective effect during allergic airway inflammation in various mouse experiments (26, 35–37). For example, mice treated with the SCFA propionate showed dampened allergic airway inflammation (26). Additionally, Theiler et al. showed that SCFA limit human eosinophil trafficking and survival, thereby limiting allergic airway inflammation (36). A study with 301 one-year-old children revealed that those with the highest levels of the SCFA butyrate and propionate in feces had significantly less atopic sensitization and a reduced risk to develop asthma later in live (38). A 7-day randomized study in 17 adults with stable asthma demonstrated for the first time improvement of asthma symptoms upon soluble fiber supplementation (39). Only recently, a study identified a novel and protective pathway along the gut-lung axis. The metabolization of L-tyrosine by bacteria in the gut increases the concentration of p-cresol sulfate (PCS), which has been shown to be protective against HDM-induced allergy by interfering with epidermal growth factor receptor (EGFR) and TLR4 signaling (40). Whether and to which extent locally produced GC synthesis in the lung contributes to the protective action of gut-derived metabolites remains to be elucidated.

In this context, the research of Bouguen et al. on ulcerative colitis (UC) is especially interesting (41). Our group already showed that type 1 cytokines, namely TNF, directly induce intestinal GC synthesis to ameliorate colitis while type 2 immune response in the model of oxazolone-induced acute colitis did not trigger GC synthesis due to the lack of TNF. Eventually, Bouguen et al. linked extra-adrenal GC synthesis in the intestine with the expression and activity of the SCFA receptor peroxisome proliferating factor gamma (PPARγ), a pathway which is impaired in UC but not in Crohn's disease (CD) (5, 41). UC is a chronic inflammation of the colonic mucosa. Pharmacological interventions in the treatment of IBD include anti-TNF antibodies and corticosteroids. Although very effective, their long-term use also promotes adverse side effects, calling for better solutions to combat causes that may not yet be known (42). UC, rather than CD, is associated with the upregulation of the $T_H2$ cytokines IL-5 and IL-13, as well as eosinophil infiltration (43). However, the exact role of type 2 cells in UC is still up to further investigation. Besides type 2, also type 1 and type 3 immune responses are involved in UC disease processes, thus UC is another example where the rigid $T_H1/T_H2$

dogma is not valid anymore (43). The finding that LRH-1 expression and intestinal steroidogenesis is impaired in UC patients resulting in reduced anti-inflammatory actions *via* PPARγ, provides further evidence for the regulatory role of GCs to restrict also diseases with features of a $T_H2$ immune response. The activation of PPARγ *via* SCFA may represent a possible link to the lung immune system in the gut-lung axis. The role of PPARγ in lung immunity has been characterized over the last twenty years with evidence for important regulatory functions. Schneider et al. uncovered that the induction of PPARγ through the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) critically regulates the differentiation of alveolar macrophages (44). Alveolar macrophages are part of the first line of defense system in the lung and are therefore crucial in shaping the immune response. Nevertheless, the role of PPARγ in asthma is controversial. On the one hand, PPARγagonists, e.g. rosiglitazone and troglitazone, reduce airway inflammation by the inhibition of inflammatory cells and pro-inflammatory cytokines (45). And on the other hand, the deletion of PPARγ in lung DCs protects from HDM-induced asthma development, by reducing their migration to the draining lymph nodes, pointing towards a pro-inflammatory role of this nuclear receptor (46). Therefore, PPARγ appears to have multiple roles in regulating type 2 immune responses, and its effect must be considered in a cell type-specific and context-dependent manner. However, its regulatory role *per se* seems undisputed, thus the question at this point is whether local GCs, similar to that in the intestine, affect also the activity and expression of PPARγ in the lung. Whether this hypothesis is correct and whether gut-derived SCFA can exert their anti-inflammatory activities in the lung *via* this pathway remains to be investigated. A possible model of regulation is depicted in **Figure 1**. The gut synthesizes local GCs that induce the expression of PPARγ with beneficial effect on gut homeostasis. In addition to that, the gut microbiota synthesize SCFA that not only activate intestinal PPARγ but also exert anti-inflammatory effects in the lung, resulting in reduced type 2 cytokine expression and consequently less tissue destruction and airway remodeling in allergic airway inflammatory diseases. Since the application of synthetic GCs has similar anti-inflammatory effects, it is very likely that also locally produced GCs in the lung exert a protective effect on type 2 immune responses (**Figure 1**). In any case, an interplay between SCFA and local GCs in the regulation of immune homeostasis along the gut-lung axis develops as an attractive new idea.

## The Perspective of GCs to Regulate Pathogenic Crosstalk in the Atopic March

Atopic march is a progressive atopy, which describes the increased likelihood of individuals with inflammatory diseases of the skin in infancy to develop allergic airway or gastrointestinal inflammations, like asthma or food allergy, later in life (47–49). The recent discoveries of local GC synthesis in the skin provide novel ways for understanding prevalent inflammatory skin diseases and their possible regulation by keratinocyte-derived GCs. Although many inflammatory skin disorders involve local dysregulation of the skin immune system, they have the potential to progress into systemic diseases affecting diverse tissues and organs, such as the diseases involving the atopic march.

Atopic dermatitis (AD) represents one of the most common chronic inflammatory skin diseases, next to psoriasis. It is

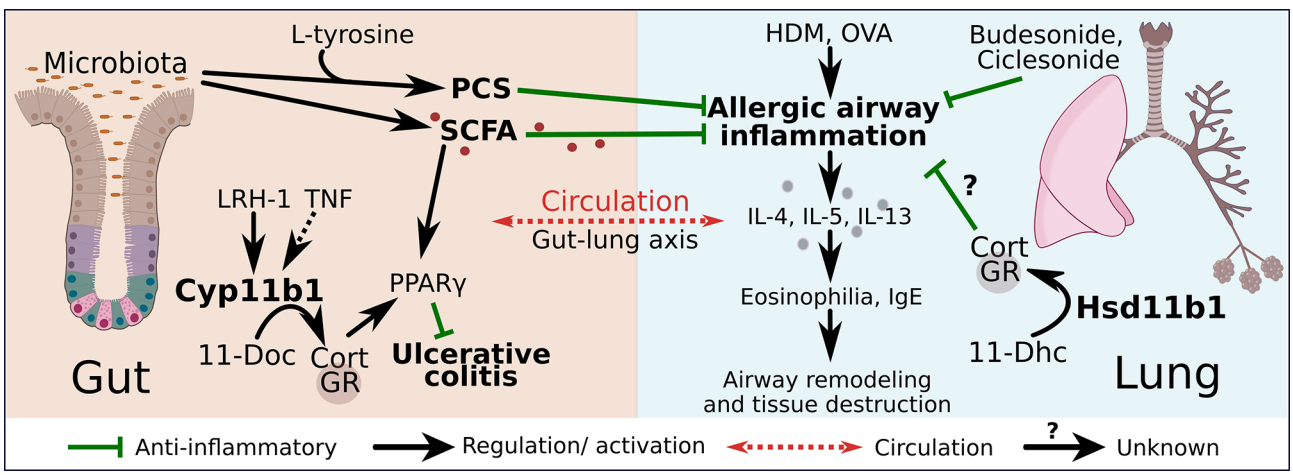


**FIGURE 1** | Regulation of type 2 responses by GCs along the gut-lung axis. The production of gut–derived GCs is regulated by liver receptor homolog-1 (LRH-1) and inducible *via* tumor necrosis factor (TNF). 11β-hydroxylase (Cyp11b1) catalyzes the conversion of 11-deoxycorticosterone (11-Doc) to corticosterone (Cort). GC signaling induces the expression of peroxisome proliferating factor gamma (PPARγ), which exerts anti-inflammatory effects on ulcerative colitis. Short chain fatty acids (SCFA) and p-cresol sulfate (PCS) are produced by the gut microbiota and exert anti-inflammatory actions on allergic airway inflammation. House dust mite (HDM) or ovalbumin (OVA)–induced allergic airway inflammation leads to increased type 2 cytokine interleukin (IL)-4, IL-5, IL-13 release, airway eosinophilia, increased immunoglobulin E (IgE) levels and finally to airway remodeling and tissue destruction. Gut-derived PCS and SCFA as well as synthetic GCs (budesonide, ciclesonide) suppress the allergic airway inflammation. The lung produces GCs in an 11β-hydroxysteroid dehydrogenase (Hsd11b1)-dependent manner through the conversion of 11-dehydrocorticosterone (11-Dhc), which may contribute to the local regulation of allergic airway inflammation.

characterized by chronic, pruritic eczematous skin lesions and is associated with dominant type 2 immune responses, including elevated $T_H2$-type cells and IgE serum levels. IL-4 and IL-13 are known to be essential for the pathogenesis of AD and are mainly secreted by $T_H2$ cells (50). However, recent studies have shown that $T_H2$ cytokines are also relevantly produced by basophils and ILC2s, which critically prime the skin immune system in the developing phase of AD (51–53). In addition to the immune dysregulation, defective skin barrier functions also play a major role in allergic skin sensitization, mostly because of impaired keratinocyte differentiation and defective barrier protein development. These circumstances often lead to increased antigen exposure due to increased epithelial permeability, involving microbial dysbiosis by dominant skin colonization and invasion of *Staphylococcus aureus*. Both, dysregulated type 2 immune responses and defective skin barrier fuel into an inflammatory feed-forward loop, which drive the chronic skin inflammation in AD. Its propagation and progression are specifically controlled by the complex interplay between keratinocytes, sensory neurons, and type 2 immune cells ($T_H2$ cells, DCs, ILC2s, mast cells and basophils), as well as their associated cytokines within the epithelia-immune microenvironment (50). Detailed underlying mechanisms are still not completely understood, but increasing evidence suggests that inflammatory AD conditions play a major role in allergic sensitization.

In fact, AD conditions represent the basis for the progression of several other atopic diseases, including allergic rhinitis, asthma and food allergy, but also eosinophilic esophagitis and allergic conjunctivitis, which define the so-called atopic march (54–56). These atopic diseases progress at different epithelial barriers of various anatomic sites, such as the upper and lower airway tract as well as from the oral-esophagus to the lower gastrointestinal organs (55). Mostly, the prevalence of infantile AD precedes these diseases of atopic march, which increase with age while AD skin conditions withdraw in the long-term (54). Thus, allergic sensitization through local inflammatory circuits in AD skin specifically implicates secondary atopic manifestations at mucosal barrier sites of the lung and the gastrointestinal tract, paving the way for further diseases of the atopic march. In addition to the clinical therapy of these diverse atopic diseases, several approaches aim to prevent the onset of atopic march through ameliorating cutaneous AD conditions and preventing skin sensitization in the first place (55). Besides the preventive disease management and the use of calcineurin inhibitors, acute inflammation and disease exacerbations are efficiently treated using several low- to high-class potent synthetic GCs for atopic march-affected epithelial sites in AD, asthma and food allergy-associated anaphylaxis. However, as discussed above, the very same epithelial barrier sites are also the source of local GCs, which contribute to the control of local inflammation (1, 4, 5, 13, 57–61). Despite that, our understanding of how local GC-mediated immunoregulatory circuits impact type 2 inflammation remains incomplete. Epithelial barrier-derived GCs were shown to exert individual roles in maintaining an autonomous and local immunoregulatory circuit in each of these tissues (1, 5, 12, 13). As already mentioned, oxazolone-induced acute colitis failed to trigger LRH-1-regulated and TNF-dependent GC synthesis in the intestine, similar to the lung where ovalbumin-induced airway hypersensitivity was ineffective in promoting local GC synthesis (5, 13). Whereas in MC903-induced AD, which also causes a type 2 skin inflammation, local GC synthesis in keratinocytes was indeed induced, it did not appear to ameliorate the pathogenesis of AD (12). Additionally, formation of lymph node (LN)-resident $CD44^+$ $CD69^+$ T cells and OVA-specific immune responses in MC903-induced AD skin conditions did not show significant alterations in mice with deficient skin GC synthesis indicating that AD development and skin sensitization is not directly restricted by local skin GCs (12). In contrast, deficient keratinocyte GC synthesis in AD skin inflammation resulted in decreased IL-4 levels in skin associated with ameliorated itchiness. Thus, absence of skin GCs indirectly resulted in the elevation of yet unknown inhibitory factors opposing IL-4-associated inflammatory responses in the skin (12). Here, our understanding of local GC biology in type 2 inflammatory skin diseases is still limited and further studies are warranted to investigate the underlying pathways. Specifically, possible regulation of the alarmins, such as TSLP, IL-33 or IL-25, by skin GCs and their impact on epithelia-type 2 immune cell interactions may unravel so far unrecognized or underestimated regulatory pathways. In vitro studies already revealed an inhibitory effect of low concentrations of exogenous GCs on the release of TSLP from keratinocytes in the presence of the type 2 cytokines IL-4 and IL-13 (62). In addition to that, mice with a constitutive GR deletion in keratinocytes show enhanced expression levels of TSLP, IL-33 and other genes involved in inflammatory skin disorders (63). Accordingly, it is likely that local skin-derived GCs influence the production and signaling of these cytokines. It must be, however, noted that pleiotropic effects mediated by GCs may be determined by their bioavailability within specific tissues and target cells, and also depend on individual tissue and cell type sensitivity and expression level of the GR (15, 64). This may also explain why clinical application of potent high-class GCs are efficient in the treatment of acute allergic reactions in skin, and at mucosal sites of the airway and intestine despite the inefficiency of endogenous local GCs to completely shut down experimental type 2 inflammation. In this regard, a recent study described an experimental model for atopic march using mice with HDM-induced chronic AD skin conditions and subsequent allergic airway inflammation upon HDM challenge (65). This study revealed that co-activation of both GR and PPARγ using dexamethasone and the PPARγ-ligand rosiglitazone was more efficient in suppressing AD skin inflammation than single nuclear receptor activation (65). However, GR/PPARγ-mediated amelioration of the preceding AD skin inflammation was not able to completely abrogate the development of allergic airway inflammation and the pathogenesis of atopic march, but asthma severity was alleviated associated with reduced IL-23/IL-17A axis, neutrophils and monocytes (65). Despite the progression of the atopic inflammation in the lung, topical GCs specifically inhibited local skin immune responses

associated with the suppression of type 3 response and thereby reduced the severity of experimental asthma. Thus, these results further indicate the therapeutic relevance of GCs to reduce disease severity, which originates in processes dependent on the skin-immune environment (65). On the contrary, the study further confirms the insensitivity of $T_H2$ cells towards GCs in allergic sensitization and inflammation (65). Especially the persistence of a $T_H2$ subset, which displays an inflammatory memory phenotype in non-lesioned AD skin, was suggested to play a major role in the recurrence of AD conditions in patients treated with the monoclonal anti-IL-4Rα antibody dupilumab (66).

These interesting findings rise the questions to which extent keratinocyte-derived GCs are capable of modifying the initial progress of atopic march diseases in the lung and intestine during early skin sensitization phases and draining lymph node priming (12, 65). Specifically, it is of interest to explore the impact of skin GCs on developing and chronic AD, and whether and how they affect the differentiation of $T_H2$ cells and innate type 2 immune cells in the skin. An overproduction of local GCs may compromise type 1 immune responses, which in general restrict cutaneous type 2 immune cells, thereby indirectly favoring the expansion of $T_H2$ cells. How these processes modulate the progression of the atopic march remains to be investigated. **Figure 2** shows how the different organs potentially communicate during the progression of atopic march. Dysregulated local GC synthesis by reduced Cyp11b1 levels in the skin could favor the development of AD that develops during atopic march to food allergy and oral-esophageal, gastrointestinal anaphylaxis. A functional local GC synthesis in the intestine could potentially counteract this process by inhibiting the expression of pro-inflammatory cytokines. And in addition to that the local GC synthesis in the lung could possibly counteract the development of allergic airway inflammation in association with atopic march by suppressing the IL-23/IL-17 axis and the recruitment of neutrophils and monocytes (**Figure 2**).

Taken together, the cutaneous GC synthesis represents a local regulatory circuit, which may not only regulate the local skin immune system but also affects other tissues *via* the crosstalk with the HPA axis and the systemic GC response. Specifically, a previous study demonstrated that UV stimulates the release of neuroendocrine mediators from the local HPA axis in the skin, which influences the systemic HPA axis and thus regulates global body homeostasis (67). This finding furthermore reveals the complexity of crosstalk mechanisms and reciprocal interactions of mediators controlling both local- and global-derived GC secretion. And thus implicating a relevant role of skin-originated mediators in controlling mucosal inflammation through mounting the systemic GC release. However, the possibility of mucosal-derived GCs to interact with global homeostasis is yet unexplored and its investigation is majorly challenged since local inflammation

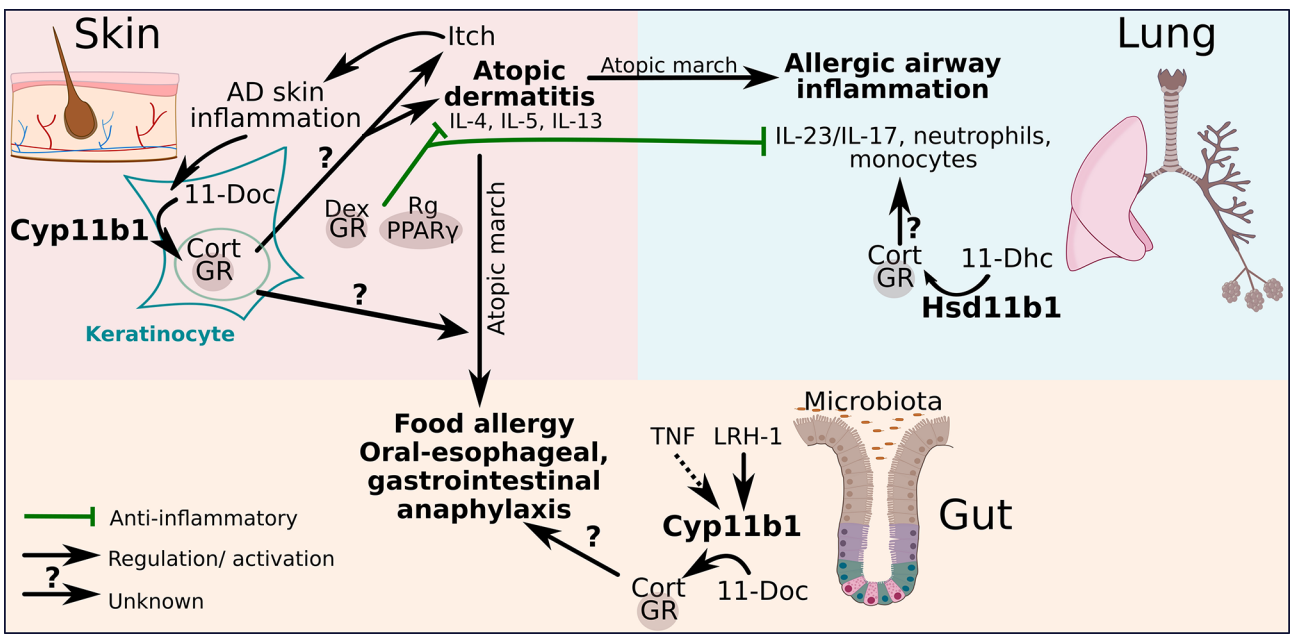


**FIGURE 2** | The perspective of GCs to regulate pathogenic crosstalk in the atopic march. Glucocorticoid receptor (GR) and peroxisome proliferating factor gamma (PPARγ)-mediated regulatory activities inhibit atopic dermatitis (AD) progression and reduce consequent atopic airway inflammation severity by inhibiting the IL-23/IL-17 axis upon topical dexamethasone (Dex)/rosiglitazone (Rg) treatment. AD skin inflammation enhances local *de novo* GC synthesis in keratinocytes through the conversion of 11-deoxycorticosterone (11-Doc) to corticosterone (Cort) catalyzed by 11β-hydroxylase (Cyp11b1). The regulatory potential of endogenous GCs on AD and associated atopic march diseases is yet still unclear. Local skin GCs have been shown to not restrict AD skin inflammation. The capacity of liver receptor homolog-1 (LRH-1)-regulated synthesis of gut-derived GCs and 11β-hydroxysteroid dehydrogenase (Hsd11b1)-dependent GC reactivation from 11-dehydrocorticosterone (11-Dhc) in the lung on atopic march diseases are unexplored.

and regulation of the tissue-immune microenvironment in skin, lung and intestine are differentially regulated due to their unique stromal-immune cell composition, tissue architecture and steroidogenic machinery.

## Regulation of Local Type 1 and 3 Immune Responses and Autoinflammatory Diseases Across and Beyond Epithelial Barriers

Of no less importance is the efficient GC therapy in the treatment of infectious diseases as well as inflammation in autoimmune and autoinflammatory disorders. Most of them are known for the aberrant production of inflammatory cytokines with the involvement of activated stromal cells and pathogenic immune cells within local circuits. In particular, key cytokines of the type 1 and type 3 immune responses, including TNF or IL-1 cytokines, and the IL-12/IFN-γ- or the IL-23/IL-17 signaling pathways, are able to activate and remodel tissue cells and recruit inflammatory phagocytes resulting in cytotoxicity and tissue damage.

The organ interplay along the gut-lung axis is also described in diseases with features of type 1 and type 3 immune responses, even though this is less well explored. For example, during influenza infection, the innate immune system senses the virus *via* pattern recognition receptors (PRRs) and initiates an adaptive immune response *via* the release of pro-inflammatory cytokines and type I interferons (IFN) (68). The activation of neutrophils, macrophage recruitment and DC maturation finally leads to the recognition and clearance of the intruder by CD8+ cytotoxic T cells and CD4+ T cells (68). Moreover, regulatory T cells and $T_H17$ cells have been shown to be important in balancing the immune response during influenza infection (68). Evidence for positive effects of SCFA in influenza infections have been obtained in mouse models. High-fiber diet protected mice from influenza infection, by regulating the balance between the innate and the adaptive immune system (69). On the one hand SCFA modulated CD8+ T cell metabolism and on the other hand the number of Ly6C- monocytes was increased, leading to enhanced alternatively activated macrophages and finally reduced neutrophil-associated immunopathology (69). Interestingly, influenza infection seems also to have a major impact on the composition and metabolism of gut microbiota (24, 25, 70, 71). The infection leads to a strong weight loss in mice, which is in part due to a lower food intake caused by inappetence (72). This reduces the production of SCFA by the gut microbiota. The lack of anti-inflammatory effects by SCFA ultimately leads to a greater risk of secondary bacterial infections, in some cases also to so-called superinfections, which massively increase the mortality rate (24, 73). Mouse models revealed that supplementation with acetate improves the potential of the immune system to control secondary superinfections upon influenza infection (24). In addition to that, respiratory infection of mice with influenza virus has been shown to cause intestinal injury in a $T_H17$–dependent manner, which was further dependent on the intestinal microbiota (25). This could explain gastroenteritis-like symptoms in human influenza patients and further strengthens the influence of the gut microbiome during disease control. Evidences for the importance of local steroidogenesis in respiratory viral infections have been observed in patients with chronic rhinosinusitis. Jun et al. observed increased expression of *HSD11B1* and *CYP11B1* as well as increased levels of cortisol in the sinus mucosa of the patients, indicating that local steroidogenesis in the conducting airways is upregulated to support balancing the immune response upon viral infection (14). In addition to that, the infection of mice with lymphocytic choriomeningitis virus (LCMV) resulted in the induction of local steroidogenesis in the intestine, indicating that GCs may also play an important role in regulating viral infections of the gut (74). However, therapeutic GC application to patients with influenza infection remains controversial. Meta-analysis of data from ten trials with patients suffering from influenza pneumonia revealed a higher mortality rate and risk of secondary infections after corticosteroid treatment compared to the placebo group (75). Yet, with regard to the current COVID-19 pandemic, Cai et al. were able to define a threshold, based on the neutrophil-to-lymphocyte ratio, above which the treatment with the synthetic GC dexamethasone is highly recommended in cases of severe COVID-19 (76). Thus, GC treatment cannot be declared as effective or ineffective across the board. Accordingly, the application of synthetic GCs needs to be carefully pondered individually in each patient to ensure the safety of the treatment. Therefore, the application of GCs in patients with influenza infection needs to be further investigated to find the balance between anti-inflammatory action and viral clearance. This need of GC application fine-tuning further strengthens the hypothesis that comparably low GC concentrations derived from local steroidogenesis may play an important role in balancing local immune responses that on the one hand allow effective viral clearance and on the other hand prevent overshooting immune responses and consequently immunopathogenesis.

Similar to the gut-lung axis, the gut-skin axis describes physiological and pathological communication, and in some cases co-morbidities. A prevalent example, also with regard to autoimmune disorders, is psoriasis. Psoriasis is a chronic inflammatory disease of the skin, which is immune cell-mediated and involves keratinocyte hyperproliferation and T cell and granulocyte infiltration. Although the underlying cause is still unclear, type 3 immune responses are known to contribute to this skin pathology. It was classically thought to be mainly driven by pathogenic $T_H17$ cells producing IL-17, IL-22 and IFN-γ, which activate epithelial cells resulting in increased production of anti-microbial peptides, inflammatory cytokines and chemokines leading to the recruitment of granulocytes. However, current research revealed multiple additional cellular sources of IL-17, e.g. secreted by γδ T cells and CD8+ T cells, among many other cell types (77, 78). Emerging evidence, reviewed by Zwicky et al., demonstrates that not IL-23-independent IL-17 signaling but specifically the IL-23/IL-17 axis mediated the pathogenic psoriasiform inflammation (78). The IL-23/IL-17 axis and the aggravated type 3 immune response is also relevant to the pathogenesis of several other autoimmune and autoinflammatory diseases, of which psoriatic arthritis and

CD were reported to be directly associated with psoriasis. These and other autoinflammatory diseases were described to show immunological parallels with similarities in genetic predispositions and are currently viewed as systemic diseases rather than local dysregulation of the immune system (79).

So far, the underlying pathways within the crosstalk of involved tissues, linking the different autoimmune and/or autoinflammatory diseases, are less clear. Several processes have been described to contribute to the interaction between the barrier tissue and the HPA axis. Beside the immunological signaling across the lymphatic system, also the microbiome appears to affect the crosstalk directly and/or indirectly (80). This mostly occurs through metabolic interactions, but also neuroendocrine molecules have been demonstrated to link organs and inflammatory diseases through local and global circuits (81). Such links seem to be crucial for local pathogenic mechanisms to trigger autoinflammation at distant sites of the body. In this regard, the study of Kiyohara et al. revealed that for example psoriasiform skin inflammation triggers pathogenic conditions in the gut (82). Specifically, alterations in intestinal microbiome and local immune response were shown to originate from skin inflammation and lead to exacerbated dextran sodium sulfate (DSS)-induced colitis (82). Additionally, transplanted feces from mice with psoriasiform inflammation was sufficient to aggravate DSS-colitis in germ-free mice compared to controls with feces from untreated mice (82). These results emphasize the association of IBD with psoriasis and further solidifies the concept of autoinflammation as a systemic disease comprising processes, which involve the interaction of microbiota with the tissue-immune microenvironment. Tissue architecture and homeostasis are organized by stromal cells, which emerge to be relevant GC-mediated regulators of local and possibly global inflammation (83–87). Accordingly, in CD patients local GC *de novo* synthesis and reactivation was shown to be downregulated indicating their role in disease regulation (61, 88, 89). In the skin, both AD and psoriasiform skin inflammation enhanced local synthesis of GCs in keratinocytes (12). But apparently keratinocyte-derived GCs exert potent control only over type 3 immune responses in the skin as keratinocyte-deficient GC synthesis resulted in aggravated psoriasiform inflammation and the development of spontaneous skin inflammation (12). Another study by Psarras et al. further emphasized the concept that dysregulated stromal cells may represent a major contributor to and trigger of the pathogenesis of autoimmune disorders and challenges the immune cell-mediated point of view (90). They revealed keratinocytes to be the major source of type I IFN in At-Risk individuals in preclinical autoimmunity stage and in systemic lupus erythematosus (SLE) patients with established autoimmunity (90). Thus, keratinocytes and their potential to control local skin-immune microenvironment *via* autocrine GCs may directly restrict their own inflammatory potential as GCs are well described to efficiently suppress type I IFN expression and signaling pathway (91–93). Unraveling regulatory pathways in the skin may not only advance our knowledge on the regulation of local inflammatory processes but also impact our current understanding how systemic diseases such as SLE develop.

Intestinal barrier homeostasis is not only affected by distant inflammatory processes in the skin, but also contributes itself to central nervous system (CNS) autoimmunity, as recently demonstrated (94). Gut homeostasis, comprising of reciprocal intestinal IL-17 signaling and microbiome-host interaction, was shown to be required for the susceptibility to the CNS autoimmune model experimental autoimmune encephalomyelitis (EAE) (94). Despite this finding, an earlier study unraveled the interaction between gut microbiota and intestinal IL-17R signaling, which regulates commensal dysbiosis, restricts $T_H17$ formation and controls susceptibility to EAE autoimmune inflammation (95).

These studies demonstrate the complexity of interactions and mechanisms within the barrier microenvironment and their potential to trigger pathogenic inflammation across several organs. This is particularly true when synthetic GCs and several biologicals are clinically deployed to shut down local immunological flares. Targeting inflammatory mediators using monoclonal antibodies against IL-17, TNF or IL-12 has led to clinical benefits for some disorders, and low efficacy or even undesirable side-effects for others, also known as paradoxical effects (78, 79, 96). These paradoxical effects indicate that some cytokines exert tissue-specific actions resulting in regulatory effects that seem to be unique to specific barrier tissues. In addition to the distinct cytokine network signaling, various regulatory and tolerance-inducing mechanisms are mediated by the activity of GCs in tissue cells, among many other factors. This challenges our current view of how inflammatory diseases may develop and urge us to consider tissue-specific regulatory processes and pathways. **Figure 3** aims to illustrate the complexity of this interorgan crosstalk and points out possible links and pathways between the gut, the lung and the skin. GCs that are produced by the adrenal glands are released into the blood stream and act systemically on all organs. The possible interaction of systemic and local GCs is currently unexplored but a very interesting future research subject. Cytokines and mediators from the HPA axis could trigger local GC synthesis in the skin with anti-inflammatory effects on psoriasis and consequently on psoriatic arthritis. It is already known that psoriasis also leads to intestinal dysbiosis, which could lead to colitis and interfere with the production of anti-inflammatory SCFA. Local gut-derived GCs exert their anti-inflammatory effect in acute inflammation, Crohn's disease and colitis. SCFA produced in the gut have beneficial effects on influenza infection in the lung, whereas the effect of local GCs in the lung on influenza infection is currently unknown. However, their role is important in acute inflammation and also in chronic rhinosinusitis, and it is therefore very likely that they also exert their anti-inflammatory function in viral infections (**Figure 3**).

Overall, local GCs have been shown to specifically restrict type 1 and 3 inflammations at various barrier tissues, indicating their important role in balancing microbiome-host interaction and cytokine responses within the tissue microenvironment. Pathologies at barrier tissues leading to inflammation at various distant sites of the body highlight the importance of

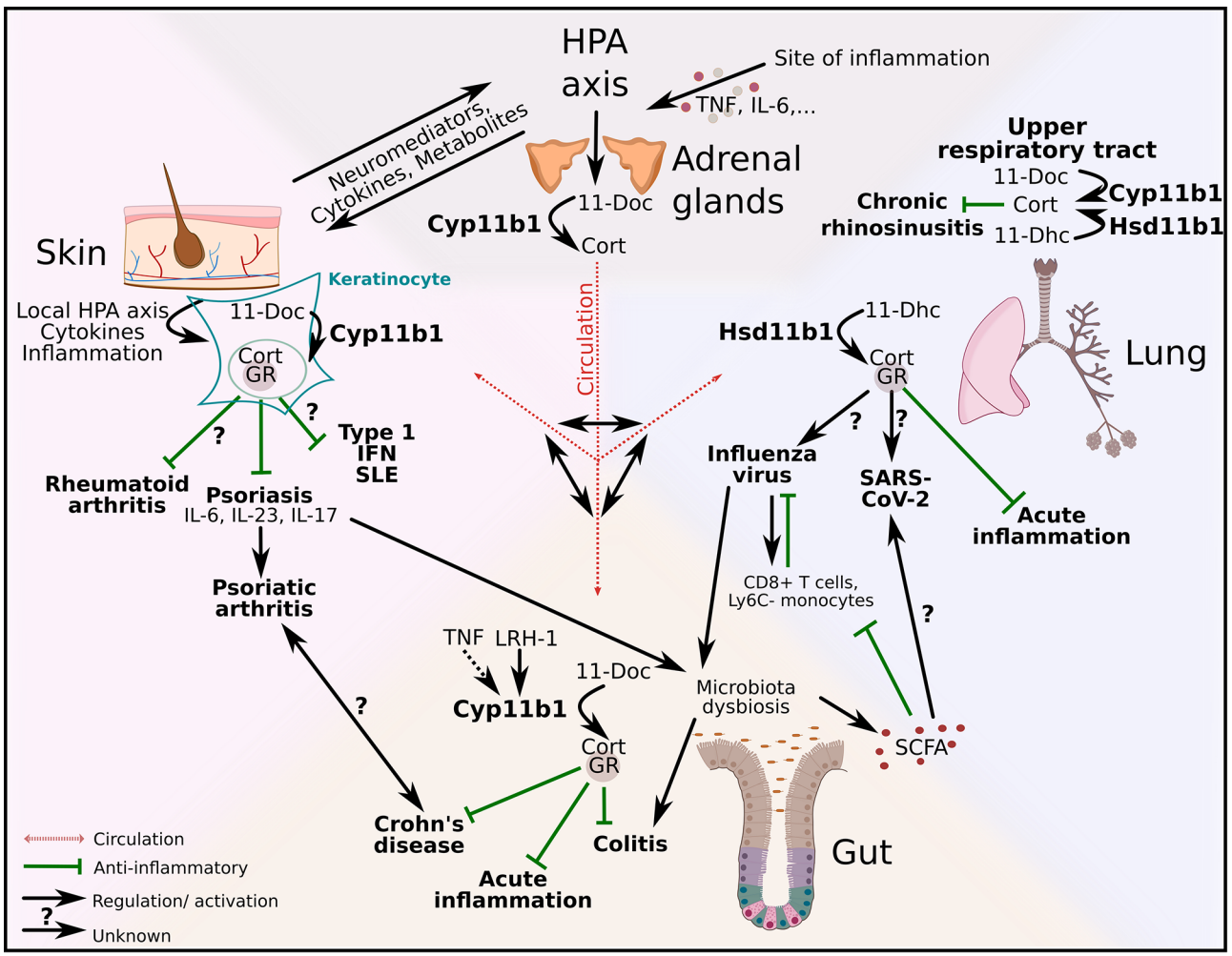


**FIGURE 3** | Regulation of local type 1 and 3 immune responses and autoinflammatory diseases across and beyond epithelial barriers. Adrenal-derived GCs are regulated *via* the hypothalamus-pituitary-adrenal (HPA) axis. Inflammation triggers the HPA axis *via* cytokines (e.g. tumor necrosis factor (TNF), interleukin (IL)-6) and *de novo* synthesized GCs act systemically *via* the circulation. Additionally, HPA axis–skin communication occurs *via* neuromediators, cytokines and metabolites. GC synthesis in keratinocytes is regulated *via* a local HPA axis. Whereas the effect of local GCs on rheumatoid arthritis is currently unknown, GC-GR interactions have anti-inflammatory effects on psoriasis. In addition to that psoriasis alters the gut microbiota with substantial effects on intestinal homeostasis. The production of gut–derived GCs is regulated *via* liver receptor homolog-1 (LRH-1) and inducible *via* TNF. 11β-hydroxylase (Cyp11b1) catalyzes the conversion of 11-deoxycorticosterone (11-Doc) to corticosterone (Cort). Intestinal GCs exert anti-inflammatory effects on acute inflammation, experimental colitis and Crohn's disease. Additionally, short chain fatty acids (SCFA) produced by the gut microbiota, have positive effects on influenza infection by modulating CD8+ T cell metabolism and increasing the number of Ly6C- monocytes. However, influenza virus has negative effects on the gut microbiota and can thereby limit the SCFA production. The influence of SCFA on other viral infections, like SARS-CoV-2, is currently not known. The synthesis of lung-derived GCs is catalyzed by 11β-hydroxysteroid dehydrogenase (Hsd11b1) and in the upper airways also by Cyp11b1. Local GCs inhibit acute lung inflammation. GC synthesis in the upper respiratory tract is upregulated in chronic rhinosinusitis patients and potentially exerts anti-inflammatory effects. The capacity of lung-derived GCs on skin-associated diseases and *vice versa* the effect of skin-derived GCs on lung disease in this context are unexplored.

local GCs in controlling these processes, and directs future research to unravel their tissue-specific regulatory processes. Extra-adrenal GCs may therefore not only maintain local tissue homeostasis but may also hold key in preventing the development of systemic autoimmune and autoinflammatory diseases, which at a first glace may appear unrelated to local barrier tissue inflammation.

## CONCLUSION

In conclusion, research on immunoregulatory networks at epithelial barriers and interorgan crosstalk contributes to a better understanding of the complexity of our immune system and opens up possibilities for novel treatments of chronic, inflammatory and autoimmune diseases. The importance of

the gut microbiome, in particular their production of anti-inflammatory metabolites like SCFA, for gut, lung and skin health and their regulatory function in a variety of inflammatory and autoimmune diseases could represent an interesting connection to local GC synthesis and regulatory pathways. The diseases we have been discussing here (asthma, influenza infection, UC, CD, AD, psoriasis) affect a large part of the population, and therefore it is even more important to better understand their causes and the regulatory mechanisms of the body opposing them, opening new perspectives in their treatment. Even if these diseases are usually not fatal, they still limit the quality of life of those affected and especially with regard to the atopic march, the path of chronic suffering starts at a very early age.

This review highlights several areas, where the role of local GC synthesis in immune regulation and the interorgan crosstalk has been largely unaddressed. Clearly, we see great potential in their exploration to fill our knowledge gaps and discover new signaling and regulatory pathways. For the scientific progress, it is indispensable today to consider local regulations of the immune system also in a systemic context in order to better understand their interrelationships and to apply the knowledge effectively in the clinics.

## AUTHOR CONTRIBUTIONS

VMM wrote the manuscript and generated the figures. TSP wrote parts of the manuscript, TB overviewed the research on extra-adrenal GC synthesis, and edited and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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# Glucocorticoid-Induced Exacerbation of Mycobacterial Infection Is Associated With a Reduced Phagocytic Capacity of Macrophages

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Glucocorticoids are effective drugs for treating immune-related diseases, but prolonged therapy is associated with an increased risk of various infectious diseases, including tuberculosis. In this study, we have used a larval zebrafish model for tuberculosis, based on *Mycobacterium marinum* (*Mm*) infection, to study the effect of glucocorticoids. Our results show that the synthetic glucocorticoid beclomethasone increases the bacterial burden and the dissemination of a systemic *Mm* infection. The exacerbated *Mm* infection was associated with a decreased phagocytic activity of macrophages, higher percentages of extracellular bacteria, and a reduced rate of infected cell death, whereas the bactericidal capacity of the macrophages was not affected. The inhibited phagocytic capacity of macrophages was associated with suppression of the transcription of genes involved in phagocytosis in these cells. The decreased bacterial phagocytosis by macrophages was not specific for *Mm*, since it was also observed upon infection with *Salmonella* Typhimurium. In conclusion, our results show that glucocorticoids inhibit the phagocytic activity of macrophages, which may increase the severity of bacterial infections like tuberculosis.

**Keywords: glucocorticoids, *Mycobacterium marinum*, macrophage, phagocytosis, zebrafish, tuberculosis**

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## HIGHLIGHTS

Using a zebrafish tuberculosis model, we show that glucocorticoids increase the severity of the bacterial infection, and decrease the phagocytosis by macrophages. This may explain the glucocorticoid-induced increase in susceptibility to tuberculosis in humans.

## INTRODUCTION

Glucocorticoids (GCs) are a class of steroid hormones that are secreted upon stress. The main endogenous GC in our body, cortisol, helps our bodies adapt to stressful situations and for this purpose it regulates a wide variety of systems, like the immune, metabolic, reproductive, cardiovascular and central nervous system. These effects are mediated by an intracellular receptor, the glucocorticoid receptor (GR), which acts as a ligand-activated transcription factor.

Synthetic GCs are widely prescribed to treat various immune-related diseases due to their potent suppressive effects on the immune system. However, prolonged therapy with these pleiotropic steroids evokes severe side effects, such as osteoporosis and diabetes mellitus (1, 2). Importantly, the therapeutic immunosuppressive effect of GCs may lead to infectious complications because of the compromised immune system (3–5). Similarly, after chronic stress an increased susceptibility to infectious diseases has been observed, due to the high circulating levels of cortisol. In order to better understand these complex effects of GCs, more research is required into how GCs influence the susceptibility to infections and the course of infectious diseases.

Tuberculosis (TB) is the most prevalent bacterial infectious disease in the world, caused by the pathogen *Mycobacterium tuberculosis* (*Mtb*). Despite the efforts made to reach the "End TB Strategy" of the World Health Organization, *Mtb* still infects approximately one-quarter of the world's population and caused an estimated 1.5 million deaths in 2018, which makes it one of the top 10 causes of death globally (6, 7). The major characteristic of *Mtb* infection is the formation of granulomas containing infected and non-infected immune cells (8). Most *Mtb*-infected people develop a latent, noncontagious infection and do not show any symptoms, with the bacteria remaining inactive, while contained within granulomas (9, 10). About 5-10% of the carriers develop a clinically active TB disease associated with a loss of granuloma integrity (9, 11). Among those TB patients, the majority manifest a lung infection and around 20% shows infection in other organs like the central nervous system, pleura, urogenital tracts, bones and joints, and lymph nodes (12). Antibiotics are currently the mainstay for TB treatment, but since antibiotic resistance is rising and an effective vaccine against latent or reactivated TB is still lacking, alternative therapies to control TB are needed (13).

GCs are known to modulate the pathogenesis of TB, but their effects are highly complicated. The use of GCs is considered as a risk factor for TB. Patients who are being treated with GCs have an approximately 5-fold increased risk for developing new TB (14), and treatment with a moderate or high dose of GCs is associated with an increased risk of activation of latent TB (15–17). Consequently, a tuberculin skin test (TST) for screening latent TB is recommended before starting GC therapy (14). Moreover, chronic stress which is associated with increased circulating levels of the endogenous GC cortisol, has been shown to be associated with a higher incidence of TB (18).

Despite the generally detrimental effects of GCs on TB susceptibility and progression, certain types of TB patients are treated with GCs. Chronic TB patients may require GCs for treatment of other disorders, and it has been shown that adjunctive GC therapy may have beneficial effects. Traditionally, adjunctive GC with standard anti-TB therapy has been used for prevention of inflammatory complications in patients with tuberculous meningitis, pericarditis, and pleurisy (19–22). It has been reported that adjunctive GC therapy could improve the probability of survival in tuberculous meningitis and pericarditis (23–26). In case of pulmonary TB, the most common form of TB, adjunctive GC therapy is recommended in advanced tuberculosis since broad and significant clinical benefits have been demonstrated (27, 28).

Although GCs are being used for adjunctive therapy, the beneficial effects of GC treatment are still under debate. For tuberculous pleurisy TB, the efficacy of GCs is still controversial and for meningitis and pericarditis, information on the GC effects is still incomplete (22, 26, 29, 30). A review regarding clinical trials for pulmonary TB showed that, although adjunctive GC therapy appears to have short-term benefits, it is not maintained in the long-term (31). An explanation for the complexity of the effects of GC therapy in TB has been offered by Tobin et al. (2012). They showed that patients suffer from TB as a result of either a failed or an excessive immune response to the mycobacterial infection, and that only the subset of TB meningitis patients with an excessive response, showing a hyperinflammatory phenotype (in their study as a result of a polymorphism in the *LTA4H* gene), benefited from adjunctive GC therapy. It was suggested that GCs may also be beneficial for similar subgroups of patients suffering from other forms of TB (32).

The complex interplay between GC actions and TB underscores the need for a better understanding of the effects of GCs on mycobacterial infection. In the present study we have studied these effects using *Mycobacterium marinum* (*Mm*) infection in zebrafish as a model system. *Mm* is a species closely related to *Mtb* that can infect zebrafish and other cold-blooded animals naturally, causing a TB-like disease (33). Infection of zebrafish larvae with *Mm* provides an animal model system that mimics hallmark aspects of *Mtb* infection in humans and is widely used for research into mechanisms underlying the course of this disease (34–36). Like *Mtb, Mm* is able to survive and replicate within macrophages and, in later stages of infection, induces the formation of granulomas (37). The transparency of zebrafish at early life stages makes it possible to perform non-invasive long-term live imaging, which has been used to reveal the earliest stages of granuloma formation (38). In addition, the availability of different transgenic and mutant zebrafish lines and the efficient application of molecular techniques allow us to exploit this zebrafish *Mm* infection model optimally to study both the host factors and bacterial factors involved in mycobacterial infection processes (33, 34, 39). For example, zebrafish studies revealed that infected macrophages can detach from a granuloma and facilitate dissemination to new locations (38). Moreover, the study of an *lta4h* mutant zebrafish line showed that the polymorphism in the *LTA4H* gene is associated with the susceptibility to mycobacterial diseases and the response to adjunctive GC therapy in human, representing a prime example of translational research (32, 40).

The zebrafish has proven to be a suitable model for studying the effects of GCs, since the GC signaling pathway is very well conserved between zebrafish and humans. Both humans and zebrafish have a single gene encoding the GR, and the organization of these genes is highly similar (41–43). Both the human and the zebrafish gene encodes two splice variants, the α-

isoform, the canonical receptor, and the β-isoform, which has no transcriptional activity (42). The DNA binding domain (DBD) and ligand binding domain (LBD) of the canonical α-isoform of the human and zebrafish GR share similarities of 98.4% and 86.5% respectively (42). The zebrafish GR α-isoform, hereafter referred to as Gr, mediates GC effects that have traditionally been observed in humans and other mammals as well, like the effects on metabolism (44) and the suppression of the immune system (45). This makes the zebrafish an ideal model to study the mechanisms of GC action *in vivo* (46, 47). In a recent study, we have demonstrated that GCs inhibit the activation of the immune system in zebrafish larvae upon wounding (48). Treatment with a synthetic GC attenuated the migration of neutrophils, and inhibited the differentiation of macrophages towards a pro-inflammatory phenotype without affecting the migration of the latter cell type.

In the present study, to investigate the functional consequences of the previously observed GC effects on immune cells, we have investigated how GCs modulate the course of an *Mm* infection in zebrafish larvae. We demonstrate that beclomethasone increases the level of *Mm* infection and tissue dissemination. This increased *Mm* infection can be explained by an inhibition of the phagocytic activity of macrophages by beclomethasone, which did not affect the microbicidal capacity of these cells. The inhibitory effect of beclomethasone on phagocytosis, which most likely results from Gr interfering with the transcription of genes required for phagocytosis, results in a higher percentage of extracellular bacteria, which eventually leads to an exacerbation of the *Mm* infection.

# MATERIALS AND METHODS

## Zebrafish Lines and Maintenance

Zebrafish were maintained and handled according to the guidelines from the Zebrafish Model Organism Database (zfin.org) and in compliance with the directives of the local animal welfare body of Leiden University. They were exposed to a 14 hours light and 10 hours dark cycle to maintain circadian rhythmicity. Fertilization was performed by natural spawning at the beginning of the light period. Eggs were collected and raised at 28°C in egg water (60 μg/ml Instant Ocean sea salts and 0.0025% methylene blue). The following fish lines were used: wild type strain AB/TL, and the transgenic lines *Tg(mpeg1:mCherry-F$^{umsF001}$)* (49) and *Tg(mpeg1:eGFP$^{gl22}$)* (50).

## Bacterial Culture and Infection Through Intravenous Injections

Bacteria used for this study were *Mycobacterium marinum*, strain M, constitutively fluorescently labelled with Wasabi or mCrimson (51, 52), *Mm* mutant *Δerp* labelled with Wasabi (53), *Salmonella enterica serovar* Typhimurium (*S.* Typhimurium) wild type (wt) strain SL1344 labelled with mCherry (54, 55), and a reactive oxygen species (ROS) biosensor *S.* Typhimurium strain (*SL1344 sifBp::mCherry/pkatGp-gfpOVA*) (54, 56). The *Mm* and *S.* Typhimurium strains were cultured at 28°C and 37°C respectively and the bacterial suspensions were prepared with phosphate buffered saline (PBS) with 2% (w/v) polyvinylpyrrolidone-40 (PVP40, Sigma-Aldrich), as previously described (57). The suspension of *Mm Δerp*-Wasabi was prepared directly from -80°C frozen aliquots.

After anesthesia with 0.02% aminobenzoic acid ethyl ester (tricaine, Sigma-Aldrich), 28 hours post fertilization (hpf) embryos were injected with *Mm* or *S.* Typhimurium into the blood island (or hindbrain if specified) under a Leica M165C stereomicroscope, as previously described (57). The injection dose was 200 CFU for *Mm* and 50 CFU for *S.* Typhimurium, except for the experiments to assess activation of the ROS biosensor, where a high bacterial dose of 2000-4000 CFU was used as previously described (56).

## Chemical Treatments and Bacterial Burden Quantification

The embryos were treated with 25 μM (or different if specified) beclomethasone (Sigma-Aldrich) or vehicle (0.05% dimethyl sulfoxide (DMSO)) in egg water from 2 hours before injection to the end of an experiment. RU-486 (Sigma-Aldrich) was administered at a concentration of 5 μM (0.02% DMSO), and cycloheximide (Sigma-Aldrich) at 100 μg/ml (0.04% DMSO). If the treatment lasted longer than 1 day, the medium was refreshed every day.

For bacterial burden quantification, the embryos from the vehicle- and beclomethasone-treated groups were imaged alive using a Leica M205FA fluorescence stereomicroscope equipped with a Leica DFC 345FX camera (Leica Microsystems). The images were analyzed using custom-designed pixel quantification software (previously described by Benard et al. (58), and Image J (plugin 'Analyze Particles').

## Hindbrain Infection and Analysis of Dissemination

To assess the dissemination efficiency, the embryos were injected with 50 CFU *Mm* into the hindbrain at 28 hpf. At 2 dpi, the embryos were imaged with a Leica M205FA fluorescence stereomicroscope equipped with a Leica DFC 345FX camera. The embryos were classified into two categories: with or without disseminated infection. An embryo was considered without disseminated infection if all the bacteria were still contained in the hindbrain ventricle and considered with dissemination if bacteria were present in any other part of the embryo.

## Analysis of Microbicidal Activity

After infection at 28 hpf with *Mm Δerp*-Wasabi, *Tg(mpeg1:mCherry-F)* embryos were fixed at 44 hpi with 4% paraformaldehyde (PFA, Sigma-Aldrich) and imaged using a Leica TCS SP8 confocal microscope with 40X objective (NA 1.3). All macrophages that contained *Mm Δerp*-Wasabi in the tail region were analyzed. The level of infection inside macrophages was classified into two categories based on the number of bacteria: 1-10 bacteria or >10 bacteria, following established protocols (59, 60). To quantify the bacterial burden of *Mm*

*Δerp* infected embryos, the fluorescence intensity of the Wasabi signal was measured using image J.

## Analysis of Phagocytic Activity

To study the dynamics of bacterial phagocytosis during the early stage of infection, *Tg(mpeg1:mCherry-F)* or *Tg(mpeg1:eGFP)* embryos were intravenously infected at 28 hpf with *Mm*-Wasabi or *S.* Typhimurium-mCherry. At 5, 15 and 25 min after infection the embryos were fixed with 4% PFA, so they could later be imaged using a Leica TCS SP8 confocal microscope with 20X objective (NA 0.75). The yolk sac area was selected as the quantification area (**Figure 3A**). The number of fluorescently labelled *Mm* or *S.* Typhimurium in this area, and those present inside a macrophage, were counted in a manual and blinded way.

## TUNEL Assay

After infection at 28 hpf, *Tg(mpeg1:mCherry-F)* embryos were fixed with 4% PFA at 48 hpi and stained using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) with the In Situ Cell Death Detection Kit, TMR red (Sigma-Aldrich), as previously described by Zhang et al. (2019). For this TUNEL staining, the embryos were first dehydrated and then rehydrated gradually with methanol in PBS, and permeabilized with 10 μg/ml Proteinase K (Roche). The embryos were subsequently fixed with 4% PFA for another 20 min and stained with reagent mixture overnight at 37°C. After the reaction was stopped by washing with PBS containing 0.05% Tween-20 (PBST), the caudal hematopoietic tissue (CHT) region of the embryos was imaged using a Leica TCS SP8 confocal microscope with 40X objective (NA 1.3). The total number of fluorescently labelled *Mm* clusters and the number of these clusters overlapping with TUNEL staining were counted in a manual and blinded way.

## Fluorescence-Activated Cell Sorting (FACS) of Macrophages

Macrophages were sorted from *Tg(mpeg1:mCherry-F)* embryos as previously described (61, 62). Dissociation was performed with 150-200 embryos for each sample after 2 hours beclomethasone or vehicle treatment (started at 28 hpf) using Liberase TL (Roche) and stopped by adding Fetal Calf Serum (FCS) to a final concentration of 10%. Isolated cells were resuspended in Dulbecco's PBS (DPBS), and filtered through a 40 μm cell strainer. Actinomycin D (Sigma-Aldrich) was added (final concentration of 1 μg/ml) to each step to inhibit transcription. Macrophages were sorted based on their red fluorescent signal using a FACSAria III cell sorter (BD Biosciences). The sorted cells were collected in QIAzol lysis reagent (Qiagen) for RNA isolation.

## RNA Isolation, cDNA synthesis and Quantitative PCR (qPCR) Analysis

RNA isolation from FACS-sorted cells was performed using the miRNeasy mini kit (Qiagen), according to the manufacturer's instructions. Extracted total RNA was reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad). QPCR was performed on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) using iTaq™ Universal SYBR® Green Supermix (Bio-Rad). The sequences of the primers used are provided in **Supplementary Table 1**. Cycling conditions were pre-denaturation for 3 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and elongation for 30 s at 72°C. Fluorescent signals were measured at the end of each cycle. Cycle threshold values (Ct values, i.e. the cycle numbers at which a threshold value of the fluorescence intensity was reached) were determined for each sample. To determine the gene regulation due to beclomethasone treatment in each experiment, the average Ct value of the beclomethasone treated samples was subtracted from the average Ct value of the vehicle-treated samples, and the fold change of gene expression was calculated, which was subsequently adjusted to the expression levels of a reference gene (*peptidylprolyl isomerase Ab* (*ppiab*)).

## Analysis of ROS Production

Embryos were infected at 28 hpf with a ROS biosensor *S.* Typhimurium strain (*SL1344 sifBp::mCherry/pkatGp-gfpOVA*) which constitutively expresses mCherry, while the expression of an unstable GFP variant (GFP-OVA) is under the control of an OxyR-activated promoter, which is activated upon exposure to ROS from the host (54). Embryos were imaged using a Leica TCS SP8 confocal microscope with 40X objective (NA 1.3) at 4 hpi. An area in the CHT region, close to the site of injection, was selected for analysis. The fluorescence intensity of the GFP signal in this area, which represents ROS production, and the intensity of the mCherry signal from *S.* Typhimurium were quantified, and their ratio was calculated.

## Statistical Analysis

Statistical analysis was performed using GraphPad Prism by one-way ANOVA with Bonferroni's *post hoc* test (**Figure 1A**) or two-way ANOVA with Tukey's *post hoc* test (**Figures 1B** and **2A–C**) or two-tailed t-test (**Figures 2D**, **4–6** and **7B**) or using R Statistical Software by fitting data to a beta inflated regression (from 'gamlss' package) (63) with Tukey's *post hoc* test (**Figures 3** and **7A**).

# RESULTS

## Beclomethasone Increases Mycobacterial Infection Through Glucocorticoid Receptor (Gr) Activation

To study the effect of GC treatment on *Mm* infection in zebrafish, we pretreated zebrafish embryos with beclomethasone and infected them intravenously with fluorescently labelled *Mm*. At 4 days post infection (dpi), the bacterial burden was assessed by quantification of pixel intensities of fluorescence microscopy images. We found that the bacterial burden increased by 2.3 fold when embryos were treated with 25 μM beclomethasone compared with the vehicle-treated group (**Figures 1A, C**). Beclomethasone treatment at lower concentrations of 0.04, 0.2, 1 and 5 μM did not affect the bacterial burden. Therefore, a concentration of 25 μM beclomethasone was used in subsequent experiments. We have previously shown that this concentration

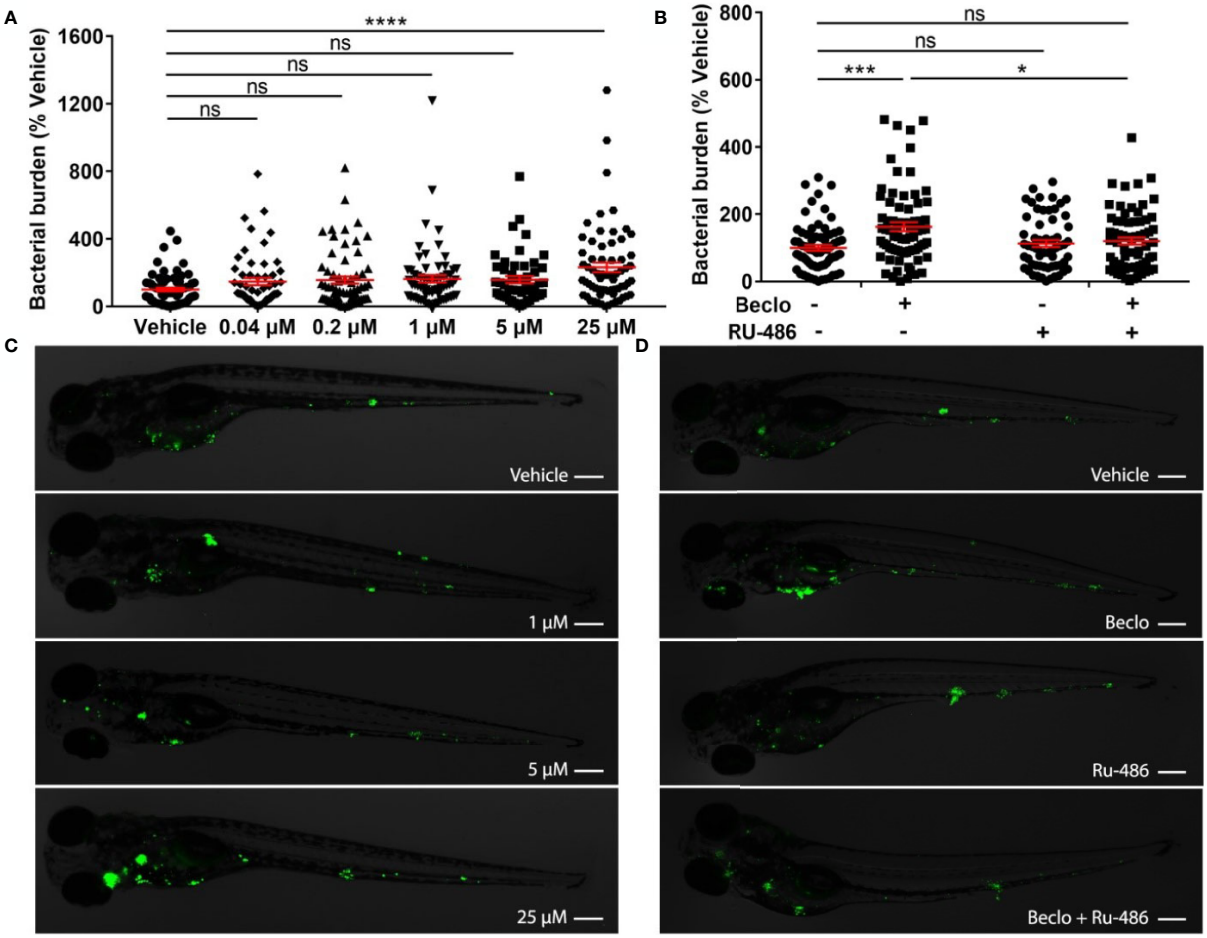


**FIGURE 1** | Effect of beclomethasone on *Mm* infection burden in zebrafish. **(A)** Bacterial burden of zebrafish larvae at four days after intravenous injection (at 28 hpf) of *Mm* and treatment with vehicle or different concentrations of beclomethasone (beclo), started at 2 h before infection. Statistical analysis by one-way ANOVA with Bonferroni's *post hoc* test revealed that the bacterial burden was significantly increased in the group treated with 25 µM beclomethasone, compared to the burden of the vehicle-treated group. **(B)** Effect of the GR antagonist RU-486 on the beclomethasone-induced increase of the bacterial burden at 4 dpi. The bacterial burden was significantly increased by beclomethasone (25 µM) treatment and this increase was abolished in the presence of RU-486. Statistical analysis was performed by two-way ANOVA with Tukey's *post hoc* test. In panels **(A, B)**, each data point represents a single larva and the means ± s.e.m. of data accumulated from three independent experiments are shown in red. Statistical significance is indicated by: ns, non-significant; *P<0.05; ***P<0.001; ****P<0.0001. **(C, D)** Representative fluorescence microscopy images of *Mm*-infected larvae at 4 days post infection (dpi), representing experimental groups presented in panels **(A, B)** Bacteria are shown in green. Scale bar = 200 µm.

effectively reduces wound-induced neutrophil migration in zebrafish as well (48).

To demonstrate that the beclomethasone-induced increase in bacterial burden was not due to a general toxicity of beclomethasone but mediated specifically by the Gr, we used the GR antagonist RU-486. The results of these experiments showed that the beclomethasone-induced increase in bacterial burden at 4 dpi was abolished when co-treatment with RU-486 was applied (**Figures 1B, D**), which indicates that the effect of beclomethasone requires activation of Gr. No significant difference was observed when the RU-486-treated larvae were compared to the vehicle-treated group. In conclusion, beclomethasone increases the level of *Mm* infection in zebrafish larvae and this effect is mediated by Gr.

## Beclomethasone Treatment Leads to a Higher Infection and Dissemination Level

Subsequently, we analyzed the effect of beclomethasone on *Mm* infection in more detail. The total bacterial burden (**Figure 2A**), the number of bacterial clusters per individual (**Figure 2B**) and the average size of the bacterial clusters (**Figure 2C**) were quantified at 1, 2, 3 and 4 dpi. The results showed that the difference in bacterial burden between the beclomethasone-treated group and the vehicle group was not significant at 1-3 dpi, but that a significant difference was observed at 4 dpi (6186.1 ± 626.5 *vs* 2870.5 ± 235.0). However, a significant increase in the number of bacterial clusters in the beclomethasone-treated group was already detected at 3 dpi (28.3 ± 1.9 vs 18.1 ± 1.5 in the vehicle group) which was sustained at 4 dpi (64.2 ± 3.5 *vs* 35.4 ± 2.6). The size of the bacterial clusters at 4 dpi

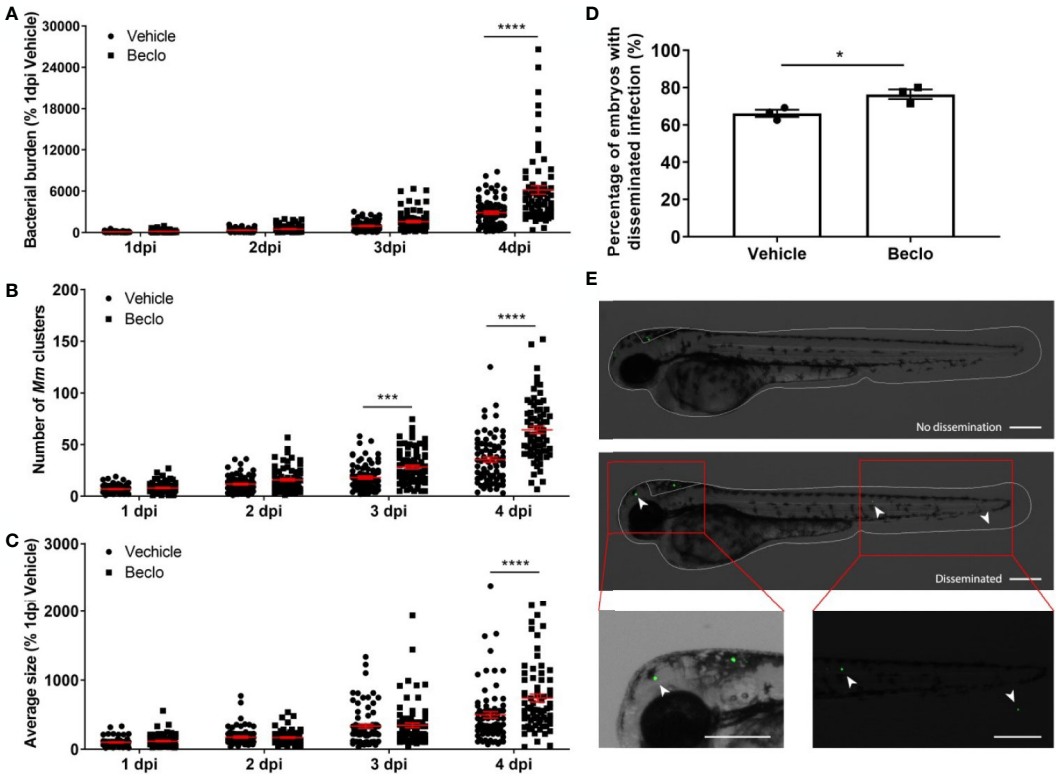


**FIGURE 2** | Beclomethasone effects on *Mm* infection progression and bacterial dissemination. **(A–C)**. Bacterial burden **(A)**, number of bacterial clusters **(B)** and the average size of bacterial clusters **(C)** were determined at 1, 2, 3 and 4 dpi following intravenous *Mm* injection (28 hpf) and treatment with vehicle or 25 μM beclomethasone, started at 2 h before infection. Significant increases due to the beclomethasone treatment were observed for all parameters at 4 dpi. For the number of bacterial clusters, the increase was also significant at 3 dpi. Statistical analysis was performed by two-way ANOVA with Tukey's *post hoc* test. Each data point represents a single larva and the means ± s.e.m. of data accumulated from three independent experiments are shown in red. Statistical significance is indicated by: ***P<0.001; ****P<0.0001. **(D)** Effect of beclomethasone on dissemination of *Mm* by hindbrain ventricle injection. Hindbrain infections were performed at 28 hpf, and at 24 hours post infection (hpi), a significantly increased percentage of larvae with disseminated Mm infection was detected in the beclomethasone-treated group compared to the vehicle group. Statistical analysis was performed by two-tailed t-test. Values shown are the means ± s.e.m. of three independent experiments with a total sample size of 27 in the vehicle-treated group and 31 in the beclomethasone-treated group. Statistical significance is indicated by: *P<0.05. **(E)** Representative images of embryos with and without dissemination of the infection upon hindbrain injection of *Mm*. Scale bar = 200 μm.

was also increased in the beclomethasone-treated group compared to the cluster size in the vehicle-treated group (741.6 ± 58.3 *vs* 498.3 ± 45.7). The increase in the number of bacterial clusters indicates an increased dissemination of the infection due to beclomethasone treatment. We confirmed this effect of beclomethasone on bacterial dissemination using hindbrain infection (**Figures 2D, E**). Following *Mm* injection into the hindbrain ventricle, 66.1 ± 2.0% of embryos in the vehicle-treated group showed disseminated infection in tissues of the head and tail at 24 hours post infection (hpi), while a significantly higher number (76.4 ± 2.6%) showed this dissemination in the beclomethasone-treated group.

## Beclomethasone Activation of Gr Inhibits Macrophage Phagocytic Activity

Since previous studies showed that increased *Mm* infection could be related to decreased phagocytic activity of macrophages in zebrafish (64), we studied the effect of beclomethasone on phagocytosis. We used the *Tg(mpeg1:mCherry-F)* line in which macrophages are fluorescently labeled, and assessed phagocytic activity of macrophages by determining the percentage of *Mm* that were internalized by macrophages in the yolk sac area within 5-25 min after infection based on previous analysis of the kinetics of this response (64) (**Figures 3A–C**). The bacteria are intravenously injected at 28 hpf, and at this developmental stage the primitive macrophages of the zebrafish embryos are primarily localized in the blood circulation (65). Phagocytosis of the injected bacteria is therefore independent of tissue migration of macrophages in this experiment. In the vehicle-treated group, the percentage of phagocytosed *Mm* was 17.4 ± 3.5% at 5 minutes post infection (mpi) and gradually increased to 41.9 ± 4.9% and 52.8 ± 5.2% at 15 and 25 mpi respectively. At each of these time points, a lower percentage of *Mm* were phagocytosed in the beclomethasone-treated group (4.6 ± 1.6% at 5 mpi, 25.7 ± 4.7% at 15 mpi and 34.0 ± 5.2% at 25 mpi). In addition, we studied the involvement of Gr in the beclomethasone-induced inhibition of phagocytosis at 5 mpi,

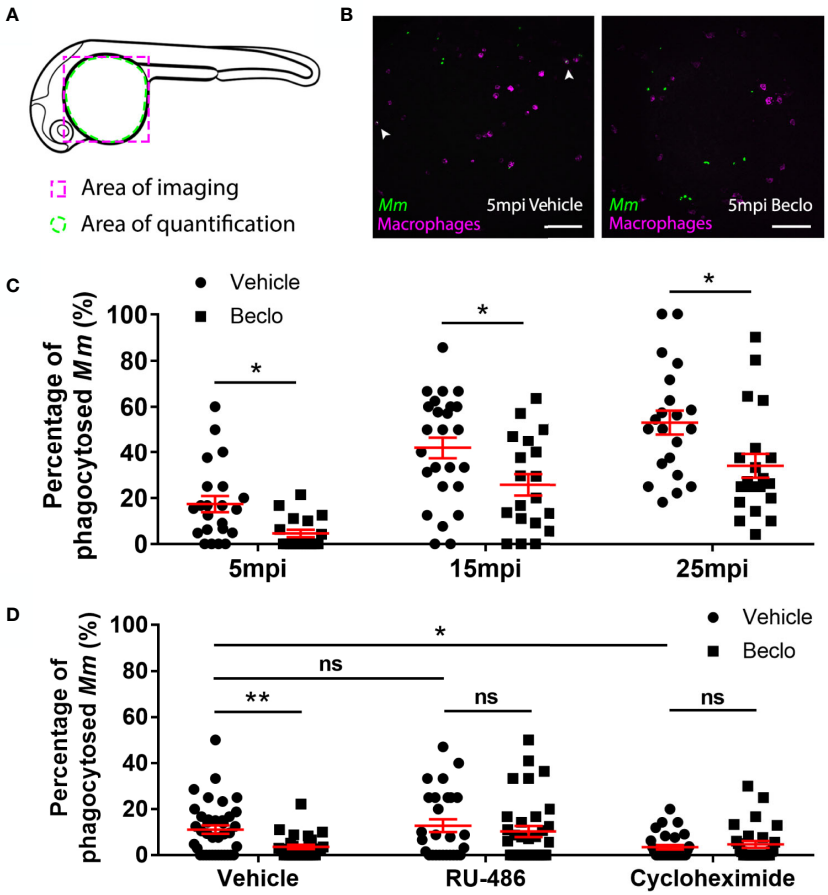


**FIGURE 3** | Effect of beclomethasone on phagocytic activity of macrophages and its dependency on Gr and *de novo* protein synthesis. **(A)** Schematic drawing of a zebrafish embryos at 28 hpf indicating the areas of imaging (purple dashed box, used for representative images) and quantification (green dashed circle) of *Mm* phagocytosis. **(B)** Representative confocal microscopy images of embryos of the *Tg(mpeg1:mCherry-F)* line injected with Mm at 28 hpf. Images were taken of infected embryos that were vehicle- or beclomethasone-treated at 5 minutes post infection (mpi). Macrophages are shown in magenta, bacteria in green. Scale bar = 100 μm. Arrowheads indicate bacterial clusters phagocytosed by macrophages. **(C)** Percentages of phagocytosed *Mm* clusters (of total number of *Mm* clusters) at 5, 15 and 25 mpi. Statistical analysis, performed by fitting data to a beta inflated regression with Tukey's *post hoc* test, showed that beclomethasone decreased this percentage at all three time points. **(D)** Effects of RU-486 and cycloheximide on the beclomethasone-inhibited phagocytic activity. Embryos were treated with vehicle or beclomethasone and received either a vehicle, RU-486 or cycloheximide co-treatment two hours before injection of *Mm* at 28 hpf, and phagocytic activity was determined at 5 mpi. The significant inhibitory effect of beclomethasone on phagocytosis was not observed in the presence of RU-486. Cycloheximide, just like beclomethasone, significantly inhibited the phagocytic activity, and the combined cycloheximide/beclomethasone treatment showed the same level of inhibition. Statistical analysis was performed by fitting data to a beta inflated regression with Tukey's *post hoc* test. In panels **(C, D)**, each data point represents a single embryo and the means ± s.e.m. of data accumulated from three independent experiments are shown in red. Statistical significance is indicated by: ns, non-significant; *P<0.05; **P<0.01.

by co-treatment with the GR antagonist RU-486 (**Figure 3D**). We found that the decreased phagocytic activity that was observed upon beclomethasone treatment was abolished when larvae were co-treated with RU-486, indicating that the inhibition of phagocytosis by beclomethasone is mediated by Gr.

Gr generally acts as a transcription factor, modulating the transcription rate of a wide variety of genes. To study whether phagocytosis could be modulated by altering the process of protein synthesis, we blocked *de novo* protein synthesis by treatment with cycloheximide (**Figure 3D**). We observed that the phagocytic activity of macrophages at 5 mpi was decreased by the cycloheximide treatment (3.4 ± 1.0% vs 11.1 ± 1.8% in the vehicle group). These data demonstrate that phagocytosis depends on *de novo* protein synthesis, and indicate that repression of transcription of specific genes may inhibit the phagocytic activity of macrophages.

## Beclomethasone Inhibits Phagocytosis-Related Gene Expression in Macrophages

To unravel the molecular mechanisms underlying the beclomethasone-induced inhibition of the phagocytic activity of macrophages, we performed qPCR analysis on FACS-sorted macrophages derived from 28 hpf larvae after 2 h of beclomethasone treatment. To determine the phenotype of the sorted macrophages, the expression of a classic pro-inflammatory gene, *tnfa*, was measured (66, 67). The levels of

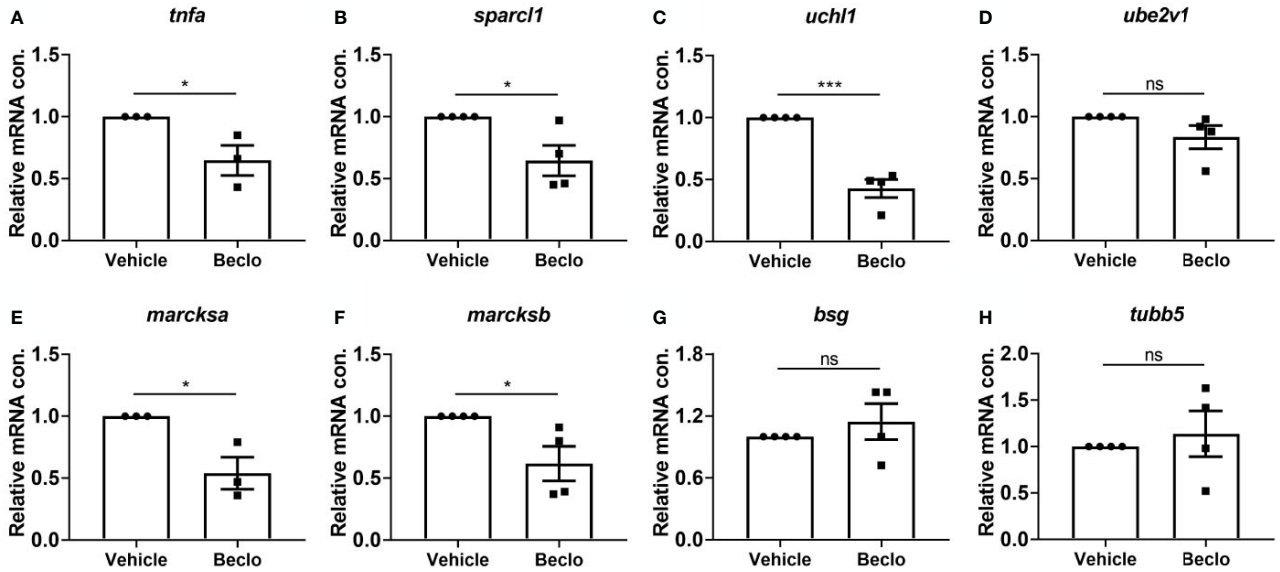


**FIGURE 4** | Effect of beclomethasone on gene expression levels in FACS-sorted macrophages. At 28 hpf, *Tg(mpeg1:mCherry-F)* embryos were treated with vehicle or beclomethasone for two hours, after which macrophages were isolated by FACS sorting. Gene expression levels were determined in the sorted cells by qPCR for *tnfa* **(A)**, *sparcl1* **(B)**, *uchl1* **(C)**, *ube2v1* **(D)**, *marcksa* **(E)**, *marcksb* **(F)**, *bsg* **(G)** and *tubb5* **(H)**. Statistical analysis by two-tailed t-test showed that the levels of *tnfa*, *sparcl1*, *uchl1*, *marcksa* and *marcksb* expression were significantly inhibited by beclomethasone treatment. Data shown are the means ± s.e.m. of three or four independent experiments, and markers show averages of individual experiments. Statistical significance is indicated by: ns, non-significant; *P<0.05; ***P<0.001.

*tnfa* expression were significantly lower after beclomethasone treatment (**Figure 4A**), in agreement with previously reported transcriptome analysis (48). In addition, we measured the expression levels of seven genes, for which a phagocytosis-promoting role has been established: *sparcl1*, *uchl1*, *ube2v1*, *marcksa*, *marcksb*, *bsg* and *tubb5* (68–70) (**Figures 4B–H**). The expression levels of four of these genes, *sparcl1*, *uchl1*, *marcksa* and *marcksb*, were inhibited by beclomethasone treatment, while the levels of the other three (*ube2v1, bsg* and *tubb5)* were not affected. These data suggest that beclomethasone inhibits the phagocytic activity of macrophages by suppressing the transcription of phagocytosis-related genes in these cells.

## Beclomethasone Treatment Results in Fewer Intracellular Bacteria, Limits Infected Cell Death, Without Affecting the Microbicidal Capacity of Macrophages

To further analyze the possible mechanisms underlying the beclomethasone-induced increase in the *Mm* infection level, we assessed the percentage of bacteria that are present inside and outside macrophages in the caudal hematopoietic tissue (CHT) at 48 hpi using *Mm* infection in the *Tg(mpeg1:eGFP)* line. The results showed that beclomethasone treatment resulted in a decreased percentage of intracellular bacteria (23.8 ± 3.0%) compared to the percentage in the vehicle-treated group (36.5 ± 3.6%) (**Figures 5A, C**). This result was in line with the observed decrease in phagocytosis at earlier stages of infection. To determine if the lower percentage of intracellular bacteria correlated with higher macrophage viability, we used terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining to detect cell death (71). In the beclomethasone-treated group, the percentage of *Mm* that were colocalized with TUNEL staining (9.4 ± 1.6%) at 48 hpi was significantly lower compared to the percentage of the vehicle group (17.2 ± 2.3%) (**Figures 5B, D**). These data suggest that the observed inhibition of phagocytosis upon beclomethasone treatment causes a decrease in the percentage of intracellular bacteria, which underlies the lower numbers of macrophages undergoing cell death as a result of the *Mm* infection.

Next, we considered the possibility that beclomethasone, in addition to inhibiting phagocytosis, might affect the microbicidal capacity of macrophages. Therefore, we injected embryos with bacteria from the attenuated *Mm Δerp* strain. The *erp* (exported repetitive protein) virulence factor is required for bacteria to survive and replicate inside acidic compartments. These *Δerp* bacteria are therefore deficient for growth inside macrophages, and assessing their numbers is considered a readout for the clearance capacity of the host, since the initial infection dose is not increased in the absence of bacterial replication (59, 60). No significant difference was observed at 44 hpi for the bacterial burden, the number of *Mm* clusters, the percentage of bacteria-containing macrophages that contained only 1-10 bacteria and the percentage of *Mm* inside macrophage in the tail region between the beclomethasone-treated group and the vehicle-treated group (**Figure 6**). These findings indicate that beclomethasone treatment does not lead to an altered microbicidal capacity of macrophages. Taken together, our results identify the inhibition of phagocytosis as the primary effect of beclomethasone during mycobacterial

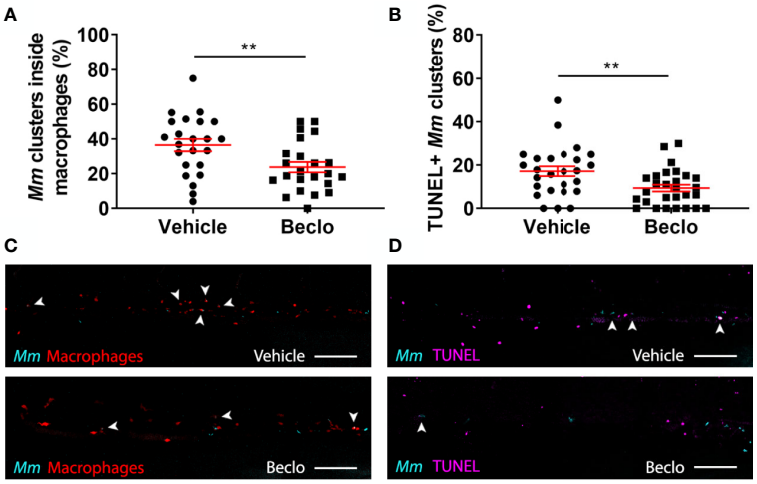


**FIGURE 5** | Effect of beclomethasone on intracellular bacterial growth and cell death. Infection was performed in *Tg(mpeg1:eGFP)* embryos at 28 hpf, a TUNEL assay was performed at 48 hpi, and the CHT region of the embryos was imaged using confocal microscopy. **(A)** The percentage of *Mm* clusters that were inside macrophages based on colocalization with the green fluorescent signal from eGFP. Statistical analysis was performed by two-tailed t-test. In the beclomethasone-treated group, the percentage of *Mm* clusters inside macrophages was significantly lower compared to the vehicle-treated group. **(B)** The percentage of TUNEL-positive *Mm* clusters. Statistical analysis by two-tailed t-test showed that the beclomethasone-treated group had a significantly lower percentage of TUNEL+ *Mm* clusters. In panels **(A, B)**, each data point represents a single embryo and the means ± s.e.m. of data accumulated from three independent experiments are shown in red. Statistical significance is indicated by: **P<0.01. **(C)** Representative confocal microscopy images of macrophage phagocytosis. Bacteria are shown in blue and macrophages in red. Arrowheads indicate intracellular bacterial clusters. Scale bar = 100 μm. **(D)** Representative confocal microscopy images of cell death (TUNEL+ cells in magenta) and *Mm* infection (bacteria in blue). Arrowheads indicate bacterial clusters overlapping with TUNEL+ cells. Scale bar = 100 μm.

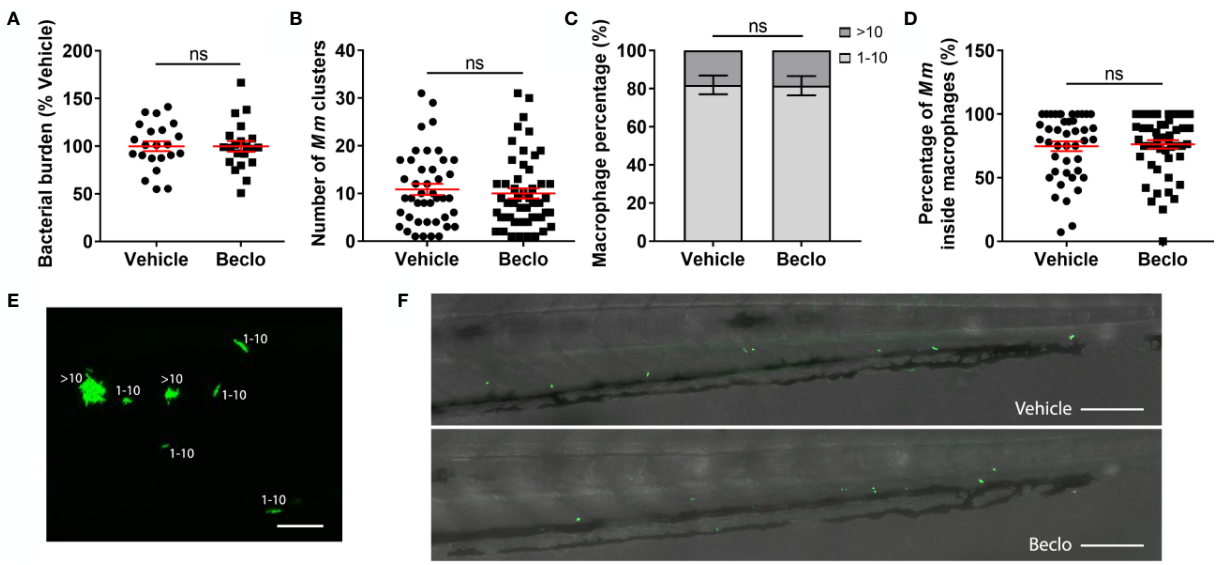


**FIGURE 6** | Effect of beclomethasone on the bacterial growth of the *Mm ΔerpA* mutant. The *Mm ΔerpA* mutant strain was injected intravenously at 28 hpf, and at 44 hpi, the bacterial burden **(A)**, the number of *Mm* clusters **(B)**, the percentage of macrophages that contained 1-10 or more than 10 bacteria (of all macrophages containing bacteria) **(C)**, and the percentage of *Mm* inside macrophages **(D)** were determined. No significant difference was observed between the vehicle- and beclomethasone-treated groups for any of these parameters. Statistical analysis was performed using two-tailed t-tests. Values shown are the means ± s.e.m. of three independent experiments, with each data point representing a single embryo. Statistical significance is indicated by: ns, non-significant. **(E)** Representative confocal microscopy image of *Mm ΔerpA* bacterial clusters (bacteria in green), indicated are clusters containing 1-10 bacteria and clusters containing more than 10 bacteria. Scale bar = 20 μm. **(F)** Representative images of the tail regions of a vehicle- and a beclomethasone-treated embryo infected with *Mm ΔerpA* bacteria. Scale bar = 100 μm.

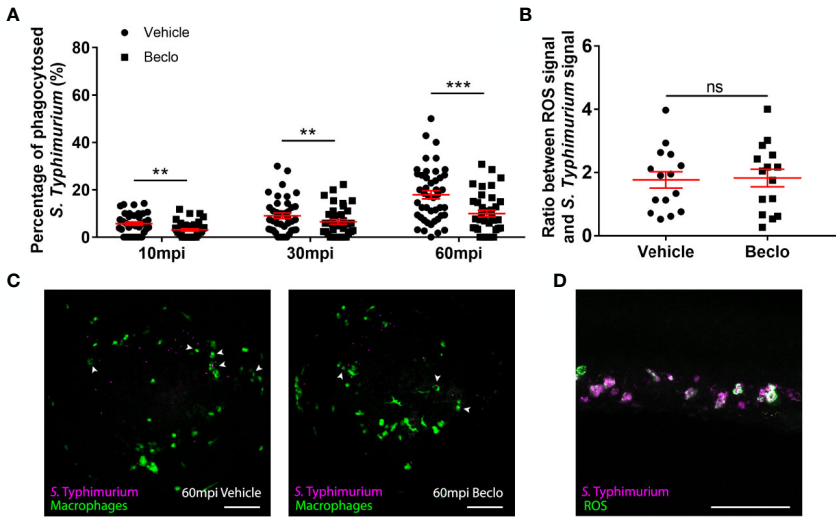


**FIGURE 7** | Effect of beclomethasone on phagocytosis of *Salmonella* Typhimurium and ROS production. At 28 hpf *Tg(mpeg1:eGFP)* embryos (vehicle- or beclomethasone-treated) were infected with *S.* Typhimurium through intravenous injection. **(A)** Percentage of phagocytosed *S.* Typhimurium wt at 10, 30 and 60 mpi. Confocal microscopy images were taken of the yolk area (as indicated in **Figure 3A**), and the percentage of bacteria inside macrophages was determined. Statistical analysis, performed by fitting data to a beta-inflated regression with Tukey's *post hoc* test, showed that the phagocytic activity of macrophages was significantly inhibited by beclomethasone treatment at 60 mpi, and not at other time points. **(B)** Ratio between ROS signal and *S.* Typhimurium signal. Embryos were injected with a ROS biosensor strain. At 4 hpi, confocal microscopy images were taken of the CHT region and the ratio between the ROS-induced fluorescent signal and the signal from a constitutively expressed fluorescent protein in the bacteria was determined. Statistical analysis was performed by two-tailed t-test, indicating no significant difference between the two vehicle- and beclomethasone-treated groups. In panel **(A, B)**, each data point represents a single embryo and the means ± s.e.m. of data accumulated from three independent experiments are shown in red. Statistical significance is indicated by: ns, non-significant; **P<0.01; ***P<0.001. **(C)** Representative confocal microscopy images of *S.* Typhimurium wt infected vehicle- and beclomethasone-treated individuals at 60 mpi. Bacteria are shown in magenta, macrophages in green. Arrowheads indicate bacteria phagocytosed by macrophages. Scale bar = 100 μm. **(D)** Representative confocal microscopy image of a vehicle-treated embryo infected with the *S.* Typhimurium ROS biosensor strain, showing the bacterial signal (magenta) and the ROS biosensor signal (green). Scale bar = 100 μm.

infection of zebrafish, which would exacerbate infection with *Mm* wt due to increased extracellular bacterial growth in line with previous results (72).

## Beclomethasone Inhibits Macrophage Phagocytosis of *Salmonella* Typhimurium and Does Not Affect Reactive Oxygen Species (ROS) Production

To study whether the beclomethasone-induced inhibitory effect on macrophage phagocytosis of *Mm* can be generalized to other bacterial infections, we analyzed the effect of beclomethasone on infection with *Salmonella* Typhimurium (*S.* Typhimurium), which is also an intracellular pathogen, but belongs to the gram-negative class. We quantified the percentages of bacteria phagocytosed by macrophages at different time points after infection in the *Tg(mpeg1:eGFP)* fish line (**Figures 7A, C**). In the vehicle group, the percentage of phagocytosed *S.* Typhimurium increased from 5.7 ± 0.7% at 10 mpi to 9.0 ± 1.2% at 30 mpi and 17.9 ± 1.7% at 60 mpi, and these percentages were significantly lower in the beclomethasone-treated group at all time points (3.1 ± 0.5% at 10 mpi, 6.5 ± 1.0% at 30 mpi and 10.0 ± 1.4% at 60 mpi). These data demonstrate that the inhibitory effect of beclomethasone on the phagocytic activity of macrophages is not specific for *Mm*, but can also be observed for a distantly related *Salmonella* species.

In order to further investigate the effect of GC treatment on the microbicidal activity of host immune cells, we analyzed the effect of beclomethasone on reactive oxygen species (ROS) production upon *S.* Typhimurium infection. To analyze ROS production, we used a ROS biosensor *S.* Typhimurium strain, which expresses GFP when it encounters ROS (54, 56). At 4 hpi, the ROS-activated fluorescent signals from the bacteria were determined and the ratio between this signal and a signal from mCherry that was constitutively expressed by the bacteria, was calculated for both vehicle- and beclomethasone-treated embryos (**Figures 7B, D**). No significant difference was observed between the two groups, indicating no effect of beclomethasone on the ROS production in immune cells upon a bacterial infection.

In conclusion, beclomethasone inhibits the phagocytosis of both Mycobacteria and Salmonella by macrophages in the zebrafish host, while no evidence for additional effects on microbicidal responses was found in either case.

# DISCUSSION

Synthetic GCs are widely prescribed to treat various immune-related diseases, but their clinical use is limited by the severe side effects evoked by prolonged therapy, including a higher susceptibility to TB (5, 14). In order to gain more insight into

the mechanism underlying this GC effect, we used the zebrafish *Mm* infection model, which mimics human TB, and studied the effect of GC treatment on the development of the infection. We showed that GC treatment increased the level of *Mm* infection, which was reflected in the overall bacterial burden, the size and number of bacterial clusters and the level of dissemination. Since we found that GC treatment inhibited the phagocytic activity but not the microbicidal capacity of macrophages, we suggest that the GC-induced increase in infection susceptibility is due to the inhibition on phagocytosis. Analysis of the transcription level of phagocytosis-related genes in macrophages suggested that the inhibition of phagocytic activity by GCs is mediated by Gr interfering with phagocytosis-related gene transcription. The lower phagocytic activity of the macrophages probably underlies the decrease in the percentage of intracellular bacteria that we observed, probably resulting in a lower level of cell death due to the *Mm* infection and exacerbated growth of the extracellular bacterial fraction. Finally, we showed that GC treatment not only limited phagocytosis of mycobacteria, but also of a Salmonella species, which suggests that the decrease in phagocytic activity may also explain the increased susceptibility to other bacterial infections that is commonly observed in patients receiving GC therapy (3–5).

Upon bacterial infections, macrophages are the first responders of the immune system. In humans, *Mtb* generally infects lungs due to its air transmission properties and in the lungs it is taken up by alveolar macrophages within the first few days. In later stages, *Mtb* replicates, translocates to secondary loci and aggregates into granulomas with other attracted immune cells (73–75). Consistently, in the zebrafish model, *Mm* is predominantly phagocytosed by macrophages within 30-60 min after intravenous infection in embryos, leading to initial stages of granuloma formation in the next few days (37, 64). The phagocytosis activity and microbicidal capacity of macrophages have been shown to be important for dealing with *Mm* infection (64, 72). Interestingly, in our study we found that the microbicidal capacity of macrophages (determined using the *Mm Δerp* and the *St* biosensor strains) was not affected by GC treatment, which suggests that the inhibition of macrophage phagocytosis is a specific effect of GCs targeted at the uptake of pathogens rather than a global suppression of anti-microbial processes in macrophages.

Our study in the zebrafish model provides *in vivo* evidence for GC interference with macrophage phagocytosis. In line with our results, it has previously been shown that GCs decrease the phagocytosis of several *Escherichia coli* strains by human monocyte-derived (THP-1) macrophages and by murine bone marrow-derived macrophages (BMDMs) (76). Similarly to our results, in this study the reduced phagocytosis activity was accompanied by a decreased expression of genes involved in phagosome formation including *MARCKS* and pro-inflammatory genes like *TNF* (76). In earlier studies, decreased macrophage phagocytosis of carbon particles was observed *in vivo*, in GC-treated rats and rheumatoid arthritis patients (77, 78). However, in other studies GC treatment has been shown to enhance the bacterial phagocytosis by macrophages. Upon GC exposure, increased bacterial phagocytic activity of human monocyte-derived macrophages was observed for *Haemophillus influenzae* and *Streptococcus pneumoniae* (79), and *Staphylococcus aureus* (80).

It could be argued that the observed effect of beclomethasone on phagocytosis, which looks more like a delay in this process than an inhibition, is unlikely to be solely responsible for the increased bacterial burden that we observed. It must be noted however that for the *Mm* infection, this burden is a result of a continuous cycle of phagocytosis, bacterial replication, cell death and subsequent phagocytosis [i.e. efferocytosis (81)], in which an effect of beclomethasone on phagocytosis may well accumulate over time. For the infection with the avirulent *Δerp* bacteria, which are simply phagocytosed and subsequently cleared, such an accumulation of the beclomethasone effect would be absent. In line with this notion, the percentage of *Mm Δerp* inside macrophages was not different between the vehicle- and the beclomethasone-treated group in our study.

Alternatively, beclomethasone may alter additional processes during the course of the infection contributing to the increased bacterial burden. One such process that can be affected by GC treatment is the migration of macrophages towards a site of infection, and such an effect would in many experimental designs be difficult to separate from effects on phagocytosis. However, in our experiments we have infected the embryos at 28 hpf by intravenous injection of bacteria, upon which all bacteria are rapidly phagocytozed by monocytes/macrophages in the bloodstream (37, 64). Migration does not play a role in this process, because the primitive macrophages of the embryo are almost all located in the circulation at this stage (65). In addition, we have previously shown that beclomethasone does not affect migration of macrophages in a wounding-induced inflammation model (48), which makes an effect of GCs on migration unlikely, although we cannot exclude a possible effect of GCs on macrophage migration in the context of an infection.

Macrophages are often divided into two functional phenotypes: a classically activated phenotype (often referred to as M1) which contributes to the anti-microbial and inflammatory response, and an alternatively activated phenotype (often referred to as M2), which can be subdivided in several different phenotypes which have been shown to be involved in the resolution of inflammation and in wound healing (67, 82). It has been well established that GCs inhibit the differentiation towards the classically activated M1 phenotype (83–86), and we have recently demonstrated in zebrafish larvae that GC treatment inhibits the differentiation of macrophages towards this pro-inflammatory phenotype upon wounding of the tail fin (48). Increased expression of *tnfa* and phagocytic activity are commonly recognized as characteristics of M1-differentiated macrophages, so the GC-induced decreases in *tnfa* expression and phagocytosis that we have observed in the present study are well in line with the inhibitory effect of GCs on the differentiation of macrophages towards an M1 phenotype.

In addition, GCs have been demonstrated to enhance the differentiation of macrophages towards a specific, alternatively activated, anti-inflammatory phenotype, which is considered to

play an important role in GCs actively promoting the resolution of inflammation (84, 87). Interestingly, this phenotype shows an enhancement of phagocytic activity, but in the GC-induced differentiation status this activity is nonphlogistic and directed at apoptotic leukocytes, thereby contributing to the resolution of the inflammation. The GC-enhanced phagocytosis of apoptotic neutrophils has been observed in differentiated THP-1 macrophages, through stimulation of a protein S/Mer tyrosine kinase dependent pathway (88–90), and in mouse alveolar macrophages (91).

Our study revealed an inhibitory effect of GCs on four phagocytosis-related genes in FACS-sorted macrophages: *sparcl-1*, *uchl-1*, *marcksa and marcksb*. Among those genes, the human and mouse homologs of *sparcl-1* and *uchl-1* were reported to have a phagocytosis-promoting activity (68, 70). In human THP-1-derived macrophages, MARCKS plays a role in cytoskeletal remodeling and phagosome formation (69). In these cells, the *MARCKS* gene expression was found to be inhibited by dexamethasone treatment, which indicates that GCs induce this effect by activating GRs in macrophages (76). According to the Transcription Factor Targets Dataset generated by Encyclopedia of DNA Elements (ENCODE) project (92), the human genes *SPARCL1*, *UCHL1*, *UCHL5* and *MARCKS* contain binding sites for GR, as determined by Chromatin Immunoprecipitation (ChIP) sequencing analysis. Together with our observation that phagocytosis is dependent on *de novo* protein synthesis, these results support the idea that GC treatment inhibits the phagocytosis activity of macrophages through GRs directly interfering with transcription of genes that stimulate the phagocytic activity of these cells.

After internalization by macrophages, *Mm* are exposed to a bactericidal environment (93). Some bacteria may be killed by macrophages, while others may proliferate mediated by virulence determinants like Erp and RD1 (59, 93, 94). When the macrophages are incapable of containing the bacteria, they undergo cell death leading to recruitment of more macrophages (38). In our study, GC treatment led to a lower percentage of intracellular *Mm* at later stages, consistent with the decreased phagocytosis at early time points, and less *Mm*-related cell death, probably as a result of the decreased number on intracellular bacteria. The GC treatment may also directly affect cell death, since in a recent study it was demonstrated that GCs inhibit necrosis of various *Mtb* infected mouse and human cell types by activating MKP-1, which suppresses a pathway involving p38 MAPK activation ultimately leading to a loss of mitochondrial integrity (95). The increased numbers of extracellular bacteria could traverse endothelial barriers directly and grow more rapidly in a less restrictive environment outside macrophages, which may explain our observation of a higher bacterial burden induced by GC treatment.

Based on our results, it may seem surprising that adjunctive GC therapy is often beneficial to TB patients, and even increases survival among tuberculous meningitis and pericarditis patients (24–26). However, many of these observed beneficial effects are either minor or under debate. This may be due to GC therapy benefiting only a subset of patients whose disease has mainly progressed as a result of an excessive inflammatory response (which can be controlled with GC therapy), rather than a failed reaction to the infection, which was demonstrated for GC-treated TB meningitis patients with specific polymorphisms in the *LTA4H* gene (32). We therefore suggest that in a subset of patients at later stages of infection, the anti-inflammatory effects of a GC treatment may outweigh a possible inhibitory effect on the phagocytic activity of the macrophages. Further research using the zebrafish model may shed light on a possible interplay between these effects, since the *Mm* infection model has been shown to have excellent translational value for human TB, including the effects of GC treatment (32, 40).

In conclusion, our *in vivo* study on the effect of GC treatment in the zebrafish *Mm* infection model shows that GCs, through activation of Gr, inhibit the phagocytic activity of macrophages, and results in more extracellular bacterial growth and a higher infection level. These results may explain why clinically prolonged GC treatment is associated with an increased risk of TB and other bacterial infections.

## DATA AVAILABILITY STATEMENT

Upon request, the raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

## AUTHOR CONTRIBUTIONS

Conceptualization: AM and MS. Methodology: YX, JX, AM, and MS. Validation: YX and MS. Formal analysis: YX and JX. Investigation: YX and JX. Resources: MS. Data curation: YX and MS. Writing - original draft: YX and MS. Writing - review and editing: YX, JX, AM, and MS. Supervision: AM and MS. Project administration: MS. Funding acquisition: YX. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.618569/full#supplementary-material

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# Gender May Influence the Immunosuppressive Actions of Prednisone in Young Patients With Inflammatory Bowel Disease

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Although the use of glucocorticoids (GC) is well established, the therapeutic response to these agents often shows important interindividual differences, in particular among young patients with inflammatory bowel diseases (IBD). Currently, GC resistance or dependence cannot be predicted by clinical or laboratory findings. The aim of this study was to investigate the association of gender and age with GC efficacy and with the expression of Glucocorticoid-Induced Leucine Zipper (GILZ). One hundred thirty patients (mean age at enrolment 12.6 years, 53 Crohn's disease, 70 males) were enrolled in this retrospective study. IBD patients with active disease despite prednisone at a daily dose of up to 2 mg/kg over a period of 4 weeks were defined as steroid resistant. Patients who initially responded but relapsed upon dose reduction were considered steroid-dependent. Total RNA was extracted from biopsies of 14 patients (9 males) and the levels of GILZ mRNA were evaluated by real-time PCR. Association between clinical response to prednisone and the considered demographic variables was evaluated using logistic regression models. After 4 weeks of treatment, 112 patients were responders to prednisone and 18 were resistant; at this time-point, resistant patients were older than responders (p=0.032). After 12 weeks, 42, 71 and 12 patients were sensitive, dependent and resistant respectively; at this time-point, females were more prone than males to develop prednisone dependence vs a good response (p=0.028) while age had no effect. Age was associated with response both at 4 and 12 weeks in the subgroups of females: resistant patients were older than sensitive ones at 4 weeks (p=0.02). Likewise, at 12 weeks of therapy, dependent patients resulted older than sensitive ones (p=0.05). No association of age with prednisone response was found in males. In a subgroup of 14 patients (5 females), GILZ mRNA expression in intestinal biopsies was higher in males (p=0.0031). Patients with unfavorable response (7) presented lower GILZ expression at disease onset in comparison to the responder group (p=0.017). Older females with IBD have a higher incidence of prednisone unfavorable response and reduced intestinal expression of the GC pharmacodynamic marker GILZ.

**Keywords: glucocorticoids, inflammatory bowel disease, gender, age, GILZ, prednisone**

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## INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic idiopathic inflammation of the intestinal tract, characterized by relapses and remissions, that comprises two disorders, Crohn's disease (CD) and ulcerative colitis (UC). The disease has a peak onset in subjects 15 to 30 years old, and its incidence is rising in the young population with approximately 25% of patients with IBD presenting before the age of 20 (1). Patients with pediatric-onset IBD tend to present with more extensive anatomic involvement, more active disease course and often require a more aggressive treatment compared to patients with adult onset disease (2). The etiology of CD and UC is often undefined and different genetic and environmental causes may result in a similar clinical outcome (3). At present, a curative therapy for IBD does not exist and the treatment aims to induce and maintain the disease in an inactive state. For this purpose, many immunosuppressants are currently employed due to the clear contribution of an uncontrolled immune response in the pathogenesis of both CD and UC (4). Despite the introduction in clinical practice of highly effective biological drugs, such as anti-TNFα agents, glucocorticoids (GCs) are still used to induce disease remission in young patients with moderate to severe active CD and UC (5, 6). GCs are steroid hormones that passively diffuse across plasma membranes and act by binding the GC receptor (GR) (7) which becomes able to translocate into the nucleus and binds as homodimer to DNA, in correspondence of GC-responsive elements (GREs), localized in the promoter region of target genes (8, 9). This binding activates the transcription of anti-inflammatory proteins such as glucocorticoid-induced leucine zipper (GILZ), interleukin-10 (IL-10), annexin 1 and enzymes involved in gluconeogenesis (10, 11), and reduces the expression of pro-inflammatory cytokines (IL-1, IL-2, IL-6, TNFα) and inflammatory prostaglandins (12).

Although the use of GCs is well established in many diseases due to their efficacy, the therapeutic response to these agents often shows important interindividual differences between patients (13–15), especially in pediatric inflammatory diseases (6). According to the clinical response, young IBD patients treated with GCs can be classified as steroid sensitive (SS) or steroid resistant (SR) within the first 30 days of treatment (16). A high percentage of patients who initially respond to GC therapy is unable to reduce steroids within 3 months of starting due to frequent relapses: these patients are classified as steroid-dependent (SD) (13, 17).

Steroid resistance is common in young patients with IBD, with an incidence of 6-20% of patients, however, a specific predictor of GC response has not yet been identified (18, 19). While resistance to GC has been widely studied both in pediatric and adult patients, few data are available for steroid dependency (17). Demographic characteristics, in particular age and gender, have been associated with GC levels (20), response (18) and side effects (21) but very few data are available on the young population affected by IBD. Moreover, the molecular mechanisms of the interindividual variability in GC response is still poorly understood in these patients (22). Polymorphisms in genes involved in the GC pathway together with the expression of candidate mRNAs and epigenetic factors have been previously reported as possible mechanisms involved in steroid resistance even though none of them had entered in the clinical practice as biomarker of response (18, 22–24).

Therefore, the aim of this study was to evaluate the association of age and gender with prednisone efficacy in a large cohort of children with IBD and to investigate gender-specific differences in GR–mediated gene expression in these patients, considering in particular GILZ expression in intestinal biopsies.

## MATERIALS AND METHODS

### Patients Characteristics

130 young patients with IBD were enrolled by the Gastroenterology Unit of the Pediatric Hospital "Burlo Garofolo" in Trieste, Italy between July 2001 and February 2019. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Ethics Committee. All subjects and/or parents gave their informed consent for inclusion before they participated in the study. The inclusion criteria were age less than 25 years, previous diagnosis of IBD and initial treatment with prednisone for 4 weeks at the standard dose (1 to 2 mg/kg/day) and 8 to 11 weeks of tapering. Steroids were administered as induction treatment in patients with ileo-colic or colonic CD when exclusive enteral nutrition was not an option and in patients with moderate or severe UC according to the pediatric ulcerative colitis activity index (PUCAI) and the pediatric Crohn's disease activity index (PCDAI) or to the physician global assessment.

Clinical response to prednisone was assessed after 4 and 12 weeks of therapy. Patients were considered responsive to treatment when a drop of at least 50% from the basal clinical score was observed. In case of clinical score unavailability, the physician global assessment definitions of "remission" and "response" or "persistent active disease" were used. Patients who initially responded to prednisone therapy but were unable to reduce steroids within 3 months of starting treatment were classified as steroid-dependent. Patients not classified as responders or dependent to prednisone, were considered resistant.

### Total RNA Isolation

Total RNA was extracted from intestinal biopsies of 14 patients using TRIzol reagent (Thermo Scientific, Carlsbad, CA, USA) according to manufacturer's instructions. The RNA concentration and purity were calculated by Nano Drop instrument (NanoDrop 2000, EuroClone, Milan, Italy).

### Quantitative Real-Time PCR

Expression levels of GILZ was evaluated by real-time RT-PCR TaqMan® analysis using the CFX96 real-time system-C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The reverse transcription reaction was carried out with the High Capacity RNA-to-cDNA Kit (Applied Biosystem, Foster

City, CA, USA) and the real-time PCR was performed in triplicate using the TaqMan® Gene Expression Assay to assess GILZ mRNA expressions, according to the manufacturer's instructions. The thermal cycling conditions for TaqMan assays were as follows: 2 min at 50°C and 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The expression levels of GILZ was evaluated using the comparative Ct method (2−ΔCt method) and normalized using the RPLP0 gene.

## Statistical Analysis

Statistical analysis was performed using the software R, version 3.6.1.

Analysis for association of clinical response, evaluated as a categorical variable, was performed by logistic regression. For these analyses, binomial models were generated using response to prednisone as the dependent variable and the clinical or demographic covariate of interest (i.e., gender and type of IBD) as the independent variable. Odds ratios were calculated from estimates of the logistic regression models.

For the analysis considering continuous variables (i.e. age and GILZ expression) linear models of the Gaussian family were used. Before applying linear models, normality of the continuous variable was assessed. All p-values < 0.05 were considered statistically significant.

# RESULTS

## Patients Enrolled

One hundred thirty IBD patients treated with prednisone were enrolled in this retrospective study; baseline characteristics of the patients are shown in **Table 1**.

## Response to Prednisone: Association With Clinical and Demographic Data

The clinical response was evaluated after treatment with prednisone 1–2 mg/kg/day and after 4 (initial response) and 12 weeks of therapy (tapering dose).

Among 130 patients enrolled, after 4 weeks of treatment, 112 (86.2%) were responder to prednisone and 18 (13.9%) were resistant. Out of the 130 patients age was available only for 93 sensitive and 17 resistant patients for a total of 110 subjects.

Interestingly, a significant association was found between the clinical response after 4 weeks of treatment and age: in particular, resistant patients resulted to be older than sensitive ones (mean age: 14.9 and 12.1 respectively, linear model p=0.032; **Figure 1**). No significant association was highlighted with sex, disease severity or type of IBD and clinical response at 4 weeks of treatment.

After tapering (12 weeks), 42 (32.3%), 71 (54.6%) and 12 (9.2%) patients were sensitive, dependent and resistant respectively. For 5 (3.8%) patients the information related to the clinical response at 12 weeks was not available for lack of scheduled visit.

Unlike what was observed with the response at 4 weeks of treatment, no significant association was found between clinical response after 12 weeks of treatment and age. A significant association was on the contrary detected between the clinical response at 12 weeks of treatment and the type of IBD; in particular, patients with UC were more GC dependent in comparison to patients with CD (logistic regression p=0.018 OR: 2.62; CI: 1.19 - 5.78). On the other hand, no significant difference was observed in the incidence of resistance according to age, disease severity or IBD type.

Among the 130 patients, 70 (53.9%) were concomitantly treated with prednisone and other medications and in particular: 55 with azathioprine, 6 with thalidomide, 3 with infliximab, 1 with adalimumab, 1 with methotrexate and 4 with topical therapy (budesonide). No association was found between the combination therapy with other immunomodulators and the response to prednisone.

Interestingly, females were more prone than males to develop prednisone dependence after 12 weeks of treatment (logistic regression p=0.028; OR: 2.44; CI: 1.10 - 5.26). Incidence of resistance was not affected by gender.

Patients were further stratified into pre-pubescent (≤ 13 years) and post-pubescent (> 13 years) categories for all analyses, but no statistical association was found, probably due to resulting sample size of the subgroups.

Multivariate analysis shows that sex (logistic regression, sensitive *vs* dependent p=0.03; OR: 2.38; CI: 1.06 - 5.26) and the type of IBD (logistic regression, sensitive *vs* dependent p=0.02; OR: 2.59; CI: 1.16 – 5.80) are independently associated with the clinical response at 12 weeks (**Table 2**).

Given the association between patient age and GC response (after 4 weeks of treatment, **Figure 1**) and the association with gender considering the clinical response after 12 weeks of treatment, we evaluated a potential effect of age on the response to prednisone in the subgroups of female and male patients. As reported in **Figure 2**, age is associated with response at 4 and 12 weeks in females. In particular, resistant patients

**TABLE 1** | Demographic and clinical characteristics of the patients enrolled in the study.

| | | All patients (n = 130) |
|---|---|---|
| Age (mean, range) | | 12.6, 1.2-22.2 |
| Gender | Female (%) | 60 (46.2%) |
| | Male (%) | 70 (53.8%) |
| Type of IBD | Crohn's disease (%) | 53 (40.8%) |
| | Ulcerative colitis (%) | 77 (59.2%) |
| Clinical Score (data available for 94 patients) | PUCAI (mean, range) | 41.4, 0-85 |
| | PCDAI (mean, range) | 34.0, 2.5-65 |

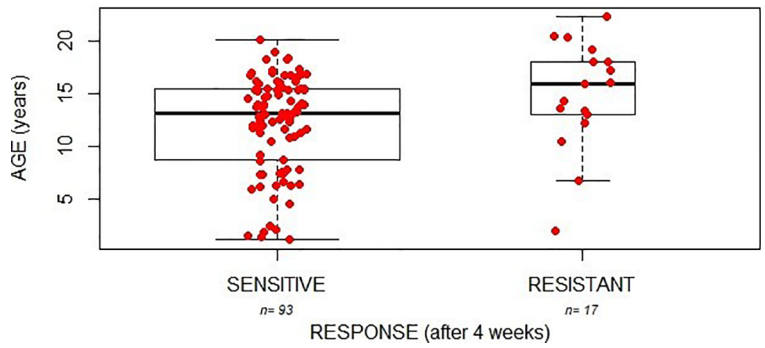


**FIGURE 1** | Initial response to glucocorticoids (4 weeks of therapy) and age of patients.

resulted to be older than sensitive ones at 4 weeks of treatment (mean age: 11.6 and 15.8 respectively; linear model p=0.02). Likewise, at 12 weeks of therapy dependent patients resulted to be older than sensitive ones (mean age: 15.5 and 12.6 respectively, linear model p=0.05; **Figure 2**) and a similar trend was observed also for resistant patients in comparison with the responder group (mean age 16.9; linear model p=0.07; **Figure 2**).

No association was found between age and the response to prednisone at both time points in the group of males (**Figure 2**).

## Gender-Specific Mechanism Controlling GC Receptor–Mediated GILZ Expression

To investigate on the gender-specific actions of prednisone in IBD patients, we evaluated in intestinal biopsies at disease onset the levels of the GC target gene GILZ in a subgroup of 14 patients (5 females, 3 CD; **Supplementary Table 1**), belonging to the same cohort of patients described above; no significant difference was observed in the demographic characteristics between the whole cohort and this subgroup. As reported in **Figure 3**, GILZ mRNA expression was lower in females in comparison to males (linear model p=0.0031), highlighting a different activity of the GC receptor between genders.

Interestingly, patients with unfavorable response (dependent + resistant) presented lower GILZ levels at disease onset in comparison to the responder group (linear model p=0.017; **Figure 4**).

Association of GILZ levels with gender and response to GCs was confirmed also by multivariate analysis (adjusted p-value respectively 0.00053 and 0.0024; **Figure 5**).

**TABLE 2** | Multivariate analysis considering all covariates significant in the univariate analysis.

| Covariate | Comparison | p-value (OR; CI) |
|---|---|---|
| Sex | Sens vs Dep | 0.03 (2.38; 1.06 - 5.26) |
| (F vs M) | Sens vs Res | 0.30 (2.00; 0.54 – 7.14) |
| Type of IBD | Sens vs Dep | 0.02 (2.59; 1.16 – 5.80) |
| (UC vs CD) | Sens vs Res | 0.53 (1.53; 0.41 – 5.62) |

# DISCUSSION

The use of GCs in young patients with IBD is mostly extrapolated from the experience in adults although many differences have been described and children are more sensitive to GC therapy-related side effects than adults (25). Despite the numerous studies conducted to investigate the underlying mechanism of resistance to GCs, no demographic, clinical or molecular biomarker is available yet to predict response or the risk of developing adverse events (4, 16). Moreover, while the molecular basis of GC resistance have been explored, the molecular mechanisms behind GC dependence are poorly investigated (5) and only few studies have been conducted in pediatric patients with IBD (17).

In our study, performed in a cohort of young patients, age and gender seem to be important contributing factors to prednisone response. In particular, resistant patients resulted to be older than sensitive ones after the initial 4 weeks of prednisone treatment, while females were more prone than males to develop dependence after tapering. A significant association was also reported between the type of IBD and clinical response at 12 weeks of treatment, but the multivariate analysis showed an independent effect of gender and the type of IBD.

The levels of endogenous GCs have been associated with age and gender, suggesting their important role in regulating GC secretion (26). Cortisol levels increase progressively with age in particular in women (20). However, the influence of age and sex on GC pharmacokinetic and pharmacodynamic properties have been reported only in adult subjects though these evidences do not suggest the need for dosage adjustments. Nowadays, the only data regarding age and gender differences in GC response in young patients with IBD were previously published by our group (18). In particular, it was reported that in IBD male patients with an age at disease onset above 7.5 years, response to GC therapy was significantly more frequent than females.

In the present study an association of age with the response to prednisone in females was highlighted: female responder patients after both 4 and 12 weeks of treatment, were significantly younger, while this effect of age was not observed in males.

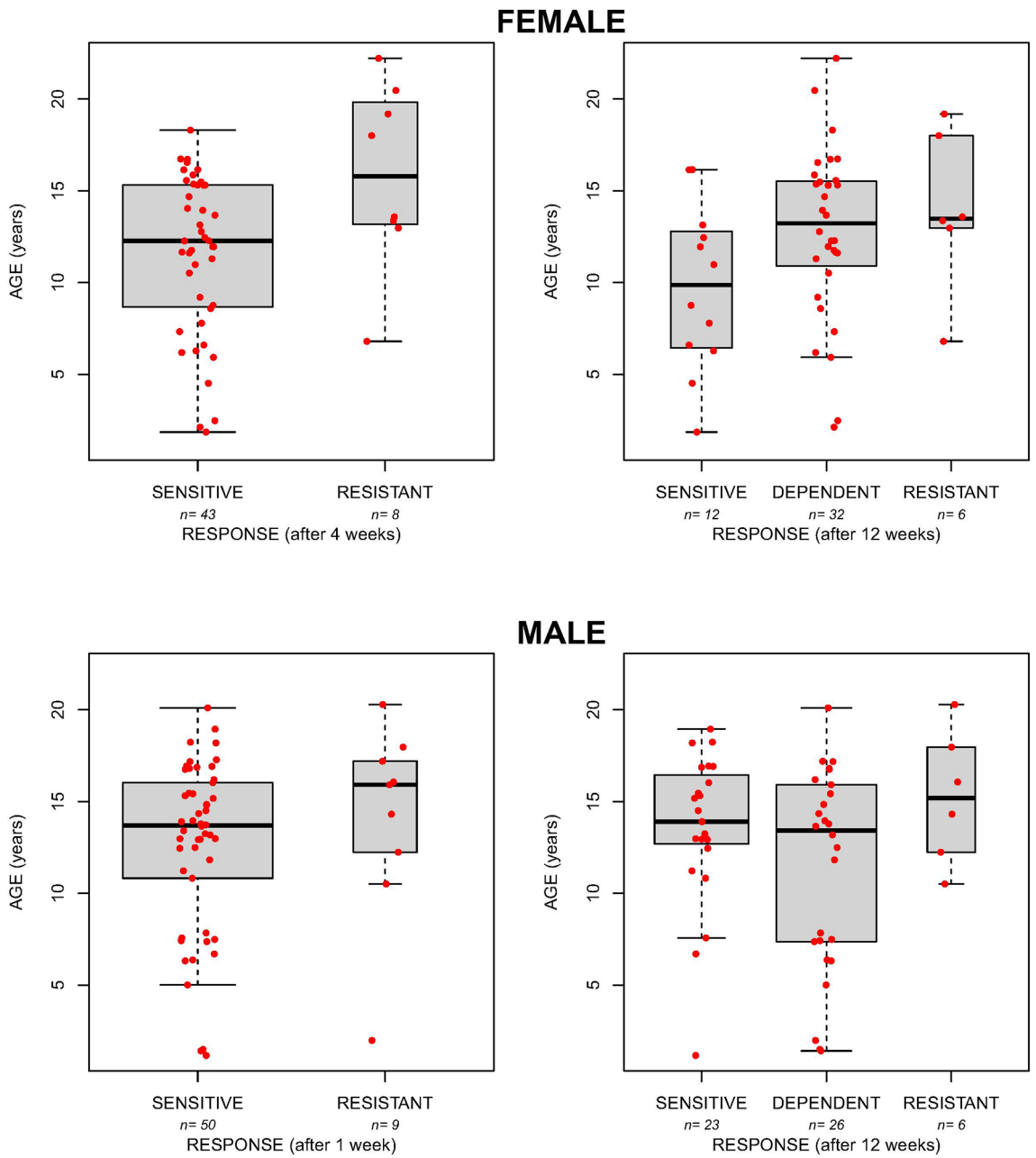


**FIGURE 2** | The boxplots show the age in female and male patients sensitive, resistant or dependent to GC after 4 and 12 weeks of therapy.

The influence of female hormonal status in IBD activity is recognized, but no studies have investigated how sex hormones modulate therapeutic response (27–30). Of interest, Altemus et al. observed a decrease in GR mRNA in the luteal phase, characterized by an increase of progesterone and, to a lesser extent, of estrogen levels (31). Moreover, the female sex steroid hormone progesterone has been shown to antagonize GC effects increasing the dissociation rate of GC from the GR and inhibiting its translocation into the nucleus (32, 33). Considering the crucial role of these hormones, GCs may not work properly in adolescent girls and young women with IBD.

To investigate on the gender-specific actions of prednisone in IBD patients, we analyzed the expression of the mRNA of the anti-inflammatory GC-induced gene GILZ in intestinal biopsies obtained before starting GC treatment of a subgroup of female and male patients of the same cohort, as a predictive pharmacodynamic marker of GC efficacy. GILZ levels were higher in males in comparison to females highlighting a different activity of the GC receptor between genders.

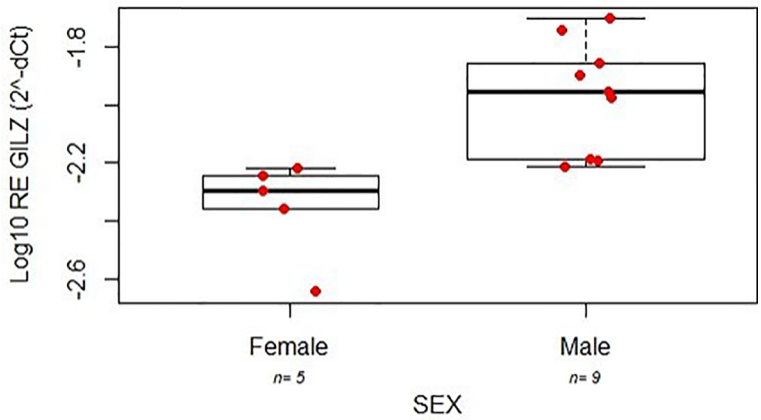


**FIGURE 3** | Expression (relative log expression) of GILZ mRNA between the two genders affected by IBD.

Sexually dimorphic GR signaling was already studied by pioneering investigations of the Cidlowski group (34–36). Results from their study indicate that estradiol antagonizes induction of GILZ gene expression providing evidence of its role also in the immune modulating functions of GCs (36).

Moreover, Cidlowski and collaborators demonstrated that ovarian hormones can influence the anti-inflammatory actions of GCs in the context of a rat adrenalectomized model of sepsis and that the anti-inflammatory actions of GCs are more effective in males (34). In particular, specific profiles of GC-regulated gene expression have been observed for females and males confirming a different gender-specific mechanism controlling GR–mediated gene expression. Even if the mechanisms that drive gender-specific effects of GC are still unclear, Cidlowski and collaborators showed a higher nuclear translocation of the GR in males after administration of the proinflammatory stimulus lipopolysaccharide compared to female mice, suggesting that the activation of GR is sex-specific.

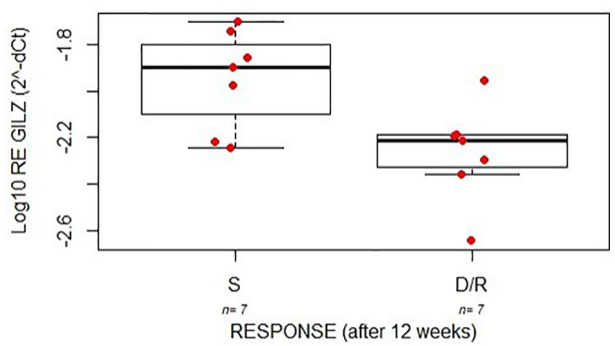


**FIGURE 4** | The boxplots indicate the expression level (relative log expression) of GILZ between good (S) and poor (D/R) responders after 12 weeks of treatment with GC in a cohort of young patients with IBD.

The importance of the transcriptional activity of the GR in GC effectiveness is confirmed by the fact that in our study patients with unfavorable response presented lower GILZ levels at disease onset in comparison to the responder group. GILZ is expressed in all peripheral blood cells and in several other non-lymphoid tissues including cell types present in the biopsies such as immune cells (T-lymphocytes and monocytes) and intestinal cells (37). This is the first study to consider the association of GILZ expression on GC sensitivity in colon biopsies from a cohort of pediatric patients with IBD and no data about the modulation of GILZ levels during GC treatment in IBD patients have been published yet. In other diseases, in particular systemic lupus erythematosus and alcohol-induced liver disease, GILZ was overexpressed in blood cells of patients under treatment with GC, but no association with the clinical response have been analyzed (38, 39).

The role of GILZ has been studied in several mouse models of IBD using dinitrobenzene sulfonic acid-induced colitis (40). In particular, the severity of colonic inflammation was diminished in transgenic mice overexpressing GILZ and exacerbated in GILZ-conditional knock-out mice with a T-cell specific GILZ deletion highlighting its anti-inflammatory effects that mimic those of GCs (41, 42). An opposite effect of GILZ was observed in oxazolone-induced colitis which is a Th2-dependent model of chronic inflammation where more severe disease was induced in mice overexpressing GILZ compared to the wild type mice suggesting that GILZ could have a protective role in Th1-mediated models of colitis (41). Further validation studies will help to explore the promising role of GILZ as marker of GC response in IBD patients as already proposed for other pathologies (43, 44) evaluating its levels also in the blood cells to avoid an invasive examination.

Our study has some limitations starting from its retrospective design. Unfortunately, no data about specific treatment administered before prednisone are available. Clinical score was not available for 36 of 130 patients before the treatment with prednisone because laboratory data from the retrospective analysis were incomplete. Moreover, the association of GILZ expression on GC sensitivity was limited to a small sample size

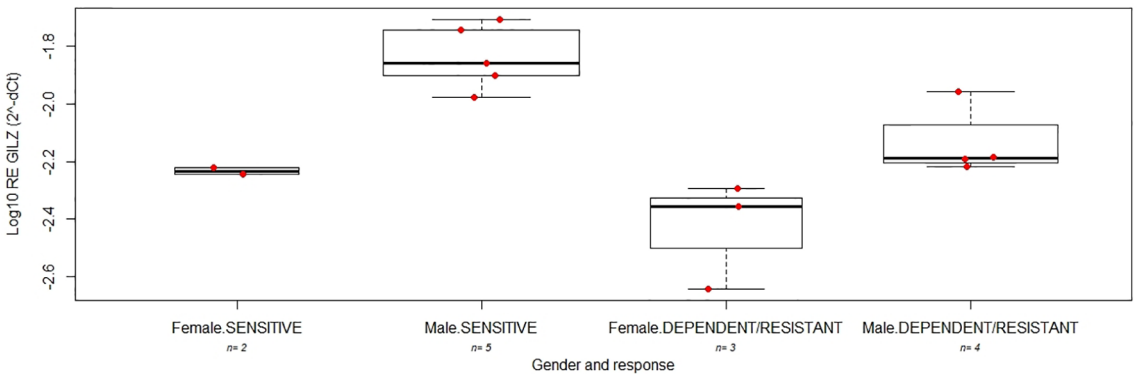


**FIGURE 5** | The boxplots indicate the expression level of GILZ between good (SENSITIVE) and poor (DEPENDENT/RESISTANT) responders after 12 weeks of treatment with GC in female and male young patients with IBD.

and mechanistic data to confirm the role of GILZ in GC response are needed.

In the present study we have demonstrated that age and gender are important variables affecting response to prednisone in young patients with IBD even though further investigation on the impact of sex hormones on therapeutic outcomes and disease course are needed. Our results support the hypothesis that the anti-inflammatory actions of GCs are reduced in female adolescents probably due to a lower activity of the GR. Furthermore, sex hormones, in particular estrogen and progesterone, and their fluctuation with age and critical phases such as puberty, might contribute to the differential sensitivity to GCs. The study is descriptive and data interpretation including links with female hormones are speculative, more in-depth investigation are therefore needed to establish the exact molecular mechanisms of these difference.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local ethical committee (Comitato indipendente per la bioetica, Istituto di Ricovero e Cura a Carattere Scientifico materno infantile Burlo Garofolo, Trieste, Italy). Approval for the study (Prot 2198; approval date: 17 September 2013) was provided. All patients participated in this study in accordance with the principles outlined in the Declaration of Helsinki, and written informed consent was obtained from each participating patient and/or their parents or guardians. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

ML and GS: study conception and design. ML, DS, and GS: acquisition, analysis, and interpretation of the data, and drafted the initial manuscript. ML, MB, and PD: acquisition of the data. GD and GS: critical discussion and study supervision. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.673068/full#supplementary-material

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# Glucocorticoid Therapy in Inflammatory Bowel Disease: Mechanisms and Clinical Practice

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Inflammatory bowel disease (IBD) comprises ulcerative colitis (UC) and Crohn's disease (CD). IBD etiopathology is multifactorial and involves alteration of immune cells and chronic activation of the inflammatory cascade against yet unknown environmental factors that trigger the disease. IBD therapy aims at improving the quality of life and reducing the risk of disease-related complications to avoid the need for surgery. There is no specific cure for IBDs, and the focus of therapy is supportive measures and use of anti-inflammatory and immunosuppressive drugs. Glucocorticoids (GCs) are powerful anti-inflammatory and immunomodulatory agents used to treat many acute and chronic inflammatory diseases. GCs remain basic treatment for moderate-to-severe IBD, but their use is limited by several important adverse drug effects. Topical administration of a second-generation of GCs, such as budesonide and beclomethasone dipropionate (BDP), represents a valid alternative to use of older, systemic GCs. Administration of second-generation GCs shows promisingly high topical activity and less systemic toxicity, but maintenance therapy with these new GCs in IBD patients is associated with multiple adverse effects. In this review, we make a comparative analysis of the efficacy of first-generation and second-generation GCs in IBD treatment. Unraveling GC biology at the molecular level to uncouple their clinical benefits from detrimental effects is important. One approach is to consider new GC mediators, such as glucocorticoid-induced leucine zipper, which may have similar anti-inflammatory properties, but avoids the side effects of GCs. This in-depth analysis can help to improve the development and the clinical outcomes of GC therapies in IBD.

**Keywords: glucocorticoids, IBD, GILZ, inflammation, drug delivery**

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## INTRODUCTION

Inflammatory bowel disease (IBD) comprises Crohn's disease (CD) and ulcerative colitis (UC). These are chronic and progressive diseases affecting the gastrointestinal tract. They arise as a consequence of a complex multifactorial etiopathogenesis that is incompletely understood, but which includes genetic predisposition and various environmental factors (1, 2). Oral systemic corticosteroids (e.g., prednisone, prednisolone) have been used to induce remission in IBD patients for more than 60 years due to their powerful anti-inflammatory effects. Second generation glucocorticoids (GCs), such as budesonide and beclomethasone dipropionate (BDP), which are

characterized by high anti-inflammatory activity, with low systemic bioavailability due to a significant first-pass effect and higher affinity for the glucocorticoid receptor (GR) compared to first generation GCs, represent an important alternative approach for IBD therapy (3). The possible advantage derives mainly from the lower adverse effects following treatment with these second-generation GCs. Nevertheless, the increase in IBD incidence and lack of long-term treatment alternatives have led to a search for new strategies and approaches.

In this review, we discuss the medical uses of synthetic GCs (e.g., second-generation GCs). We also report new perspectives in the study of GCs, focusing especially on the role of the glucocorticoid-induced leucine zipper (GILZ) protein in the clinical outcome of IBD.

## GCs

GCs are endogenous molecules (cortisol, cortisone, corticosterone) that can also be synthesized in the laboratory and widely used as treatments for several diseases. GC secretion is mediated by the hypothalamic–pituitary–adrenal (HPA) axis and is regulated by the circadian rhythm and stress, but also by inflammatory stimuli. For example, upon activation by cytokines such as interleukin (IL)-1, tumor necrosis factor, (TNF) and IL-6 (which can lead to extreme tissue damage due to uncontrollable inflammation), the HPA triggers the production and secretion of GCs. The HPA acts on inflammation by regulating genomic and non-genomic pathways, thereby blocking the activation, migration, and proliferation of immune cells of the innate and adaptive immune system (**Figure 1**) (4). Their action is mediated mainly by the GR, which is present predominantly in the cell cytoplasm, bound to a multi-protein complex of heat-shock proteins, immunophilins, kinases, and phospholipases (receptosome). After GC/GR interaction and the consequent conformational changes, the GR dissociates from the receptosome and translocates to the nucleus. The genomic action of the activated GR is elicited by a DNA-binding sequence with two zinc finger motifs. This sequence targets specific glucocorticoid-responsive elements (GREs) to control expression of many genes, such as proinflammatory mediators

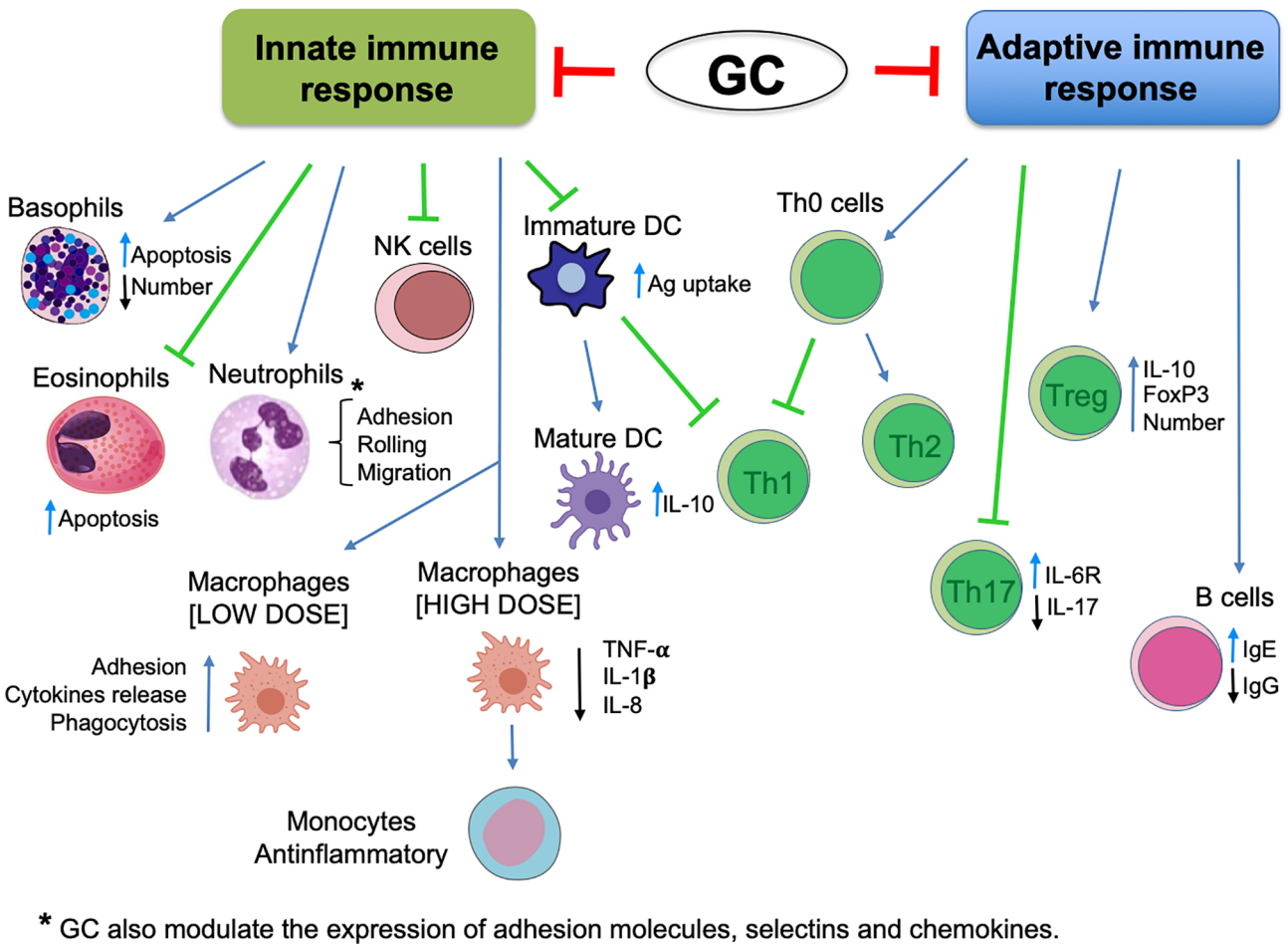


**FIGURE 1** | Glucocorticoid effects in innate and adaptive immune response (schematic).

and transcription factors [e.g., activator protein-1, nuclear factor-kappa B (NF-κB)] (5, 6). Conversely, GC/GR upregulate expression of lipocortin-1, IL-1 receptor antagonists, and Iκ-B. These transcriptional effects contribute to the anti-inflammatory and immunoregulatory actions of GCs. An important target of GC/GR transcriptional activity is GILZ, which interferes with the mitogen-activated protein kinase (MAPK) pathway and NF-kB transcriptional activity and, consequently, regulates inflammatory and immune-mediated reactions (7–10).

Nonetheless, GRE presence is not the only mechanism by which the GR regulates its transcriptional activity. The cell type-specific activities of GCs are dependent upon several other factors, including the chromatin accessibility of target genes, which modulates the availability of DNA sequences (11). Non-genomic actions also contribute to the complexity of GC/GR signaling. These are characterized mainly by a short delay of action and often involve generation of intracellular second messengers and various signal-transduction cascades, such as phospholipase activation, modulation of cyclic adenosine monophosphate, protein kinase pathways, and $Ca^{2+}$ mobilization (12, 13).

GCs have an essential role in controlling the metabolism of carbohydrates, lipids, proteins, water, $Ca^{2+}$, and other electrolytes, as well as maintaining the optimal function of the endocrine, musculoskeletal, cardiovascular, nervous, and immune systems (14–16). Prolonged therapy with GCs amplifies the physiological effects of endogenous GCs, which often lead to deleterious side effects such as hypertension, hyperglycemia, osteoporosis, Cushing's syndrome, and mood disorders (17, 18). Therefore, the anti-inflammatory and immunosuppressive clinical effects of GCs, even if they are considerable, are transitory. The influence of GCs on the complex control of cellular responses to stress and in the regulation of inflammatory processes is linked markedly also to the adverse effects that accompany their chronic use, and therapy must be discontinued.

## IBD

IBD represents a hypernym for the chronic remission and relapse of an immunologically mediated chronic and lifelong disease characterized by gastrointestinal-tract inflammation. IBD is caused by the interaction between the genetic predisposition of the individual and activation of the immune response. Various environmental factors also have important roles in IBD onset. UC and CD are chronic and debilitating diseases that are incurable (19, 20). T-helper type 2 (Th2) cells have been demonstrated to have a pathogenic role. An imbalance in Th17/T regulatory cells has been linked to UC. CD is characterized by an imbalance in the number of Th1 cells (21). However, recent researches reported a number of novel immune cell populations that correlate with disease in IBD patients indicating that there is no clean cut that UC and CD could be separated according to Th1 and Th2 action (22). The role of B cells is less clear because they mainly control mucosal homeostasis, antibody production, and co-stimulation of T lymphocytes. Experimental evidence suggests that cytokine production by B cells may also affect immune regulation (23, 24).

UC and CD show heterogeneity in terms of clinical and pathological features, and can be distinguished by their location and the nature of inflammation they cause. UC attacks mainly the colonic mucosa, whereas CD can affect the gastrointestinal tract at any level. UC causes inflammation and ulceration of the inner lining of the colon and rectum, and usually manifests with diarrhea, loss of appetite/weight, abdominal pain, fatigue, and anemia. The level of inflammation determines disease severity and the subsequent therapeutic strategy. CD is a chronic inflammatory disorder that can affect all parts of the gastrointestinal tract, but mainly influences the terminal ileum, caecum, perianal area, and colon. Several symptoms are caused by CD, which hampers confirmation of the diagnosis. Among those symptoms are weight loss, bowel obstruction, abdominal pain, fever, and chronic or nocturnal diarrhea, although less frequently than other symptoms. These are all critical parameters for the initial diagnosis (25, 26).

IBD at any age is considered a risk factor for the onset of colon cancer due to the development of chronic inflammation that causes immune-mediated tissue destruction. The disease manifests itself in genetically susceptible individuals (i.e. mutations in IBD susceptibility loci including those of STAT3, TYK2, JAK2, CARD9 and IL10RA genes) (27), as a cause or consequence to an imbalance in commensal microbes, or if different environmental factors favor dysbiosis; poor hygiene (28), unbalanced diets (29), smoking, and stress (27). Several factors have been found to impair the functions of immune cells, including lymphocyte activation, autophagy, GC response, and chemotaxis (2, 30).

Specific and curative treatment for IBD is lacking. The focus is on supportive measures as well as the use of anti-inflammatory and immunosuppressive drugs (e.g., aminosalicylates, GCs, anti-TNF agents, such as infliximab, adalimumab, certolizumab pegol and CDP571) (31). The goals of therapy are to improve the quality of life, reduce the risk of disease-related complications, and avoid the need for surgery.

## FIRST-GENERATION GCs

All GCs can be considered to be valid options when treating IBD. The most commonly used GCs are prednisone, methylprednisolone, and hydrocortisone (**Figure 2**). They can be used alone or in combination with mesalamine for the induction and maintenance of clinical remission in patients suffering from chronic IBD (32–34).

The primary purpose of GC use is to induce clinical remission. A controlled therapeutic regimen guarantees a reduction in the symptoms and side effects caused by these drugs. The most efficacious dose of prednisone is 1 mg/kg up to 40–60 mg/day, and a higher dose seems to not change therapy efficacy (35). The dose administered initially is 40–60 mg/day, but it must be reduced gradually to achieve therapy interruption

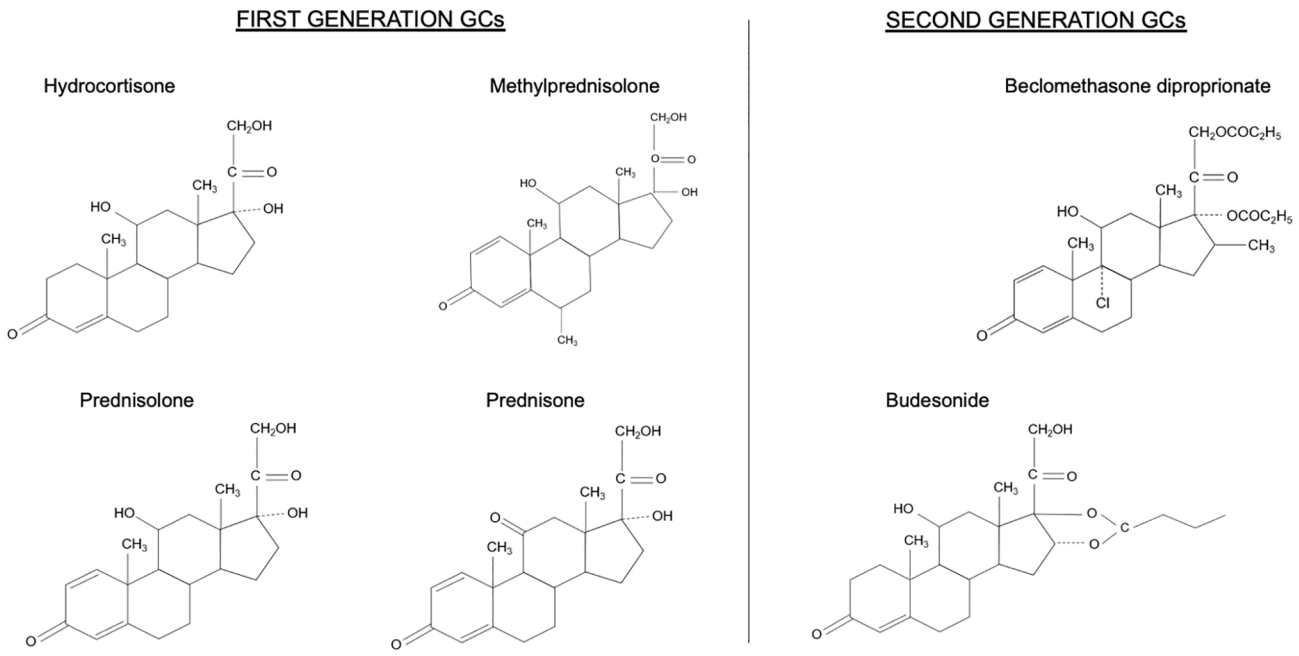


**FIGURE 2** | Chemical structures of first and second generation glucocorticoids (GCs).

while maintaining control of IBD symptoms. At the same time it has been shown that alternate-day treatment helps to reduce and prevent the adverse effects on the central nervous system. Concomitant use with other drugs (e.g., azathioprine) also permits to avoid further use of GCs, while allowing maintenance therapy (36). It is important that prednisone can be considered lifesaving in severe diseases and this is especially true of chronic cases of UC after treatments from six weeks to three months. The effects are however temporary, as patients tends to miss out on the benefits after nine months of therapy (37).

It has been shown that intake of first-generation GCs, such as methylprednisolone (12–48 mg/day), if combined with other drugs, such as sulfasalazine (3 g/day), is important for symptom improvement. It was shown that these improvements occurred faster than if patients were treated with sulfasalazine alone (3).

Another factor in the choice of treatment (and consequently the dose) is the administration route because this is dependent upon the disease location. In people suffering from UC, drug administration can be topical if proctitis is present, or topical and systemic in the case of left-side colitis. In CD, disease severity is the main criterion for GC use because patients might not respond well to treatment and might need surgery if disease severity is high (38). To note, non-response to GCs is comparable between CD and UC.

Despite the efficacy of first-generation GCs, several serious side effects can be associated with their use, including osteoporosis, hyperglycemia, hypertension, mood disorders, gastric ulcer, and increased susceptibility to infections (3, 17). This characteristic limits their long-term use. Maintenance treatment with systemic GCs is not recommended.

## SECOND-GENERATION GCs

Due to the limitations of prolonged therapy with first-generation GCs, compounds such as budesonide and BDP have been developed that maintain topical anti-inflammatory efficacy but minimize their bioavailability and, consequently, their systemic adverse effects (**Figure 2**) (3).

Budesonide undergoes hepatic inactivation before reaching the systemic circulation to diminish corticosteroid-related side effects. Several clinical trials have been conducted to establish the efficacy of oral budesonide. A randomized, double-blind controlled trial conducted in patients with mild, active CD demonstrated that budesonide, if administered at 9 mg/day for 2 months, was as efficacious as prednisone (which was used at 40 mg/day for the first 2 weeks) followed by 30 mg/day in the third month (39). Budesonide carried a lower prevalence of systemic adverse effects (33% of cases) than that of conventional GCs (55%). Budesonide use has not been reported to directly cause changes in the bone mineral density or adrenal insufficiency, however individual sensitivity may vary and therefore cause their onset. Consequently there is no direct correlation to the appearance of osteoporosis symptoms or decreased/inadequate cortisol production (40, 41). Multiple meta-analyses have demonstrated the efficacy of budesonide in inducing remission, and that a once-daily dose is just as efficacious as 3 mg taken

thrice daily. In the context of more severe disease, budesonide is not as efficacious as prednisolone in achieving remission (42).

The second-generation GC BDP is a derivative of cortisone. It has topical effects and minimal systemic activity. It is administered as a prodrug and is partially metabolized in the lower gastrointestinal tract. If used at low doses, BDP has been shown to be free of many of the deleterious side effects associated with GCs absorbed systemically. The induction of remission in CD using BDP has not been investigated in double-blind randomized trials. The efficacy of BDP in patients with UC has been well established: eight double-blind randomized trials have been published (43). Oral BDP for UC has been approved in several countries. In one randomized controlled trial, oral BDP was administered as a daily dose of 5 mg for 4 weeks, then alternated weekly for 4 weeks. A similar efficacy to that observed with prednisolone was evident, and there was no difference in the prevalence of adverse events. However, as a second-generation GC with low systemic absorption, it is associated with fewer adverse events than that of first-generation GCs (44, 45).

## NEW THERAPEUTIC GC-BASED APPROACHES IN CHRONIC IBD

Several studies have aimed to develop new delivery methods for existing drugs to improve the efficacy of therapeutic approaches by limiting the side effects associated with the use of specific drugs or resistance to therapy. Complexity of molecular pathways and networks regulating living organisms has become a central issue of molecular biology. Thus, "molecular stratification" is a new approach based on the needs of patients, their susceptibility to disease, and their gene variability. Studies are carried out on specific elements belonging to different molecular pathways. This can take the form of choosing biologically active molecules bound to cells such as erythrocytes, the targeting of selective GR agonists or the targeting of specific elements able to regulate the inflammatory reaction upon their activation. *GILZ* is a target linked to GC action, so it can be considered a new approach for the regulation of inflammatory diseases such as IBD or even as a molecular marker of inflammation (10, 24).

### Drug-Delivery Strategies in IBD Therapy

Drug-delivery systems aim to improve the pharmacological activities of drugs. They play a key part in regulating solubility, drug aggregation, low bioavailability, poor biodistribution, and reducing side effects (46).

Red blood cells (RBCs) are the largest population of blood cells. Their main function is to carry oxygen to all body tissues. To use erythrocytes as carriers of biologically active substances, pores must be formed reversibly in their membrane, through which the drug can penetrate. To achieve this aim, the cell is stimulated with external influences, such as ultrasound. Drug molecules can also enter by endocytosis in the presence of certain chemical compounds, such as hydrocortisone. Active substances of different molecular weights can be delivered by incorporation in the cell or by binding to its surface (47). The average life of RBCs guarantees a therapeutic effect for 120 days, so biologically inactive compounds such as dexamethasone-21-phosphate are used (48). In recent years, drug selection and the relationship between specific drugs and RBCs have been adapted to reduce some of the limitations that their use could present. Prodrugs, such as corticosteroid prodrugs and nucleotide prodrugs, can improve the action of RBCs as carriers. If the molecule to be delivered is inactivated, erythrocytes can transform and release it in its active form (49).

A type of colonic-delivery technology called Multi-Matrix System (MMX®) has been developed to provide controlled release of budesonide throughout the entire colon. The most common adverse effects in studies using MMX were headache, nausea, and urinary-tract infection (50).

Therapy with systemically active GCs, including budesonide MMX, is associated with suppression of endogenous cortisol levels and HPA function. Oral budesonide MMX at 9 mg/day has been shown to be significantly more efficacious than placebo and can induce remission in mild-to-moderate UC, being as efficacious as 5-aminosalicylic acid (5-ASA, which is first-line therapy for mild-to-moderate active UC) (51). GC effects appear in a shorter time, while 5-ASA takes approximately 2 weeks and therefore it cannot be used in moderate disease as a sole therapeutic regimen (52).

Another strategy for drug delivery involves nanoparticles (NPs) which are organic, inorganic or polymeric particles that ranges between 1 to 100 nanometres in size. Among synthetic polymers used in pharmaceutical formulations there are poly-L-lactic acid, polyvinyl alcohol, poly(lactic-co-glycolic acid), and polyethylene glycol (53). NP efficacy is linked mainly to rapid and preferential intestinal uptake and decreased excretion of the drug following diarrhea (one of the main symptoms of IBD). Cellular internalization of NPs is characterized by paracellular transport or endocytosis into epithelial cells. It has been shown that NPs of diameter 200 nm prepared with cetyltrimethylammonium bromide and relatively neutral charge adhered to healthy colonic tissue in controls as well as inflamed colonic tissue in a colitis model using 2,4,6-trinitrobenzene sulfonic acid (TNBS). Simultaneously, polylactide–coglycolide FK506-NPs were shown to enhance drug penetration into inflamed tissue significantly in a TNBS-induced model and oxazolone-induced colitis model. Other parameters capable of influencing uptake (in addition to diameter) must be studied in detail. *In vivo* and *ex vivo* studies have shown that the surface charge of NPs can affect targeting in the colon. Cationic systems adhere to the mucosal surface due to the interaction between the positively charged nanocarrier and the negatively charged intestinal mucosa. Anionic systems adhere preferentially to targeted sites *via* electrostatic interaction with the higher concentration of positively charged proteins (53). Use of hydrophilic and uncharged NPs is also under development. The creation of NPs bound to the surface with poly(ethylene glycol) (PEG) seem to increase NP translocation through the mucus and to ensure a substantially increased half-life. Another type of nanoformulation used for GC delivery is polymeric micelles in which PEG is present to allow an extended circulation time and

slower release of the compound (54). Zeng et al. showed that a resin microcapsule composed of 717 anion exchange resin and Eudragit S100 could target dexamethasone to the colon, improve the efficacy of UC therapy, and reduce the overall toxic side effects (55).

## Selective Glucocorticoid Receptor Agonists (SEGRAs)

Identification and development of SEGRAs is of particular interest because their therapeutic potential is uncertain (56, 57). SEGRAs are also defined as "dissociated GCs" because they could allow anti-inflammatory activities and the avoidance of metabolic effects. SEGRAs allow the mediation of only repressive effects on various controlled target genes from the GR, and not those of gene activation, because they work by operating through one of two main pathways: transactivation or transrepression (58). The concept that the activating action is responsible for the metabolic (and therefore collateral) effects, whereas immunosuppressive mechanisms are dependent upon the repressive effects, is controversial. Some SEGRA compounds have been shown to have anti-inflammatory properties with reduced collateral effects, such as Compound A [2-((4-acetophenyl)-2-chloro-N-methyl) ethylammonium-chloride))], or ZK216348 N-(4-methyl-1-oxo-1H-2,3-benzoxazine-6-yl)-4-(2,3-dihydrobenzofuran-7-yl)-2-hydroxy-2-(trifluoromethyl)-4-methylpentanamide (59). Therefore, their efficacy *in vivo* for treatment of inflammatory diseases, such as colitis, asthma, and rheumatoid arthritis, is being evaluated (60, 61).

## GILZ-Based Therapy

One possible therapeutic solution involves GILZ being induced rapidly by CGs. GILZ protein has been recognized as a downstream inducer of anti-inflammatory effects (**Figure 3**). GILZ is a protein of 135 amino acids. It is highly conserved in humans and mice, and its binding activity can interfere with MAPK/extracellular receptor kinase and protein kinase B pathways (62, 63). Its action is mediated by the binding of GRs to the GREs present in the proximal region of the GILZ transcriptional start site. At the molecular level, GILZ can interact with proteins such as NF-κB, thereby obstructing the transcription of proinflammatory mediators (64). GILZ is expressed in virtually all cells, including macrophages, granulocytes, dendritic cells and T lymphocytes, as well as other non-lymphoid tissues (10). The function of GILZ has been identified only partially and its role (like that of GCs) is being studied in several mouse models of inflammatory and autoimmune diseases. Attention has focused mostly on various transduction pathways that act on suppression of the immune system (65). Several studies have focused on the role of GILZ and analyzed its protective aspect in dinitrobenzene sulfonic acid (DNBS)-induced colitis. It has been shown that transgenic mice overexpressing GILZ had lower levels of colonic inflammation. This result was reliant on a decrease in the number of Th1 cells in the lamina-propria lymphocytes and increase in the number of Th2 cells induced by GILZ overexpression (24, 66). Also, treatment with a recombinant form of TAT-GILZ has been

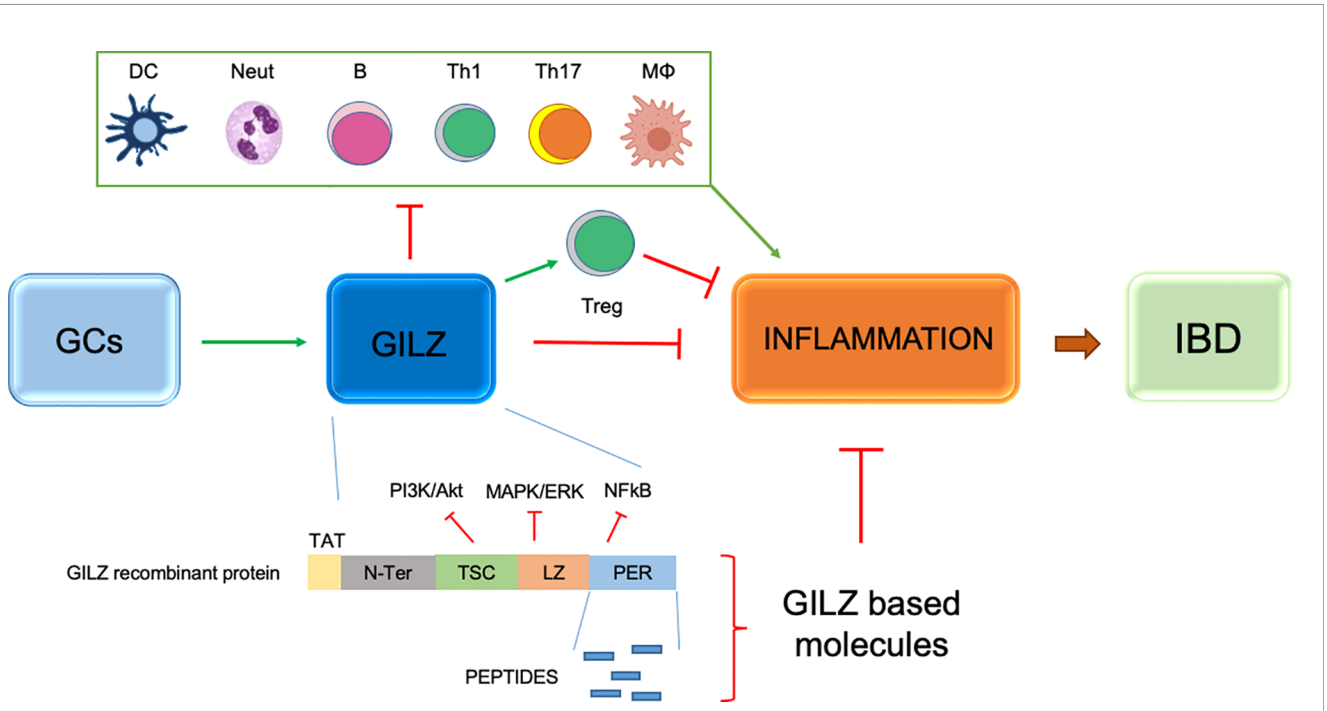


**FIGURE 3** | Schematic representation of GILZ efficacy in inflammatory processes and IBD and anti-inflammatory activity of GILZ based molecules, such as peptides deriving from the Proline anf Glutamic acid rich (PER) region of the GILZ protein and TAT-GILZ full length protein. Green arrows indicate positive regulation; red lines indicate negative regulation. DC, dendritic cells; Neut, neutrophils; B, B cells; TH1, T helper Type-1 cells; Th17, T helper Type-17 cells; MΦ, monocytes/macrophages; T regulatory cells.

studied in animals with IL-10 knockout. The presence of GILZ has been shown to lower the prevalence of spontaneous UC, thereby demonstrating a protective action of GILZ against severe inflammation (66). Moreover, we and other groups demonstrated the efficacy of GILZ based molecules (full recombinant GILZ protein or peptides) in different experimental models of inflammatory diseases. The main inflammatory target of GILZ is NF-kB. GILZ binds and inhibits p65/NF-kB subunit, thus exerting its anti-inflammatory activity (7, 64). The GILZ interaction with NF-kB is mediated by proline-rich (PXXP) domains present in proline and acid glutamic rich (PER) region of GILZ COOH-terminal portion. Consequently, the possibility to synthesize and use GILZ peptides, that include PXXP domains of the GILZ protein, could be an alternative to the administration of the entire recombinant GILZ protein with possible pharmacokinetic and pharmacodynamic advantages. Notably, no apparent toxic effects have been observed in mice upon *in vivo* administration (intraperitoneally or subcutaneously) of TAT-GILZ protein or GILZ-peptides in mice (66, 67, and Bruscoli, unpublished data). Thus, GILZ represents a new approach for IBD treatment, and to have an immunosuppressant effect comparable with that of GCs (24, 66, 68). GILZ has also has been associated with several inflammatory diseases other than IBD, such as rheumatoid arthritis and fibromyalgia (69, 70). An advantage of GILZ therapy could be that side effects have not been associated with administration of GILZ recombinant protein in cellular systems or in established *in vivo* models of IBD.

Therefore, novel drugs in inflammatory/autoimmune diseases (including IBD) can be developed based on the structure and molecular interactions of GILZ. Moreover, GILZ could be of interest for several inflammation-based degenerative diseases and possibly cancer.

## AUTHOR CONTRIBUTIONS

SB and MF wrote the article. CR substantially contributed to the conception and design of the article and interpreting the relevant literature. GM revised it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Bidirectional Crosstalk Between Hypoxia Inducible Factors and Glucocorticoid Signalling in Health and Disease

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Glucocorticoid-induced (GC) and hypoxia-induced transcriptional responses play an important role in tissue homeostasis and in the regulation of cellular responses to stress and inflammation. Evidence exists that there is an important crosstalk between both GC and hypoxia effects. Hypoxia is a pathophysiological condition to which cells respond quickly in order to prevent metabolic shutdown and death. The hypoxia inducible factors (HIFs) are the master regulators of oxygen homeostasis and are responsible for the ability of cells to cope with low oxygen levels. Maladaptive responses of HIFs contribute to a variety of pathological conditions including acute mountain sickness (AMS), inflammation and neonatal hypoxia-induced brain injury. Synthetic GCs which are analogous to the naturally occurring steroid hormones (cortisol in humans, corticosterone in rodents), have been used for decades as anti-inflammatory drugs for treating pathological conditions which are linked to hypoxia (i.e. asthma, ischemic injury). In this review, we investigate the crosstalk between the glucocorticoid receptor (GR), and HIFs. We discuss possible mechanisms by which GR and HIF influence one another, *in vitro* and *in vivo*, and the therapeutic effects of GCs on HIF-mediated diseases.

**Keywords: glucocorticoids, glucocorticoid receptor, HIF, hypoxia, inflammation**

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## GLUCOCORTICOIDS AND THE GLUCOCORTICOID RECEPTOR: A GENERAL INTRODUCTION

Glucocorticoids (GCs; corticosterone in rodents, cortisol in humans) are important steroid hormones which play a role in several fundamental physiological processes such as lipolysis (1) and gluconeogenesis (2), inflammation (3), development (4) and reproduction (5), growth (6), mood and cognition (7, 8), and cardiovascular function (9). They are mainly synthesized in the cortex of the adrenal glands by enzymatic processing of cholesterol (10). Extra-adrenal GC production in the thymus, brain, vasculature, and epithelial barriers has also been observed, where GCs primarily regulate local immune responses (11).

## The Biosynthesis of GCs, Their Regulation, and Biological Activity

The adrenal GC production is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (**Figure 1A**). Under homeostatic, unstressed conditions, the adrenal glands release GCs into the bloodstream in a circadian and ultradian rhythm characterized by peak levels in the morning in humans and in the late afternoon/early night in nocturnal animals such as mice. Upon physiological (e.g. activated immune response) and emotional stress, the activity of the HPA axis is increased. Furthermore, the production of cytokines during inflammation also activates the HPA axis which forms an important regulatory process. GCs will limit the production of most cytokines to maintain homeostasis and guarantee the survival of the host to a life-threatening impact of excessive inflammation, which can be described as a tolerance-like mechanism (12).

When the HPA axis is stimulated, the hypothalamus secretes corticotropin-releasing hormone (CRH) which subsequently induces the secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary in the bloodstream. ACTH will then bind to its receptor and stimulate the adrenal glands to synthesize and secrete GCs into circulation (13). Blood GC levels are regulated by a negative feedback loop, whereby the HPA axis is inhibited at different levels by GCs both in a genomic and non-genomic way (14). The total amount of GCs in circulation is controlled by the adrenal production, but extracellular binding proteins and intracellular enzymes regulate local GC activity. In plasma, ~90% of circulating GCs are bound by corticosteroid-binding globulin (CBG) and albumin, thereby leaving only a limited amount of circulating GCs in a free, biological active form (15). At sites of infection, proteases such as neutrophil elastases target CBG, causing the local release of bound GCs (16). Once the lipophilic GCs are released into the bloodstream, they diffuse through cell membranes to bind cytosolic glucocorticoid receptor (GR). This receptor is ubiquitously and constitutively expressed throughout the body, but exerts tissue-specific and cellular effects (17). The bioavailability of GCs in the cytoplasm is determined by the balance between active and inactive forms of GCs. Within cells, 11β-hydroxysteroid dehydrogenase (11β-HSD1/2) enzymes are responsible for the conversion of inactive cortisone/11-dehydrocortisone into active cortisol/corticosterone, and *vice versa* (**Figure 1B**). Inflammatory cytokines are able to regulate the expression of 11β-HSD1/2 enzymes, thereby modulating GC activity locally (18).

## The GR Protein Structure

The GR is a member of the nuclear receptor superfamily of transcription factors (TFs). It is a 97 kDa protein encoded by the *NR3C1/Nr3c1* gene (chromosome 5 in human, chromosome 18 in mouse). The GR protein contains an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain (LBD) (19). In the NTD, the ligand independent activation function (AF1) is present which is responsible for the binding of co-factors, chromatin modulators, and the transcription machinery (20). The DBD contains two subdomains each containing a zinc finger important for specific GR DNA binding (proximal box) and GR dimerization (distal box) (21). The hinge region provides flexibility between the DBD

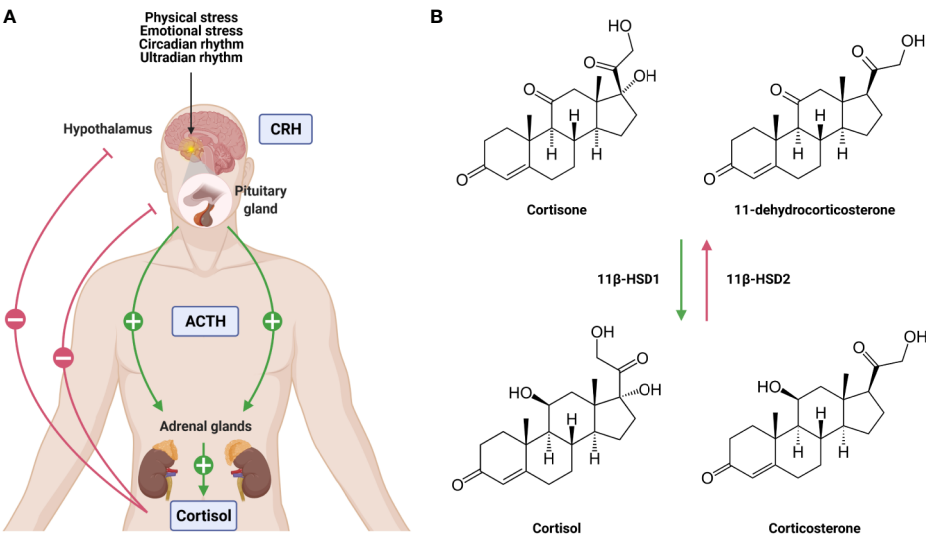


**FIGURE 1** | The hypothalamic-pituitary-adrenal (HPA) axis **(A)** and the conversion of glucocorticoids (GCs) **(B)**. **(A)** Under unstressed, homeostatic conditions GCs are released from the adrenal glands in the blood stream in a circadian and ultradian rhythm. Upon physiological and emotional stress, this release is increased. Stimulation of the HPA axis induces the secretion of corticotrophin releasing hormone (CRH) by the hypothalamus which stimulates the release of adrenocorticotrophic hormone (ACTH) by the pituitary. This will lead to the production of GCs by the adrenal glands and the secretion into the blood stream. Blood GC levels regulate the hypothalamus and the pituitary *via* a negative feedback loop. **(B)** The lipophilic GCs diffuse freely through the plasma membrane. The bioavailability of the GCs is regulated by 11β-HSD1 and 11β-HSD2 which are responsible for the conversion of inactive cortisone/11-dehydrocortisone into active cortisol/corticosterone and *vice versa*. Figures created with Biorender.

and the LBD and acts as a regulatory interface. The LBD contains a ligand binding pocket which is composed of 12 α-helices and 4 β-sheets, and the ligand-dependent AF-2 domain. GR dimerization is also provided by a dimerization interphase in the LBD (22). Further, nuclear localization (NLS), nuclear export (NES), and nuclear retention (NRS) signals have been identified in the GR and are required for the subcellular traffic of GR (**Figure 2A**) (23).

## GR Signalling

In the absence of bioactive ligand, the GR resides in the cytoplasm in a multiprotein chaperone complex. This chaperone complex is critical for the conformation of GR, in an ATP dependent manner, in which the ligand-binding cleft of GR is opened to allow GC binding with high affinity. Furthermore, the chaperone complex is important for nuclear translocation and activation of the GR. Upon ligand binding, the GR undergoes conformational changes leading to dissociation of the chaperone complex and translocation to the nucleus (24). Once in the nucleus, the GR transcriptionally activates or represses gene expression as a GR monomer or a GR dimer (or even a tetramer) usually *via* direct interaction with specific DNA sequences (25). In most tissues and in the presence of endogenous GCs during homeostasis, GR most frequently binds to DNA as a monomer and interacts with DNA *via* binding to GC response element (GRE) half-sites. When a binding site for another TF is in close proximity of the GRE half-site, both elements are able to act as a composite site where the GR monomer and the other TF might interact in a positive or negative manner (26). Two GR monomers can also bind to DNA *via* interaction with inverted negative GREs and repress gene expression by recruitment of corepressors (27). When exogenous GCs like dexamethasone (DEX) and prednisolone are administered, the binding of the GR dimer to GREs is favoured at the cost of the GR monomer. Of course, GR homodimers are also formed under certain pathophysiological conditions, e.g. when GCs rise very high in the blood. Binding of GR homodimers induces transcriptional activation of genes, which indicates that the GR monomer is the most important for physiological functions whereas the GR dimer is crucial for the pharmacological and stress functions (28). GR can also interact with specific genome regions indirectly *via* tethering with other TFs such as nuclear factor κB (NF-κB) and AP-1 (29). Additionally, GR is able to compete with other TFs for overlapping DNA binding sequences (30) of for the binding of co-factors (31, 32). Finally, GR can sequester other TFs to prevent them of binding to the DNA (**Figure 2B**) (33). Next to genomic effects, GR can also induce non-genomic effects which are not dependent on transcriptional activities or protein synthesis (34).

# THE PHYSIOLOGICAL REGULATION AND STRUCTURE OF HYPOXIA-INDUCIBLE FACTORS

The discovery of how cells are able to sense and respond to low oxygen levels, known as hypoxia, has been rewarded in 2019 with the Nobel Prize in Physiology or Medicine, for Drs. Ratcliffe, Kaelin and Semenza. The regulation of oxygen homeostasis and the access to an adequate oxygen supply is crucial for the survival of all aerobic organisms, including humans. During oxygen deprivation induced by low levels of haemoglobin or insufficient blood flow to specific organs, cells modulate their protein activity or change their transcriptional and post-transcriptional organisations (35). Cells will activate multiple genes involved in diverse biological processes such as cell survival and proliferation (36), glucose metabolism (37), and angiogenesis (38).

## Oxygen Sensing and the Importance of HIF Proteins

The human body senses oxygen levels by different mechanisms. First, the carotid body located in the carotid artery senses oxygen and carbon dioxide levels in the blood. When the carotid body detects a decrease in blood oxygen levels, it transduces a signal to stimulate breathing, thereby increasing the acquisition of oxygen from the air (39). Second, an unexpected and novel role for the mouse olfactory system has been revealed as a peripheral oxygen-sensing system that enables mice to rapidly assess the oxygen level in the environment before the arterial blood becomes hypoxic (40). Also, oxygen-dependent enzymes such as 2-oxoglutarate-dependent [2-OG, also known as α-ketoglutarate (α-KG)] oxygenases require oxygen for their activity. When oxygen levels decrease, their activity is inhibited. Prolyl hydroxylases (PHDs) are the best-characterized 2-OG-dependent oxygenases which negatively regulate hypoxia inducible factors (HIFs) (41), and thus operate as an "oxygen sensor" system. Additionally, mitochondria are also considered as a site of cellular oxygen sensing since they are responsible for the majority of oxygen consumption within cells. Oxygen is used at the terminal complex in the electron transport chain (ETC), where oxygen serves as the acceptor of protons, which have been stripped from metabolites in the ETC, forming $H_2O$. The production of mitochondrial reactive oxygen species (ROS) signals upon hypoxia was firstly demonstrated by Chandel et al. They have shown that HIF1α is stabilized upon hypoxia by the generation of ROS by the mitochondrial ETC (42), and more specifically, the ROS generated by complex III (43). A possible explanation how mitochondrial ROS stabilize HIF proteins is by inhibiting PHDs and Factor inhibiting HIF1 (FIH). Hydroxylases can be modified post-translationally by redox signals. Since PHD2 is able to interact with other proteins (44), ROS signals can influence PHD2 activity by changing these protein-protein interactions. Another possibility is the oxidation of cysteine residues or attacking iron ($Fe^{2+}$) atoms (45, 46), which are important for PHD and FIH function, by ROS leading to the inactivation of these oxygen-dependent enzymes.

The master regulators involved in oxygen homeostasis and important for development, physiology, and disease are HIFs. They are members of the basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) transcription factor superfamily. HIFs are heterodimers containing an α- and β-subunit. Three α-subunits are found in mammals, namely HIF1α, HIF2α, and HIF3α, which are oxygen-sensitive as they accumulate in hypoxia. The β-subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed

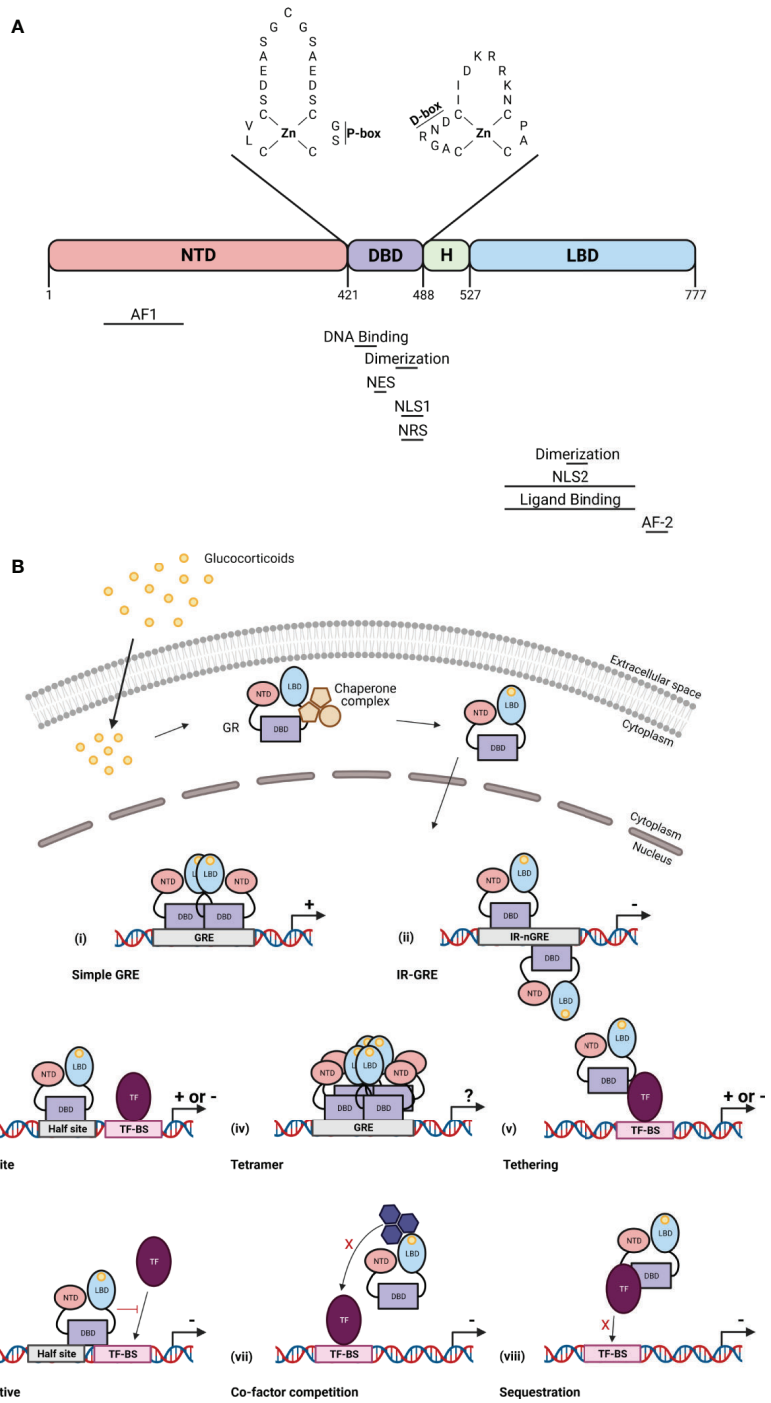


**FIGURE 2** | The glucocorticoid receptor (GR) protein **(A)** and its activation and function **(B)**. **(A)** The structure of the GR protein containing an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region (H), and a ligand-binding domain (LBD). The DBD contains two zinc fingers important for specific GR DNA binding (P box) and GR dimerization (D box). Regions important for the GR function are indicated below the protein structure. AF, activation function; NLS, nuclear localization signal; NES, nuclear export signal; NRS, nuclear retention signal. **(B)** GCs diffuse through the cell membrane and bind the GR in the cytoplasm. This causes a change in the chaperone complex followed by its release and the translocation of the GR to the nucleus where it induces transactivation (+) or transrepression (–) of gene transcription as a GR monomer or a GR homodimer. The GR transactivate gene expression by binding to glucocorticoid receptor element (GRE) as a dimer (i), or can transrepress gene expression by binding to inverted repeat negative GREs (ii). The GR further transrepresses the expression of genes *via* binding to composite elements (iii), by tethering (v), by competing for DNA binding sites (BS) (vi), by competing for co-factors with other TFs (vii) or by sequestrating TFs (viii). GR can also function as a tetramer (iv), but its function is unknown. Figures created with Biorender.

and not affected by hypoxia. HIF1α and HIF1β contain an N-terminal bHLH and PAS domain, responsible for DNA binding and heterodimerization, respectively, and C-terminal transactivation domains (N-TAD and C-TAD). N-TAD is mandatory for target gene specificity and the stability, while C-TAD interacts with co-activators e.g. p300 and CREB binding protein (CBP) required for full HIF activity and regulation of HIF target gene expression. The oxygen sensitivity of HIFα is conferred by an internal oxygen-dependent degradation domain (ODDD). Besides these domains, two nuclear localization signals (N-NLS and C-NLS) are present, which direct the HIF protein to the nucleus. HIF2α exhibits high structural similarity with HIF1α, but they differ in their transactivation domains leading to differences in target gene specificities. HIF1α is ubiquitously expressed, while HIF2α expression is more cell type specific like hepatocytes, adipocytes, endothelial cells, cardiomyocytes, interstitial cells, kidney glomeruli and neurons (47–52). HIF3α is the dominant-negative regulator of the HIF pathway. Due to the absence of the ODDD, HIF1β is constitutively expressed in an oxygen-independent manner (**Figure 3A**).

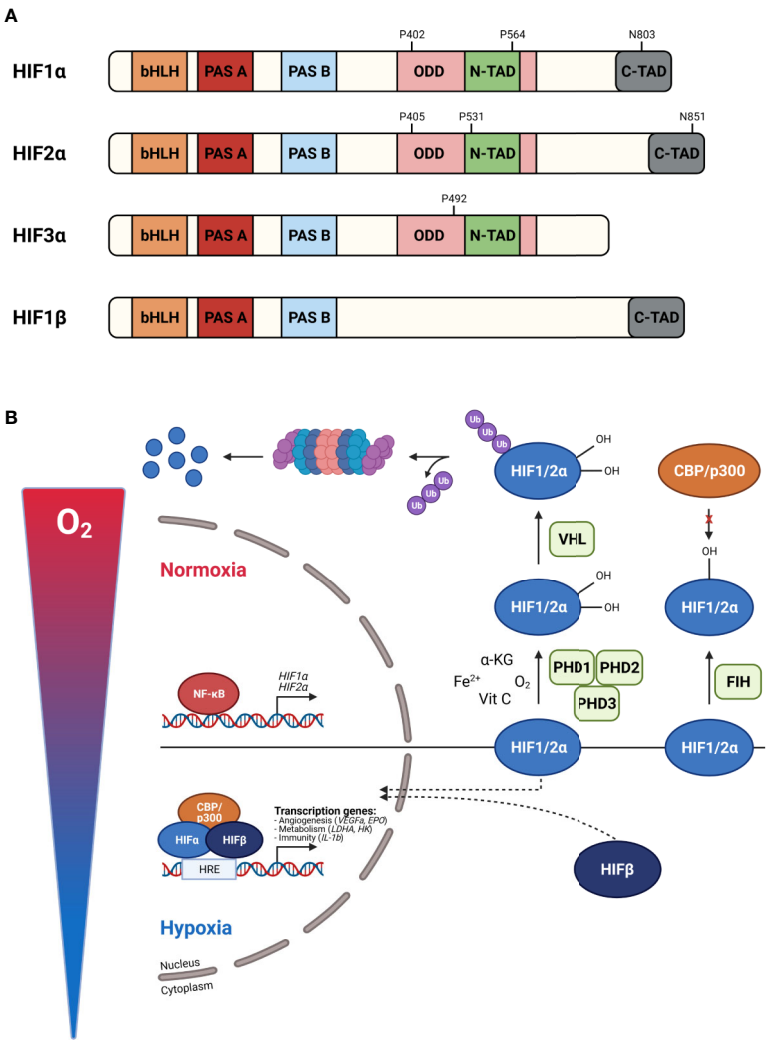


**FIGURE 3** | Protein structure of hypoxia inducible factors (HIFs) **(A)** and their activity under normoxic and hypoxic conditions **(B)**. **(A)** HIF proteins are basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) transcription factors build up by an α-subunit and β-subunit. HIF1α consist of a bHLH and PAS domain, necessary for DNA binding and homodimerization, respectively. Further, an oxygen-dependent domain (ODDD) and two transactivation domains (N-TAD and C-TAD) are present. The stability and target gene specificity are determined by the N-TAD which overlaps with the ODDD. The C-TAD regulates the interaction with co-activators thereby activating gene transcription. HIF1α and HIF2α differ within their transactivation domain, while HIF3α is known as the dominant-negative regulator. Due to the absence of the ODDD, HIF1β is constitutively expressed. **(B)** In normoxia, prolyl hydroxylases (PHDs) hydroxylate proline (P) residues in the ODDD leading to the binding of the von Hippel-Lindau protein (pVHL) followed by ubiquitination and degradation by the 26S proteasome. The factor inhibiting HIF1 (FIH) hydroxylates asparagine (N) residues in the C-TAD, preventing the interaction of the C-TAD with co-activators thereby inhibiting gene transcription. When oxygen levels decrease, PHDs are inactivated, the HIFα subunits are stabilized, dimerize with HIFβ and translocate to the nucleus. After CBP/p300 are recruited, gene transcription is induced by binding to hypoxia-responsive elements (HREs). Figures created with Biorender.

## HIF Protein Regulation

In physiological oxygen conditions, HIFα proteins are constantly produced and degraded by the 26S proteasome. When oxygen is present, PHDs hydroxylate proline (Pro) residues present in the ODDD (Pro 402 and 564 for HIF1α, Pro 405 and 531 for HIF2α) inducing an ubiquitination reaction by the E3 ubiquitin ligase Von Hippel–Lindau protein (pVHL) followed by 26S proteasome-mediated degradation. The activity of PHDs relies on the presence of oxygen, $Fe^{2+}$, α-KG and ascorbic acid (also known as vitamin C). Each PHD has a different affinity for a certain HIF. HIF1α is mainly regulated by PHD2, while PHD1 and PHD3 are the master regulators of HIF2α (53–55). Mouse studies have reported that PHD2 deficiency causes defects in the developing heart and placenta leading to embryonic lethality between E12.5 and E14.5 (56, 57). Deletion of PHD1 and PHD3 is not lethal and only has tissue specific effects, because of their role in cellular metabolism in skeletal muscle and blood pressure in the central nervous system, respectively (58, 59). Hydroxylation of asparagine (Asn) residues in the C-TAD (Asn 803 for HIF1α and Asn 851 for HIF2α) by FIH prevents the interaction of C-TAD with co-activators causing the inhibition of the transcriptional activity of HIFα. Under hypoxic conditions, or in the absence of the cofactors $Fe^{2+}$, α-KG or vitamins, PHDs are inactivated and HIFα hydroxylation is inhibited. The availability of HIFα can also be regulated at a transcriptional level *via* a crosstalk with other signalling pathways. For example, NF-κB is able to bind the HIF1α promotor and induces its transcription (60). Once the α-subunits are stabilized, they dimerize with HIF1β and translocate to the nucleus. After co-factor recruitment, the heterodimeric complex binds to hypoxia responsive elements (HREs) containing the core sequence RCGTG (R: A/G) leading to the activation of target genes (**Figure 3B**).

# BIDIRECTIONAL CROSSTALK BETWEEN HIF AND GR

## The Direct Effect of Hypoxia/HIF on GR Function and *Vice Versa*

The presence of a crosstalk between hypoxia-dependent signalling pathways and GCs and their receptor have been described in several *in vitro* studies (**Figure 4**). Kodama et al. have been the first to provide evidence of an interaction between HIF and GR. They have identified that hypoxia-dependent gene expression and HRE activity in HeLa cells is enhanced by ligand-dependent activation of GR after DEX stimulation. Furthermore, they show that the LBD is essential for the induction of HRE-Luc activity in HeLa cells upon DEX stimulation, since the HRE-Luc activity is not altered when HeLa cells are transfected with a GR plasmid lacking the NTD or in the presence of the point mutation A458T causing impaired GR dimerization. Although they have no direct evidence of a clear protein-protein interaction between the GR LBD and HIF1, colocalization of GR and HIF1 in distinct compartments of the nucleus are shown (61). Subsequently, a functional role for hypoxia and HIF1α in the regulation of GR mRNA and protein expression and the associated increased GC activity has been shown by Leonard et al. in human proximal tubular epithelial cells (HK-2 cells), indicating that there is an obvious crosstalk between HIF and GR. The upregulation of GR might occur through the binding of HIF1α to one or more HRE sites in the NR3C1 promotor, thereby enhancing GR transcription (62). These results were confirmed in another study where GR expression is upregulated in mouse pituitary AtT-20 cells in hypoxic conditions by increased HIF1α mRNA and protein expression levels (63).

In contrast to the previous studies, Wagner et al. demonstrate that the effect of hypoxia on human hepatoma HepG2 cells in combination with DEX stimulation leads to reduced HIF1α DNA binding activity and HRE activity in a dose-dependent manner. Also, the expression of endogenous HIF1α target genes is decreased. This indicates that DEX attenuates HIF1α activity in a GR-dependent manner, since these effects are not present in a GR deficient human hepatoma Hep3B cell line and are restored upon transient expression of GR. They also report an unusual distribution of HIF1α in the cytoplasm, which suggests a problem with nuclear translocation of HIF1α (64).

## The Consequences of Hypoxia on the Anti-Inflammatory Actions of GCs

Next to the direct effect of hypoxia/HIF on GR and *vice versa*, several *in vitro* studies describe the effect of hypoxia on the anti-inflammatory actions of synthetic GCs. Hypoxia plays a key role in chronic lung diseases e.g. asthma and chronic obstructive pulmonary disease (COPD). The alveolar epithelial cells are directly exposed to changes in oxygen pressure in the arterial blood and to inhaled steroids (65). The exposure of A549 cells (immortalized human alveolar epithelial cells) to hypoxic conditions causes a downregulation of both GR mRNA and protein levels (66) and inhibits nuclear translocation of GR (67), which is in contrast with the results of Leonard et al. (62). This suggests that hypoxia has cell-type-specific effects on GR mRNA and protein levels (68). In the presence of normal oxygen levels, DEX inhibits the production of IL-8 by A549 cells when stimulated with lipopolysaccharide (LPS). However, hypoxia significantly attenuates the DEX-mediated inhibition of IL-8 production in these cells (66). Charron et al. also demonstrate a reduced anti-inflammatory effect of DEX: stimulation of A549 cells with the pro-inflammatory cytokine IL-1β leads to increased CXCL8 production, which can be repressed by DEX, but in hypoxia the DEX response is less effective (**Figure 4A**). They show that this reduced DEX effect might be linked to the binding of HIF1α to HREs present in the promotor of histone deacetylase 2 (HDAC2), which is normally recruited by an activated GR to repress NF-κB mediated transcription in airway epithelial cells. However, binding of HIF1α to the promotor leads to reduced *HDAC2* transcription and less suppression of NF-κB mediated transcription of genes with known pro-inflammatory functions (69).

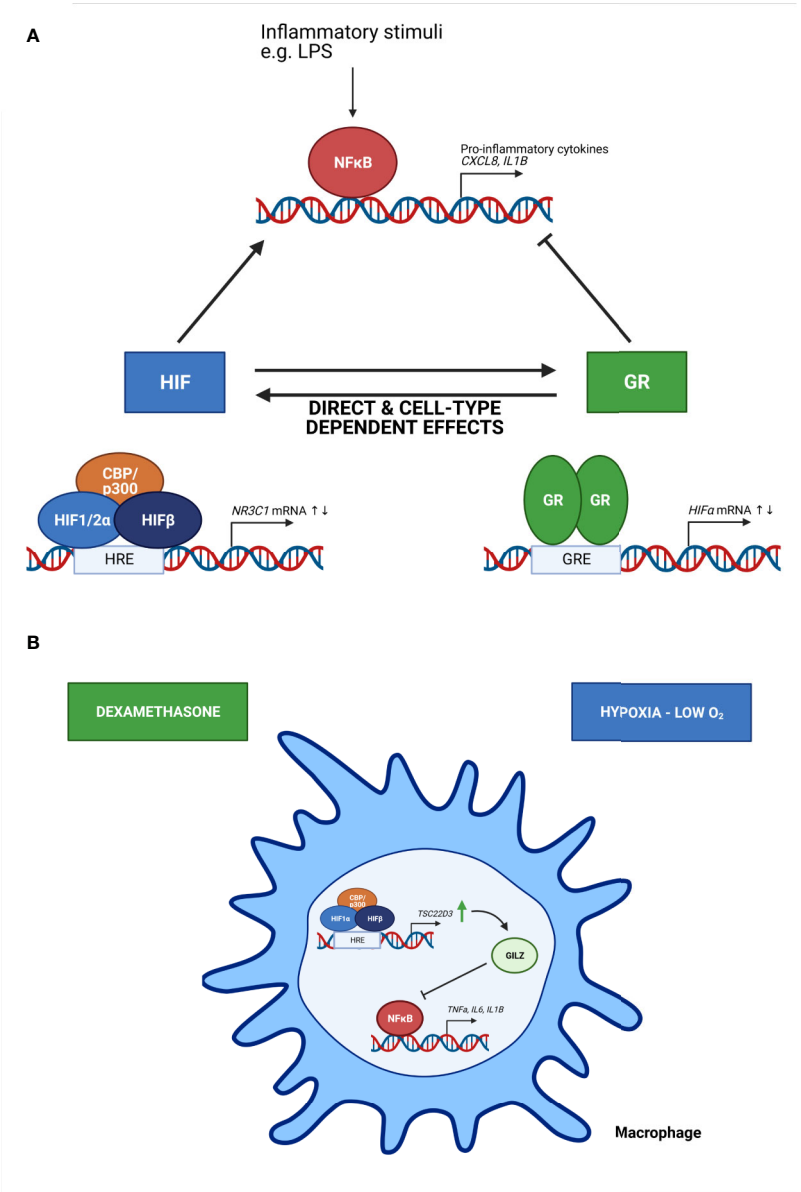


**FIGURE 4** | The crosstalk between HIF and GR and its effect on NF-κB activity **(A)** and the effect of oxygen availability on immune cells **(B)**. **(A)** Based on *in vitro* studies, HIF and GR have direct and cell-type dependent effects on each other, thereby increasing or repressing the transcription of *HIF* mRNA and *N3RC1* mRNA genes. Inflammatory stimuli like lipopolysaccharide (LPS) induce NF-κB mediated transcription of genes encoding pro-inflammatory cytokines. HIF is known to stimulate this NF-κB mediated gene transcription, while GR will repress this. **(B)** Upon hypoxic conditions, the expression of GC-induced leucine zipper (GILZ) encoded by the *TSC22D3* gene is upregulated, thereby inhibiting the expression of pro-inflammatory cytokines mediated by NF-κB. When macrophages are pre-treated with dexamethasone, a synthetic GC, the effects are amplified. Figures created with Biorender.

## Immune Cells and the Role of Oxygen Availability

Oxygen availability is also important for the functional behaviour of immune cells. Hypoxia is able to activate monocytes, macrophages and dendritic cells by altering their gene expression and cytokine secretion (70–73). GC-induced leucine zipper (GILZ) is encoded by the *TSC22D3* gene, which is known as a DEX-inducible gene and is highly expressed in cells of the immune system (74). GILZ is able to inhibit the activation of macrophages and the production of pro-inflammatory cytokines and inflammatory mediators upon exposure to LPS (75). The exposure of macrophages to hypoxic conditions significantly upregulates GILZ mRNA expression, thereby inhibiting the production and secretion of pro-inflammatory cytokines IL-1β and IL-6. When pre-treated with DEX these effects are amplified. These results indicate that GILZ prevents the overactivation of immune cells and overproduction of

pro-inflammatory cytokines in hypoxic microenvironments and might be involved in systemic adaptation to hypoxia (**Figure 4B**) (76). In A549 cells, hypoxia induces the expression COX-2 *via* NF-κB, which is suppressed by DEX. The inhibitory effects of DEX on hypoxia-induced COX-2 is mediated by GILZ *via* a physical interaction between GILZ and HIF1α (77).

Macrophages are known to be key players in inflammatory diseases. Next to their protective immunological function, they also induce the production of pro-angiogenic cytokines and growth factors such as vascular endothelial growth factor (VEGF-A) and basic fibroblast growth factor (FGF-2) thereby promoting angiogenesis (78). Upon hypoxia, the polarization of macrophages is promoted towards the activated, anti-inflammatory M2 phenotype, and not to the pro-inflammatory M1 phenotype. This causes an increased the expression of VEGF and decreased the release of pro-inflammatory cytokines, thereby regulating macrophage functions including tumour promotion in cancer (79). It is known that GCs are angiostatic and are used to tread angiogenesis-related diseases such as solid tumours. They regulate angiogenesis *via* the suppression of proliferation, migration and sprouting in endothelial cells and by reducing the expression or secretion of cytokines and proteins involved in angiogenesis (80). Since both hypoxia and GCs are involved in angiogenesis and influence the macrophage function e.g. by inducing GILZ expression, it is possible that the hypoxic tumour environment in combination with GC treatment also causes the upregulation of GILZ expression and thereby preventing overactivation of immune cells. However, further investigation is necessary to elucidate the connection between hypoxia, GCs and GILZ expression and macrophage polarization. Must be added at the end of this paragraph: An overview of the most essential key studies concerning the bidirectional crosstalk between GCs and HIF can be found in **Table 1**.

## The *In Vivo* Crosstalk Between HIF and GR and Its Role in Inflammation

From these *in vitro* studies, a dynamic interaction between oxygen concentration and GR function mediated through HIF1α has become clear (**Figure 4A**). Also *in vivo* studies using zebrafish have identified new activators of the HIF signalling pathway in the liver e.g. betamethasone (BME) and DEX, synthetic GR agonists. These GCs activate HRE reporters in a GR dependent manner (81), although *via* a non-transcriptional route since these HIF transcriptional responses were still present when the GR DBD harbours a point mutation (R443C; R484C in mouse and R477C in human), a missense mutation in the DBD thereby largely eliminating the transcriptional activity of GR (96). They suggest a mechanism by which GCs stabilize HIF proteins *via* the degradation of pVHL (81). Upregulation of the HIF signalling in *vhl*$^{-/-}$ zebrafish represses the GR activity and dampens its responsiveness to BME. Also endogenous cortisol levels were reduced, most likely due to HIF-mediated downregulation of POMC activity. The inhibition of the HIF pathway leads to a significant increase in both GR activity and cortisol levels (97). These results are confirmed by Marchi et al. They have also demonstrated the repression of GR activity and its reduced responsiveness to exogenous GCs when HIF protein levels are upregulated in *vhl*$^{-/-}$ zebrafish. Furthermore, cortisol levels were also reduced suggesting that HIF signalling can act both at the level of the hypothalamus by inhibiting *Pomc* expression and intracellularly by blocking the transcriptional activity of GR itself (82). A recent

**TABLE 1** | Overview of key studies concerning the bidirectional crosstalk between GC signalling and HIF mediated pathways.

| | Purpose | Results | References |
|---|---|---|---|
| *In vitro* | DEX effect on HeLa cells under hypoxic conditions | Induction of hypoxia-dependent gene expression<br>Increased HRE-luciferase activity<br>The LBD of GR is necessary for HRE-luciferase activity | (61) |
| | Exposure of HK-2 cells or AtT-20 cells to hypoxia | Upregulation of GR mRNA and protein levels due to binding of HIF1α to HREs present in the *NR3C1* promotor | (62, 63) |
| | The effect of GCs on HIF1α function (HepG2 cells) | Attenuation of HIF1α activity upon hypoxia and DEX stimulation as a results of reduced DNA binding and HRE activity associated with problems with HIF1α nuclear translocation | (64) |
| | Characterization of the hypoxic effect on GR levels and its anti-inflammatory actions in A549 cells | Hypoxia causes a time-dependent downregulation of GR mRNA and protein levels and inhibits GR nuclear translocation<br>The anti-inflammatory effect of DEX is attenuated when A549 cells are exposed to hypoxia and stimulated with LPS or IL-1β | (66, 67, 69) |
| | Effect of chemical hypoxia ($CoCl_2$) and/or DEX on RAW264.7 cells | GILZ is upregulated by hypoxia and is further increased upon DEX stimulation to prevent overactivation of immune cells (macrophages) and overproduction of pro-inflammatory cytokines (inhibition of IL-1β and IL-6 production) | (76) |
| *In vivo* | How is hypoxia affecting the endogenous GC pathway and vice versa in zebrafish larvae? | GCs stabilize HIF *via* pVHL degradation<br>HIF represses GR activity and the GR response to exogenous GCs (e.g. BME) in *vhl*$^{-/-}$ zebrafish larvae<br>Cortisol levels are reduced by HIF due to repression of POMC expression and intracellular blocking the transcriptional activity of GR | (81, 82) |
| GCs in AMS | Prophylactic effect of GCs when ascending to high altitude | Administration of GCs (DEX and prednisolone) prior to ascending to high altitude reduces the symptoms of AMS (suppresses inflammatory pathways, reduces vascular permeability and vasoconstriction, improves arterial oxygenation and induces a better antioxidant-oxidant balance) | (83–88) |
| Perinatal hypoxia and GCs | Effect of GCs during perinatal hypoxia | Neonatal hypoxia leads to the activation of the HPA axis in the neonates and causes higher GC levels<br>GCs can have neuroprotective effects on neonatal HI-induced brain damage<br>Ideal timing, dose and duration of GCs is necessary to prevent neurotoxic effects | (89, 90)<br>(91–93)<br>(94, 95) |

study by Watts et al. propose HIF1α as a vital regulator of steroidogenesis by controlling the expression of enzymes involved in steroid production (98).

In the presence of inflammation, an important role for the GR-HIF1α signalling pathway has been implicated by Lu et al. (99). Immune mediated hepatic injury (IMH) caused by LPS leads to a decrease in GR protein levels in myeloid derived suppressor cells (MDSCs), which are known to be negative regulators of the immune response (100). However, when these MDSCs are treated with DEX, GR expression is restored followed by an ameliorated mortality and reduced inflammatory insults in IMH. This implies that the GR signalling pathway in MDSCs is a potential therapeutic target in treating innate immune cell-mediated hepatic injury. They also elucidate the suppression of HIF1α and HIF1α mediated glycolysis in MDSCs thereby promoting the immune suppressive activity in MDSCs (99). Pulmonary arterial hypertension (PAH) is a progressive and life-threatening disease with poor prognosis characterized by pulmonary vasoconstriction and increased pulmonary vascular resistance leading to right ventricular failure, fluid overload and eventually death (101). The infiltration of inflammatory cells e.g. T cells, B cells, macrophages and dendritic cells is typically present in the pulmonary vascular lesions of PAH patients (102). A critical role for serum GC regulated kinase 1 (SGK-1) in the pro-inflammatory response in hypoxia-induced PAH is demonstrated. In the absence of SGK-1, the hypoxia-induced PAH development and pulmonary arterial remodelling is ameliorated, and the production of pro-inflammatory cytokines such as TNFα and IL-6 are inhibited. This suggest that SGK-1 plays a critical role in PAH development and might be a potential therapeutic target (103).

Based on these *in vitro* and *in vivo* studies, a bidirectional crosstalk between GR and HIF is present. However, more studies using animal models will be necessary to elucidate this interaction in more detail and how important this crosstalk is in inflammatory disease models. Further understanding of the GR-HIF crosstalk might lead to the development of alternative therapeutic strategies.

## IMPACT OF GCs ON ACUTE MOUNTAIN SICKNESS AND THE IMPORTANCE OF NF-κB

Hypoxia and inflammation are unequivocally linked (104). Just as hypoxia causes inflammation by stimulating NF-κB gene transcription and the production of pro-inflammatory cytokines, inflamed tissue can also become hypoxic (105). The increased demand for oxygen and decreased oxygen supply are the main reasons why tissue becomes hypoxic during inflammation. The metabolic activity following an immunogenic insult is enlarged in the inflamed tissue and requires an increased synthesis of inflammatory cytokines and enzymes leading to a higher oxygen demand (106). Additionally, the influx of immune cells at the site of inflammation leads to more oxygen consumption and cellular hypoxia (105). Another cause of hypoxia during inflammation is disrupted oxygen delivery due to vascular dysfunction (107). Therefore, both an increased oxygen consumption and decreased oxygen delivery finally lead to tissue hypoxia during inflammation.

### The Prophylactic Effect of Synthetic GCs When Ascending to High Altitude

Millions of people live permanently at high altitude. The inhabitants living at the highest altitude are found in La Rinconada in the Southern of Peru, where the altitude is 5100 m and only 7000 people live there. However Cerro de Pasco in Peru with an altitude of 4300 m has up to 75000 inhabitants and more than 1 million people reside at El Alto in Bolivia (altitude 4100 m) (**Figure 5**) (108). In the recent years, the interest in carrying out activities at high altitudes has increased. People travel to regions of high altitudes (i.e. higher than 2500 m) for work, permanent residence, sports and tourism. However, when ascending to high altitude, people are exposed to hypobaric hypoxia. Dependent on the length and time spent at high altitude, this can result in the development of high altitude illnesses encompassing pulmonary and cerebral syndromes which occur in non-acclimatized individuals shortly after rapid ascent (109, 110). Acute mountain sickness (AMS) is the most common syndrome and is characterized by headache and can also be accompanied by nausea, loss of appetite, disturbed sleep, fatigue and dizziness within 12h after ascent. A grading system that is used for the diagnosis of AMS is the Lake Louis self-assessment questionnaire (LLS) (111–113). AMS is a phenomenon of systemic hypoxia, vascular leakage and increased levels of circulating pro-inflammatory cytokines which can lead to the development of high altitude pulmonary edema (HAPE) or high altitude cerebral edema (HACE) and are a potentially fatal consequence (109, 114).

Several clinical trials to prevent AMS have been performed in which acetazolamide and synthetic GCs such as DEX, budesonide and prednisolone were used. Acetazolamide has been the first drug used in a prophylactic manner, however symptoms were only partly controlled and the drug caused some undesirable side effects (83). Prophylactic treatment with GCs is able to reduce the symptoms of AMS when administered prior to ascending to high altitude (83–88). Both DEX and prednisolone are able to suppress inflammatory pathways (115), reduce vascular permeability and vasoconstriction (116), improve arterial oxygenation (88) and induce a more favourable antioxidant-oxidant balance (117). Budesonide, in contrast to DEX and prednisolone, is not recommended for the prevention of AMS, although it is able to reduce the heart rate and increases the oxygen saturation (118, 119). Next to synthetic GCs, clinical trials using non-steroidal anti-inflammatory drugs (NSAIDs) e.g. ibuprofen against AMS suggest that NSAIDs might be a safe and effective alternative medicine in the prevention of AMS (120). Further, large randomized controlled clinical trials are necessary to look in more detail to the prophylactic effect of budesonide in AMS. The fact that GCs prevent AMS may suggest that AMS is in fact nothing else than a hypoxia-induced general inflammatory response. Also, more clinical trials comparing the benefits of NSAIDs to synthetic GCs in the prevention of AMS are recommended.

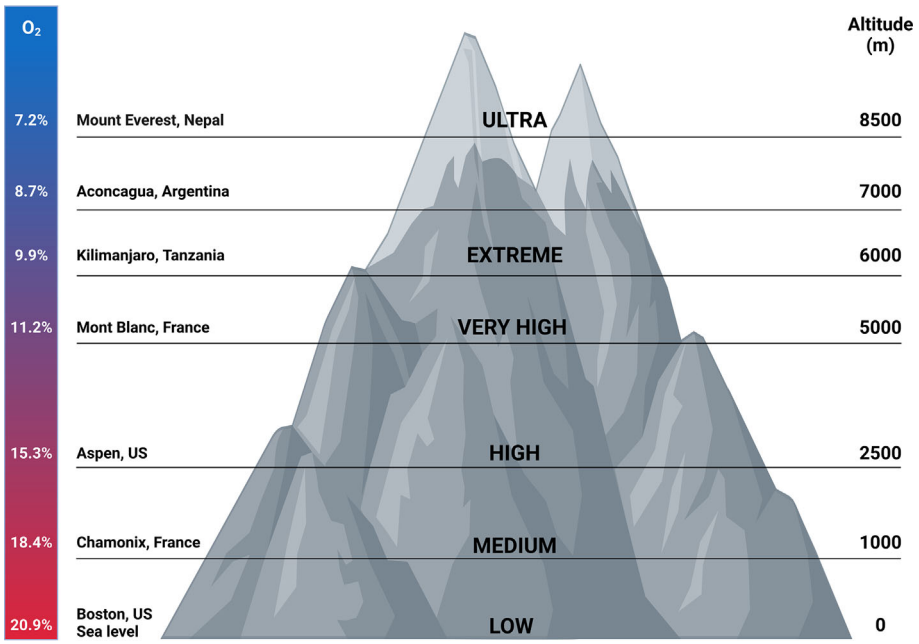


**FIGURE 5** | Correlation between altitude and oxygen concentrations. Millions of people live permanently at high altitude. Also travelling to regions of high altitudes has gained interest throughout the years. In this figure, oxygen concentrations correlated with certain altitudes are depicted. In addition, the grade of hypoxia present at certain places worldwide are shown. Figures created with Biorender.

## How Does Hypoxia Cause Generalized Inflammation?

### Physical Interaction Between NF-κB and HIF Proteins

When humans ascent to high altitude and are exposed to hypoxic conditions, hypoxia itself is able to promote several TFs such as NF-κB thereby stimulating the production of pro-inflammatory cytokines like TNFα and IL-6 (121). NF-κB is a family of TFs composed of RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100). Different stimuli are able to activate these different subunits e.g. bacterial LPS, viral pathogens, cytokines and growth factors. NF-κB activation requires the degradation of the inhibitory proteins such as IκBs. Once they are phosphorylated by the IκB kinase (IKK) complex, IκBs are degraded by the proteasome and the NF-κB subunits can translocate to the nucleus to activate their target genes. One of these target genes is the HIFα subunit, thereby increasing its transcription (122). Several studies have reported the physical interaction between HIF and NF-κB. Hypoxia itself is also able to stimulate the activation of NF-κB. In the presence of hypoxia, PHD1 activity is inhibited thereby decreasing PHD-dependent hydroxylation of IKKβ, since IKKβ contains a conserved motif for PHD hydroxylation. This results in the phosphorylation of IKKβ and liberation of NF-κB from the cytoplasm (123). It is shown that HIF1α binds to RelA in EGF-induced cells (124). Also a novel and specific interaction between NF-κB essential modulator IKKγ (NEMO) and HIF2α has been reported by Bracken et al. This interaction enhances the transcriptional activity of HIF2α in normoxia in which NEMO promotes CBP/p300 recruitment to HIF2α (125). HIF1β is also able to interact physically with NF-κB. Namely, in CD30 stimulated cells, HIF1β interacts with RelB and p52 subunits thereby promoting NF-κB mediated transcription (126). Next to hypoxia, HIF is also induced by growth factors such as insulin-like growth factor 1 and platelet-derived growth factor, cytokines like TNFα and IL-1, and ROS, all of which are activators of NF-κB (**Figure 6**). This learns us that NF-κB functions as a direct modulator of HIF expression by regulating basal, TNFα and hypoxia-induced HIF expression (127–129). More elaborate studies will be necessary to determine whether the physical interaction between HIF and NF-κB is dependent on DNA-binding or is the result of protein-protein interactions. This is important, as more precise intervention strategies, involving GR or not, can then be developed.

### Systemic Inflammation in Response to High Altitude

It has been shown that HIF1α also plays an important role in promoting the expression of NF-κB regulated inflammatory cytokines in macrophages after LPS (130). In humans ascending to high altitude, Hartmann et al. have reported elevated levels of the pro-inflammatory cytokine IL-6, the inflammatory marker IL-1 receptor antagonist (IL-1ra) and C-reactive protein (CRP) in the blood compared to normal levels. This demonstrates the presence of moderate systemic inflammation in response to high altitude (131). Also other studies have shown that exposure to hypoxia of rodents and

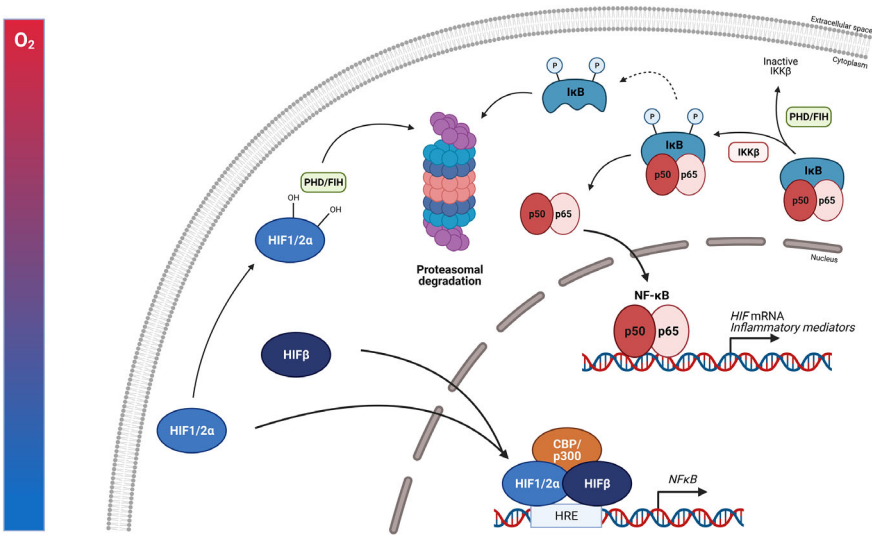


**FIGURE 6** | NF-κB protein regulation and the influence of HIF. The expression of NF-κB is induced by inflammatory stimuli and hypoxia. Inactive NF-κB is composed of a p50 and p65 subunit. The activation of this complex requires the degradation of inhibitory protein IκB. The phosphorylation of IκB by the β subunit of the IκB complex (IKKβ) leads to the degradation of IκB by the 26S proteasome. Once degraded, the NF-κB heterodimer can translocate to the nucleus where it can activate the transcription of inflammatory genes as well as HIF. PHDs and FIH can regulate the activation of NF-κB by controlling the IKKβ activity. Upon hypoxia, HIF heterodimers can translocate to the nucleus, bind to HREs and induce the transcription of numerous genes including NF-κB. Figures created with Biorender.

humans causes increased vascular leakage and oxidative stress with elevated NF-κB expression in the lungs followed by significantly higher levels of pro-inflammatory cytokines IL-1, IL-6 and TNFα (132–134). Circulating chemokines known to recruit and activate leukocytes during inflammation such as macrophage inflammatory protein 1α (MIP-1α) and monocyte chemoattractant protein 1 (MCP-1) are also significantly upregulated in HAPE susceptible individuals (fast ascending mountaineers with previous episodes of HAPE) which is suggestive for chronic inflammation (135). DEX is able to block the production of MCP-1 generated by hypoxic alveolar macrophages to increase capillary permeability (136, 137). It also blocks the formation of migration inhibitory factor (MIF) (138). The high recurrence rate of HAPE susceptibility might point towards a genetic predisposition linked with PAH. For example, a missense mutation in the *JAK2* gene represents a good candidate gene for PAH and the possible development of HAPE upon ascending to high altitude. Also variants in the *CYP1B1* and *HRG* gene have been identified in HAPE susceptible mountaineers (139). Therefore, the identification of more candidate gene polymorphisms in the pathogenesis of HAPE in combination with its prevention and treatment will be of great importance in the better understanding of the disease and formulating new strategies to deal with it.

A recent prospective observational trial has shown that several pro-inflammatory cytokines and chemoattractant proteins are increased after 24h when non-acclimatized humans ascend to high altitude and can be associated with an increased LLS and the clinical symptoms for AMS. In blood samples of these individuals, levels of IL-1β, MCP-1 and its target protein VEGFA are increased at high altitude (140). When AMS further develops into HACE, both in humans and rats the high-altitude hypoxia causes an increase in circulating TNFα, IL-1β and IL-6 cytokines. Also the stress hormone CRH is significantly increased. In rats, this has been associated with the upregulation of pro-inflammatory cytokines which can be blocked with a CRH receptor type 1 (CRHR1) antagonist. Based on these findings, one could suggest that the hypoxia-activated CRH and its CRHR1 signalling might be important for the induction AMS and its pro-inflammatory response (141).

In general, ascending to high altitude of non-acclimatized humans causes the development of AMS, which can further develop into HAPE or HACE. This is clearly associated with the induction of a pro-inflammatory response, most likely linked with a higher NF-κB activity and perhaps the involvement of CRH and the CRHR1 signalling. Small scale clinical trials suggest a prophylactic effect of acetazolamide and GCs such as DEX and prednisolone against AMS probably by reducing NF-κB activity and the production of pro-inflammatory cytokines (**Figure 7**). It would be of great interest to conduct large scale clinical trials with healthy individuals ascending to high altitude which could provide new insights into the body's reaction to hypoxia. The exploration of possible mechanisms responsible for the adaptation to acute hypoxia might be of great added value to improve the understanding of causes and consequences of hypoxia in critical illness. Also the mechanism on how GR is able to inhibit HIF mediated effects in AMS, at the level of HIF itself or more downstream on HIF triggered inflammatory pathways might also be important in critically ill and septic patients and lead to the identification of new therapeutic targets.

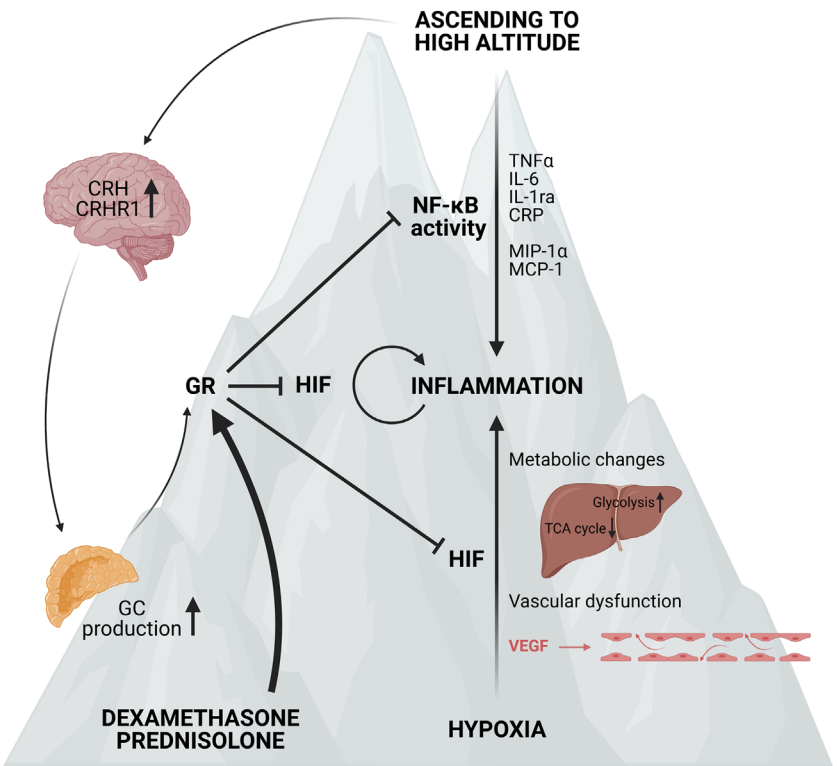


**FIGURE 7** | Acute mountain sickness (AMS) and the prophylactic effect of synthetic GCs. Ascending to high altitude can lead to the development of AMS. This is characterized by elevated NF-κB expression and higher levels of po-inflammatory markers such as TNFα, IL-6, IL-1ra, CRP and chemokines like MCP-1 and MIP-1α. Next to the inflammatory response, the expression of CRH and its receptor CRHR1 in the hypothalamus is also elevated, leading to increased GC production by the adrenal glands and stimulation of the GR. When ascending to high altitude, the hypoxic response is also characterized by metabolic changes and vascular dysfunction induced by the vascular endothelial growth factor (VEGF) contributing to the inflammatory response. When pre-treated with synthetic GCs such as dexamethasone and prednisolone, the GR is stimulated and the NF-κB and HIF mediated responses are inhibited and the symptoms of AMS are reduced. Figures created with Biorender.

## OXYGEN DEPRIVATION DURING BIRTH AND THE ROLE OF GCs

Hypoxia during the period before and during parturition, and also after birth is one of the most common causes of neonatal morbidity followed by admission to the intensive care unit and neonatal mortality. Causes of foetal hypoxia might include unhealthy behaviour and the presence of chronic diseases in pregnant women (e.g. cardiovascular diseases, diabetes and anaemia). Also impairment of the foetal-placental barrier and exposure to harmful environments can lead to foetal hypoxia (142, 143). Postnatal hypoxia can appear from birth until days to weeks after parturition and is caused by a variety of cardiovascular and pulmonary disorders e.g. lack of lung development following preterm birth or patent ductus arteriosus in which the ductus arteriosus fails to close after birth (144, 145). Hypoxia in the premature neonate is generally associated with a harmful conditions requiring mechanical ventilation and oxygen therapy, GCs and other supplementary therapies (146, 147). The adaptation of neonates to hypoxia requires a coordinated physiological response, which includes an increase in the release of GCs, but not aldosterone, from the adrenal cortex. This implies a zone-specific adaptation of the adrenal glands to hypoxia (148). *In vitro* studies have shown that adrenal cells isolated from hypoxic neonatal rats display increased steroidogenesis due to hypoxia-induced changes to the steroidogenic enzyme activity rather than an alterations in the expression of steroidogenic enzymes (149). This is in contrast with adult rodents where hypoxia leads to a decreased expression of steroidogenic enzymes (150) and HIF1α is a central regulator of steroidogenesis (98).

### The Effect of Neonatal Hypoxia on the Activity of the HPA Axis

To gain further insights into the effect of neonatal hypoxia on the HPA axis, Raff et al. exposed suckling rats to hypoxia from birth until 5-7 days of age. This results into increased basal GC levels while no differences in endogenous plasma ACTH levels are detected (89, 90). The augmentation in steroidogenesis appears to be partly mediated by an increase in intracellular controllers of mitochondrial cholesterol transport, namely the steroidogenic acute regulatory (StAR) and peripheral-type benzodiazepine receptor (PBR) proteins (89). Since GCs induce a negative feedback loop at the level of the hypothalamus and pituitary, most likely when the hypothalamus is exposed to a direct stimulus e.g. ether vapours, this

increased GC levels will inhibit the ACTH response to this stimulus (90). Also when perinatal hypoxia is induced in pregnant dams, maintaining them in a hypoxic environment during parturition and maintaining pups in hypoxia until 2 weeks of age, the GC response to an acute stressor in adult rats is significantly higher. This enhanced GC response is linked to higher CRH mRNA levels in the paraventricular nucleus (PVN) of the hypothalamic cells (151). The exposure of pregnant rats to hypoxia during the gestational period important for the maturation of the hippocampus induces long-term impairments in the GC system of the progeny. The exposure of pregnant females to adverse effects such as hypoxia causes an excess of GCs produced and a weakened negative feedback control of the HPA axis activity, resulting in higher baseline GC levels (152). This is associated with decreased nuclear GR protein levels (153) and reduced expression of GR dependent genes in the hippocampus of newborn rats. Additionally, the exposure to hypoxia during foetal development causes an age-related depletion of GR in the liver and is accompanied with a reduced efficacy of GC mediated processes such as the inability to maintain normal glucose levels by the liver (152).

## The Effects of GCs on Hypoxia-Ischemia Induced Neonatal Brain Injury

Next to GR, it is clear that HIF1α also plays a role in hypoxia-ischemia (HI) induced neonatal brain injury. The inhibition of HIF1α after a HI-induced injury results in neuroprotective effects by maintaining the blood-brain-barrier (BBB) integrity and reducing brain edema (154). On the contrary, when HIF1α is induced in a severe neonatal HI model, the BBB permeability is attenuated by the inhibition of VEGFA (91). It is clear that the exposure to perinatal and postnatal hypoxia causes a higher GC response, however a more detailed understanding of the mechanism and the timing of the HPA axis response to acute hypoxia remains to be further elucidated. Furthermore, the exposure to hypoxia during the perinatal period does have long-term pathophysiological effects that persist until adulthood. Next to that, a role of GR and HIF1α in HI-induced brain injury in neonates is obvious. It is likely that GR and HIF1α influence the adaptive response to a HI-induced insult thereby causing metabolic, apoptotic and inflammatory differences.

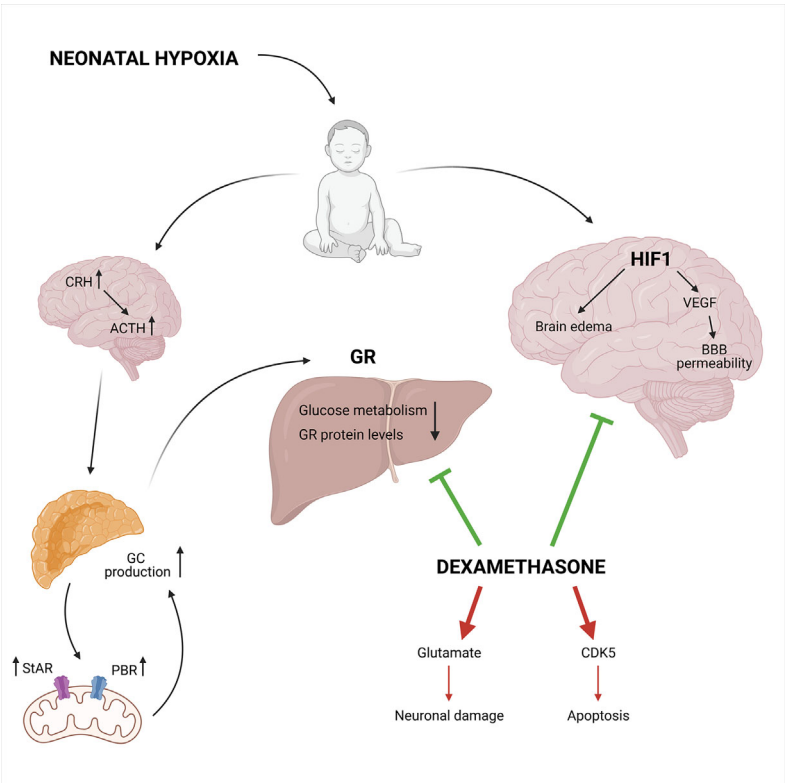


**FIGURE 8** | The neuroprotective and neurotoxic effects of synthetic GCs during neonatal hypoxia. Neonatal hypoxia activates the HPA axis leading to increased GC production, partly mediated by a higher StAR and PBR expression involved in the mitochondrial cholesterol transport. This leads to decreased GR protein levels and reduced glucose metabolism. On the other hand, HIF1 is involved in the hypoxia-ischemia induced brain injury *via* the induction of VEGF and increases blood-brain barrier permeability in combination with brain edema. Neuroprotective effects can be induced by DEX *via* the inhibition of HIF1 mediated brain injury and the inhibition of the GR mediated effects in the liver. Next to the neuroprotective effects, DEX also has neurotoxic effects. It can lead to increased glutamate levels and CDK5 expression causing neuronal damage and apoptosis, respectively. Figures created with Biorender.

## Neuroprotective Mechanisms of GCs

Perinatal hypoxia results in a significant increase in hypoxia-induced brain infarct size in neonates. The question remains whether treatment of neonatal HI-induced brain injury with exogenous GCs like DEX provide neuroprotective or neurotoxic effects. It is shown by Tuor et al. that DEX-mediated alterations in metabolism have a protective effect against neonatal HI-induced brain damage (92, 93). Additionally, the inflammatory response in HI injury is attenuated when neonates are treated with GCs by significantly reducing TNFα production (155). GCs also provide neuroprotective effects by inhibiting cleaved caspase-3. Hence, treatment of neonates with exogenous GCs affects downstream apoptotic pathways and decreased neuronal cell death in the brain injury. Furthermore, DEX also exerts neuroprotective effects by activating phosphorylated Akt, which is important in neuronal pro-survival signals (156). In general, the protective effects of DEX focuses more on the mechanisms that cause damage to neuronal cells rather than decreasing ROS (92, 157). When DEX is administered *via* an intracerebroventricular (ICV) injection prior to hypoxia-ischemia (HI), a concentration-dependent neuroprotective effect of DEX on HI-induced brain injury is present in which the VEGF pathway is partially involved (158). This suggests a direct, local neuroprotective effect of the activation of GR in the neonatal brain.

## Neurotoxic Mechanisms of GCs

However, when exposed to foetal hypoxia the neuroprotective effect of DEX in the neonatal brain is abrogated, most likely due to the downregulation in GR protein levels in the developing brain. This implies a role for GR in the increased vulnerability to HI-induced brain injury in the neonate due to foetal stress (153). The dosing and duration of GC treatment determines the neuroprotective or neurotoxic outcomes. For example, chronic exposure to GCs before HI-induced brain injury results in exacerbated neuronal cell death and white-matter injure (159). GCs also cause an accumulation of glutamate in the brain

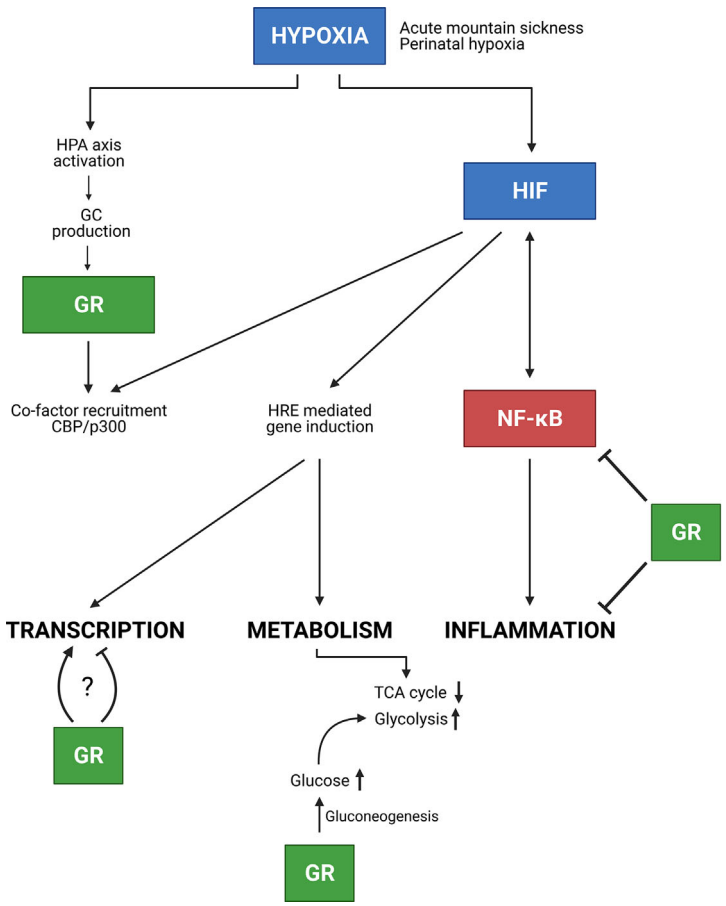


**FIGURE 9** | Conclusion. In the presence of hypoxia e.g. due to AMS or neonatal hypoxia, the HPA axis is activated thereby increasing GC production and GR mediated effects. Next to GR, HIF is involved in NF-κB inflammatory responses, in which these responses can be inhibited by GR. Furthermore, HIF mediated transcription is stimulated, but GR competes with HIF in the recruitment of co-factors e.g. CBP/p300. Whether GR stimulates or inhibits HIF mediated transcription requires further investigation. Finally, hypoxia also causes metabolic changes. The GR promotes gluconeogenesis thereby increasing glucose levels which will be used during glycolysis for appropriate energy levels in hypoxic conditions. Figures created with Biorender.

thereby inducing cytotoxicity and neuronal damage, which contributes to the detrimental effects in neonatal brain injury (160). Also, GCs enhance apoptotic cell death *via* overexpression of cyclin-dependent kinase 5 (CDK5), which is an important kinase for cell cycle regulation (**Figure 8**) (161).

In conclusion, evidence is provided that treatment of neonatal HI-induced brain injury with DEX can be either neuroprotective or neurotoxic (94, 95). In most cases, when DEX is administered systematically and chronically, neurotoxicity remains present, but when DEX is administered directly into the brain, it becomes neuroprotective. The ability of GCs to suppress neuroinflammation caused by hypoxia is thus dependent on dosing, timing and duration after the initial injury.

## CONCLUSION

The bidirectional crosstalk between GR and HIF is clearly highlighted based on the studies described above. *In vitro* studies have shown cell-type specific effects of exogenous GCs on hypoxia-dependent gene expression and HIF1α activity upon hypoxia, which is associated with effects on the nuclear translocation and DNA binding of HIF. Conversely, hypoxia is also able to exert an effect on the GR mRNA and protein levels by influencing GR nuclear translocation or due to the binding of HIF1 to HREs present at the *NR3C1* promotor. Only a few *in vivo* studies using zebrafish as a model organism have addressed the direct interaction between HIF and GR, thereby showing that HIF represses GR activity and its responsiveness to exogenous GCs. Moreover, HIF signalling reduces GC production by acting on POMC expression at the level of the hypothalamus. Further *in vivo* studies will be necessary to elucidate the precise crosstalk between these two complex signalling pathways.

GCs are known to be strong anti-inflammatory mediators. However, in this review, it is shown that hypoxia is able to affect these anti-inflammatory effects by influencing the DEX mediated suppression of pro-inflammatory cytokine production. On the contrary, hypoxia leads to higher GILZ levels in macrophages when exposed to an inflammatory stimulus to prevent overactivation of immune cells and overproduction of pro-inflammatory cytokines. This teaches us that a balance between GR and HIF mediated signalling is required upon inflammatory conditions and that it will be necessary to further investigate the interplay between HIF and GR in disease models such as IMH, PAH and sepsis. Also the link with NF-κB signalling is of utmost importance (**Figure 9**). The exploration of possible mechanisms responsible for the adaptation to acute hypoxia might be of great added value to improve the understanding of causes and consequences of hypoxia in critical illness. Also the mechanism on how GR is able to inhibit HIF mediated effects in AMS, at the level of HIF itself or more downstream on HIF triggered inflammatory pathways might also be important in critically ill and septic patients and lead to the identification of new therapeutic targets.

Since hypoxia is one of the most common causes of morbidity and mortality in neonates, a more complete understanding of what is the mechanism behind these hypoxic conditions in neonates will be of great added value. It is necessary to find the ideal dose, timing and during of GC treatment so that neurotoxic effects of GCs can be eliminated. Considering the importance of HIF1α during neural development, it could be relevant to further elucidate the use of PHD inhibitors or HIF stabilizers during neonatal hypoxia.

Overall, a clear interaction between HIF, GR and NF-κB is shown both *in vitro* and *in vivo*. It will be of importance to identify the role of specific HIF isoforms in several diseases and how this is connected with GR and/or NF-κB. Based on these results, specific targeting of certain HIF isoforms in inflammatory disease models could lead to the identification of new therapeutic targets.

## AUTHOR CONTRIBUTIONS

TV wrote the draft, and RB and CL supervised, corrected, and finalized the paper. All authors contributed to the article and approved the submitted version.

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# Protection of Antigen-Primed Effector T Cells From Glucocorticoid-Induced Apoptosis in Cell Culture and in a Mouse Model of Multiple Sclerosis

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Induction of T cell apoptosis constitutes a major mechanism by which therapeutically administered glucocorticoids (GCs) suppress inflammation and associated clinical symptoms, for instance in multiple sclerosis (MS) patients suffering from an acute relapse. The sensitivity of T cells to GC action depends on their maturation and activation status, but the precise effect of antigen-priming in a pathological setting has not been explored. Here we used transgenic and congenic mouse models to compare GC-induced apoptosis between naïve and antigen-specific effector T cells from mice immunized with a myelin peptide. Antigen-primed effector T cells were protected from the pro-apoptotic activity of the synthetic GC dexamethasone in a dose-dependent manner, which resulted in their accumulation relative to naïve T cells *in vitro* and *in vivo*. Notably, the differential sensitivity of T cells to GC-induced apoptosis correlated with their expression level of the anti-apoptotic proteins Bcl-2 and Bcl-$X_L$ and a loss of the mitochondrial membrane potential. Moreover, accumulation of antigen-primed effector T cells following GC treatment *in vitro* resulted in an aggravated disease course in an adoptive transfer mouse model of MS *in vivo*, highlighting the clinical relevance of the observed phenomenon. Collectively, our data indicate that antigen-priming influences the T cells' sensitivity to therapeutically applied GCs in the context of inflammatory diseases.

**Keywords: glucocorticoids, apoptosis, T cells, antigen-priming, adoptive transfer EAE**

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## INTRODUCTION

Glucocorticoids (GCs) have been used as immunosuppressive and anti-inflammatory agents for more than 70 years (1) and have become indispensable for the management of a variety of diseases. In some fields such as dermatology and ophthalmology, GCs are mostly applied in topical form, whereas systemic administration is customary in the treatment of lymphoma and various autoimmune diseases. This also concerns multiple sclerosis (MS) patients suffering from an acute relapse, who commonly receive high-dose intravenous methylprednisolone pulse therapy to ameliorate clinical symptoms (2).

Most of our current knowledge on the mechanisms of GCs in neuroinflammatory diseases stems from the analysis of experimental autoimmune encephalomyelitis (EAE), a frequently used animal model of MS (3). It has turned out that therapeutic GCs mainly act on T cells in this disease whereas GC nanoformulations such as PEGylated liposomes and hybrid nanoparticles rather target myeloid cells (4–6). GCs engage a variety of mechanisms to modulate T-cell function in neuroinflammation. They alter the expression and secretion of cytokines and chemokines, downregulate adhesion molecules, and redirect T cell migration (4, 5, 7, 8). In addition, they induce apoptosis in developing and mature T cells, which shapes the thymocyte repertoire (9) and contributes to the effectiveness of GC therapy (10). In contrast, the role of regulatory T cells (Treg) as targets of GC action remains controversial (4, 11).

Induction of T-cell apoptosis by GCs requires DNA-binding-dependent transcriptional regulation (12), presumably of pro-apoptotic genes such as Bim and PUMA (13), and is mediated *via* the intrinsic apoptotic pathway (14). The sensitivity of T cells to GC-induced apoptosis depends on their maturation and differentiation stage. While GCs efficiently cause cell death of $CD4^+CD8^+$ double-positive (DP) thymocytes, $CD4^+$ and $CD8^+$ single-positive (SP) thymocytes turned out to be highly resistant (15–17). Differential sensitivities to GC-induced apoptosis have also been noticed for peripheral T cell subpopulations such as Th1, Th2 and Th17 cells (18), whereas contradictory results have been obtained for Treg cells (19–21). Experiments in cell culture further indicated that activated T cells are less prone to GC-induced apoptosis than naïve T cells. Such a dichotomy was found for both murine (22, 23) and human T cells (24, 25). However, little information is available about the impact of GCs on antigen-specific effector T cells that have been activated in a pathological context *in vivo* rather than by artificial treatment with anti-CD3/CD28 antibodies, PMA/ionomycin or ConA *in vitro*. In order to close this gap, we made use of transgenic mice expressing green or red fluorescent proteins as well as CD45.1/2 congenic mice to distinguish between individual T cell populations, and a protocol to generate antigen-specific effector T cells *in vivo* by immunizing 2D2 transgenic mice expressing a MOG-specific TCR. Using this approach, we could demonstrate that antigen-primed effector T cells are protected from GC-induced apoptosis *in vitro* and *in vivo*, and that this effect is relevant in the context of a model neuroinflammatory disease in mice.

# MATERIALS AND METHODS

## Mice

C57BL/6J and B6.SJL-PtprcaPepcb/BoyJ (CD45.1-congenic) mice were originally purchased from Charles River (Sulzfeld, Germany) and bred in the animal facility of the University Medical Center Göttingen. The following mouse strains were also housed in the University Medical Center Göttingen animal facility: 2D2 transgenic mice (26), Act-GFP mice (27), and RFP knock-in mice (28). 2D2 and RFP knock-in mice were intercrossed to obtain mice expressing both the MOG-specific 2D2 TCR transgene and RFP. The mice were kept in individually ventilated cages under specific-pathogen-free conditions and supplied with food and water *ad libitum*. All animal experiments were performed in accordance with ethical standards of animal welfare and approved by the responsible authorities of Lower Saxony (*Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit*).

## Animal Experimentation

Mice were immunized s.c. with 50 µg $MOG_{35-55}$ in Complete Freund's Adjuvant (CFA) and injected twice with 200 ng pertussis toxin (PTX) i.p. on day 0 and 2 as described previously (4). The draining lymph nodes were collected on day 12 post immunization in the case of C57BL/6J mice and on day 10 post immunization for 2D2/RFP mice. To investigate GC effects *in vivo*, 2.5 x $10^6$ restimulated $RFP^+$ antigen-primed T cells and 8-10 x $10^6$ purified $GFP^+$ naïve T cells were injected i.v. into the tail vein of CD45.1-congenic C57BL/6 recipient mice. Three days later, 5 mg/kg or 20 mg/kg Dex (Urbason soluble 32 mg; Sanofi, Paris, France) or PBS as a control were injected i.p. On day 4 after cell transfer, inguinal and mesenteric lymph nodes and spleen were collected, single cell suspensions prepared and analyzed by flow cytometry.

## T-Cell Isolation and Cell Culture

$CD3^+$ T cells were isolated from lymph nodes and spleen with the EasySep™ Mouse T Cell Enrichment Kit (Stemcell Technologies, Cologne, Germany) according to the manufacturer's protocol. Cell purity was routinely determined by flow cytometry and always exceeded 90%. Antigen-specific restimulation of T cells was achieved by seeding cell suspensions from draining lymph nodes of immunized animals in 96-well round bottom plates at 5 x $10^5$ cells/well in 100 µL of restimulation medium consisting of advanced RPMI 1640 (Gibco 51800-035; Thermofisher Scientific, Darmstadt, Germany), 10% fetal calf serum (PAA Laboratories A15-043; Thermofisher Scientific), 1% penicillin/streptomycin (Gibco 150063), 2 mM L-glutamine (Gibco 25030-024), and 0.0002% β-mercaptoethanol. In addition, 25 µg/mL $MOG_{35-55}$ peptide (wildtype T cells) or 10 µg/mL $MOG_{35-55}$ peptide (2D2 T cells) provided by the Charité (Berlin, Germany), 25 ng/mL recombinant mouse IL-12 (R&D Systems, 419-ML; Wiesbaden, Germany) and 20 µg/mL anti-IFNγ antibody (XMG1.2, Biolegend, 505847; Uithoorn, The Netherlands) were added. The cell suspension was incubated at 37 °C and 5% $CO_2$ for 48 hrs, after which it was transferred into 96-well flat bottom plates. Dex at concentrations ranging from $10^{-9}$ M to $10^{-6}$ M or restimulation medium alone as a control were added to the wells, followed by 24 hrs incubation at 37 °C and 5% $CO_2$.

## Flow Cytometry

All antibodies and reagents for extracellular staining were obtained from BioLegend unless otherwise indicated: anti-CD3ε (145-2C11), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD19 (6D5), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD62L (Mel14), AnnexinV, streptavidin, and LIVE/DEAD stain (Thermofisher Scientific). Antibodies and reagents were

directly conjugated with FITC, AF647, PE, PerCPCy5.5, PE-Cy7, APC, APC-Cy7, BV421, BV650, BV785 or biotin. Extracellular staining was performed as previously described (29). For intracellular antigen detection, cells were fixed with 4% PFA for 20 min after completion of surface staining, followed by 30 min of incubation with Perm buffer (BD Biosciences, Heidelberg, Germany) and 60 min with an anti-Bcl-2 antibody (clone REA356, conjugated with APC; Miltenyi Biotec, Bergisch Gladbach, Germany), an anti-Bcl-$X_L$ antibody (clone 7B2.5, conjugated with biotin; SouthernBiotech, Birmingham, AL, USA) in combination with streptavidin, an anti-Bim antibody (polyclonal, conjugated with AF647, antibodies-online, Aachen, Germany) or an mIgG2b isotype control antibody (clone MPC-11, conjugated with APC or biotin; BioLegend), all diluted in Perm buffer. The mitochondrial membrane potential was measured with the help of the MitoProbe™ TMRM Kit (Thermofisher Scientific) according to the manufacturer's instructions. All measurements were performed with a Cytoflex® flow cytometry device (Beckmann Coulter, Krefeld, Germany) and analyzed using Cytexpert® (Beckmann) or FlowJo® (Tree Star, Ashland, OR, USA; version 10.7.1.) software.

## Transfer EAE

Lymph node cells from immunized C57BL/6J mice were cultured as described above in the absence or presence of $10^{-6}$ M or $10^{-7}$ M Dex for the last 24 hrs of the culture period. Afterwards, 2.5 x $10^6$ cells were transferred i.p. into C57BL/6J recipient mice (30). The induction of transfer EAE did not require application of PTX. Animals were monitored daily for clinical symptoms by measuring their weight and scoring disease progression as follows: 0 = normal; 1 = reduced tone of tail; 2 = limp tail, impaired righting; 3 = absent righting; 4 = gait ataxia; 5 = mild paraparesis of hindlimbs; 6 = moderate paraparesis; 7 = severe paraparesis or paraplegia; 8 = tetraparesis; 9 = moribund; 10 = death.

## Statistical Analysis

All data were analyzed by One-way ANOVA followed by a Newman-Keuls Multiple Comparison test. Analyses were performed with GraphPad Prism software (San Diego, CA, USA). Data are depicted as scatter dots plots with individual data points and a horizontal line representing the mean, or as XY plot with the mean ± SEM. Levels of significance are as follows: n.s., $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

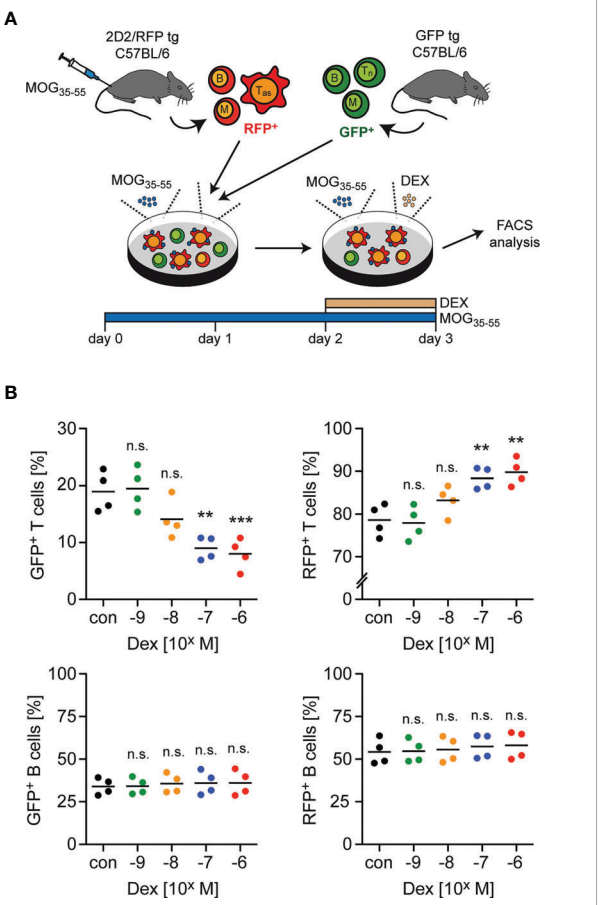


**FIGURE 1** | Enrichment of antigen-primed effector T cells after GC treatment *in vitro*. **(A)** Setup of the experimental model. **(B)** Percentages of live/dead$^-$ (naïve) GFP$^+$ T cells (upper left panel) as well as live/dead$^-$ (antigen-primed) RFP$^+$ T cells (upper right panel) after 3 days of cell culture in the presence of the indicated concentrations of Dex for the last 24 hrs. In control (con) conditions, no Dex was added to the cell culture. Percentages of GFP$^+$ B cells (lower left panel) and RFP$^+$ B cells (lower right panel) were determined in the same cultures. Each dot corresponds to cells originating from one individual mouse, the horizontal line represents the mean. N=4 (biological replicates from individual mice). Statistical analysis was performed by One-way ANOVA followed by a Newman-Keuls Multiple Comparison test. Levels of significance (depicted for the comparison between control and Dex-treated cultures): n.s. = not significant; **$p < 0.01$; ***$p < 0.001$. $T_{as}$, antigen-specific T cells; $T_n$, naïve T cells; B, B cells; M, myeloid cells.

# RESULTS

## Antigen-Priming Protects T Cells From GC-Induced Apoptosis

GC therapy of MS patients and EAE mice induces T cell apoptosis but the differential sensitivity of antigen-primed effector and naïve bystander T cells to the pro-apoptotic activity of GCs has not been explored. To address this issue, we initially established an *in vitro* model that mimics the situation encountered in the treatment of neuroinflammatory diseases (**Figure 1A**). Lymph node cells mostly composed of antigen-primed T cells with a MOG-specific TCR were isolated from 2D2/RFP double-transgenic C57BL/6 mice previously immunized with $MOG_{35-55}$ peptide. These cells were then co-cultured for 3 days in the presence of the same antigen with lymph node cells from GFP transgenic mice containing naïve T cells expressing a natural TCR repertoire. During the last 24 hours of co-culture, Dex was added at concentrations ranging from $10^{-9}$ M to $10^{-6}$ M to induce apoptosis while leaving T cell differentiation largely unaffected. Flow cytometric analysis revealed that the abundance of naïve GFP$^+$ T cells amongst live cells was gradually diminished with ascending concentrations of Dex, whereas the percentage of antigen-primed RFP$^+$ effector T cells was concomitantly

increased (**Figure 1B**). In contrast, no such effect was observed for other cell types also contained in these lymph node cell preparations. More specifically, the percentages of $GFP^+$ and $RFP^+$ B cells amongst live cells for instance remained unaltered after the addition of Dex (**Figure 1B**). We conclude that antigen-primed effector T cells are more resistant to GC-induced apoptosis than naïve T cells.

## Reduced Sensitivity of Antigen-Primed T Cells to Apoptosis Induction

Next, we explored whether the enrichment of antigen-primed effector T cells after Dex treatment was a direct consequence of an altered sensitivity to apoptosis induction. Using the same experimental setup as before, we found that the percentage of $AnnexinV^+$ cells amongst $GFP^+CD4^+$ naïve T cells strongly increased in a dose-dependent manner after Dex treatment, whereas the percentage of $AnnexinV^+$ cells amongst $RFP^+CD4^+$ effector T cells increased only very moderately (**Figure 2A**). To further unveil the mechanisms underlying the T cells' differential sensitivity to GC-induced apoptosis, we investigated the expression of Bcl-2 and Bcl-$X_L$ in naïve $GFP^+CD4^+$ and antigen-primed $RFP^+CD4^+$ T cells. Flow cytometric analysis revealed that antigen-priming increased the levels of both anti-apoptotic proteins compared to the naïve state regardless of whether the cells were treated with Dex or not (**Figure 2B**). In contrast, the levels of the pro-apoptotic molecule Bim were comparable in both cell types (data not shown). Therefore, the elevated expression of anti-apoptotic proteins presumably explains the observed protection of effector T cells from GC-induced apoptosis.

To corroborate our findings, we also measured the mitochondrial membrane potential, as Bcl-2 family members have been reported to exert their apoptosis-modifying activity *via* this organelle (31). Lymph node cells, mostly composed of antigen-primed T cells with a MOG-specific TCR, were isolated from 2D2 transgenic C57BL/6 mice previously immunized with $MOG_{35-55}$ peptide. These cells were co-cultured for 3 days in the presence of the same antigen with lymph node cells from CD45.1-congenic mice containing primarily naïve T cells. During the last 24 hours of co-culture, the cells were either treated with $10^{-6}$ M Dex or left untreated (**Figure 3A**). Under control conditions, a more pronounced loss of the mitochondrial membrane potential, as indicated by a reduced staining with TMRM, was observed for naïve $CD45.1^+CD4^+$ T cells in comparison to antigen-primed $CD45.2^+CD4^+$ T cells (**Figure 3B**). This difference was even stronger after Dex treatment and correlated well with the expression levels of the anti-apoptotic molecules Bcl-2 and Bcl-$X_L$ in these cells. Collectively, our results underscore that naïve T cells are the preferential target of apoptotic cell death after GC treatment.

## Enrichment of Antigen-Primed T Cells Coincides With Their Activation State

In the last step, we investigated late activation/memory markers as an alternative strategy to distinguish between naïve and

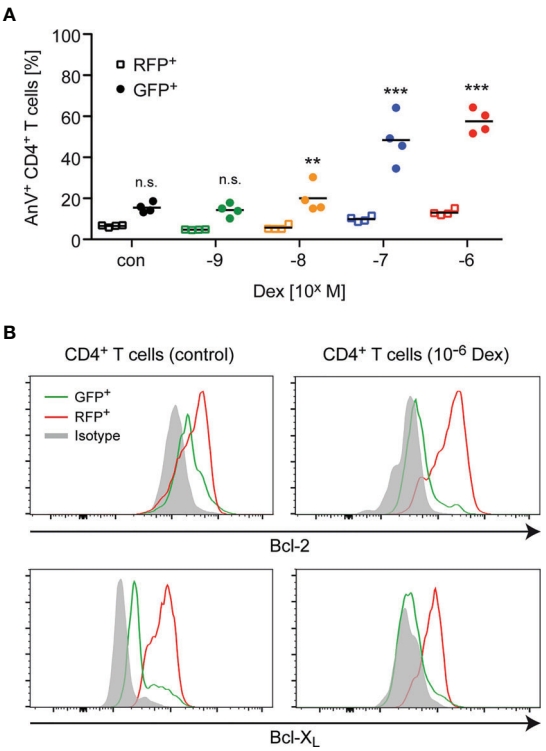


**FIGURE 2** | Apoptosis sensitivity of naïve and antigen-primed effector T cells. **(A)** Percentages of $AnnexinV^+$ ($AnV^+$) cells amongst (naïve) $GFP^+$ and (antigen-primed) $RFP^+CD4^+$ T cells after 3 days of cell culture in the presence of the indicated concentrations of Dex for the last 24 hrs. No Dex was added to the cell culture under control (con) conditions. The experimental setup corresponds to the one illustrated in **Figure 1A**. Open symbols refer to $RFP^+$ cells and filled symbols refer to $GFP^+$ cells. N=4. Statistical analysis was performed by One-way ANOVA followed by a Newman-Keuls Multiple Comparison test. Levels of significance (depicted for each comparison between $RFP^+$ and $GFP^+$ cells): n.s. = not significant; $^{**}p < 0.01$; $^{***}p < 0.001$. **(B)** Intracellular stainings of Bcl-2 or Bcl-$X_L$ in (naïve) $GFP^+$ and (antigen-primed) $RFP^+CD4^+$ T cells cultured in the absence (control) or presence of $10^{-6}$ M Dex for the last 24 hrs according to the experimental scheme depicted in **Figure 1A**. Staining with an mIgG2b antibody served as an isotype control. One representative analysis for each condition is depicted as overlayed histograms.

effector T cells. Here, we used total lymph node cells from C57BL/6 wildtype mice previously immunized with $MOG_{35-55}$ and restimulated them for 3 days in cell culture. During the last 24 hours, Dex was added at ascending concentrations ranging from $10^{-9}$ to $10^{-6}$ M (**Figure 4A**). In agreement with our earlier findings, the abundance of naïve $CD44^{low}CD62L^+$ cells amongst live $CD4^+$ T cells decreased from approximately 60% to 20% with increasing concentrations of Dex, while $CD4^+$ T cells with an activated phenotype ($CD44^{high}CD62L^-$) were concomitantly enriched amongst live cells (**Figure 4B**). In summary, our data suggest that upregulation of anti-apoptotic proteins after activation specifically protects effector T cells from GC-induced apoptosis.

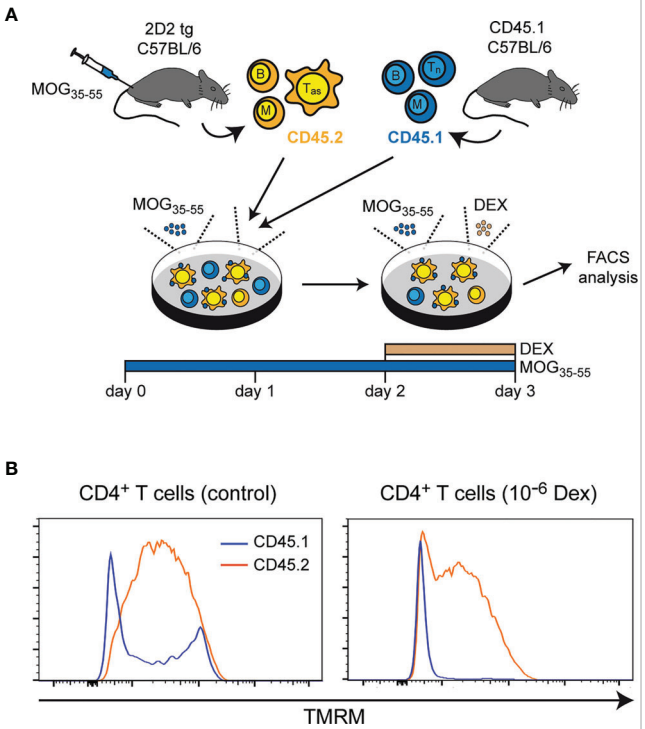


**FIGURE 3** | Mitochondrial membrane potential in naïve and antigen-primed effector T cells. **(A)** Setup of the experimental model. **(B)** Intracellular stainings of (naïve) CD45.1$^+$ and (antigen-primed) CD45.2$^+$CD4$^+$ T cells with tetramethylrhodamine methylester (TMRM) after culturing the cells in the absence (control) or presence of $10^{-6}$ M Dex for the last 24 hrs. One representative analysis is depicted for each condition as overlayed histograms. $T_{as}$, antigen-specific T cells; $T_n$, naïve T cells; B, B cells; M, myeloid cells.

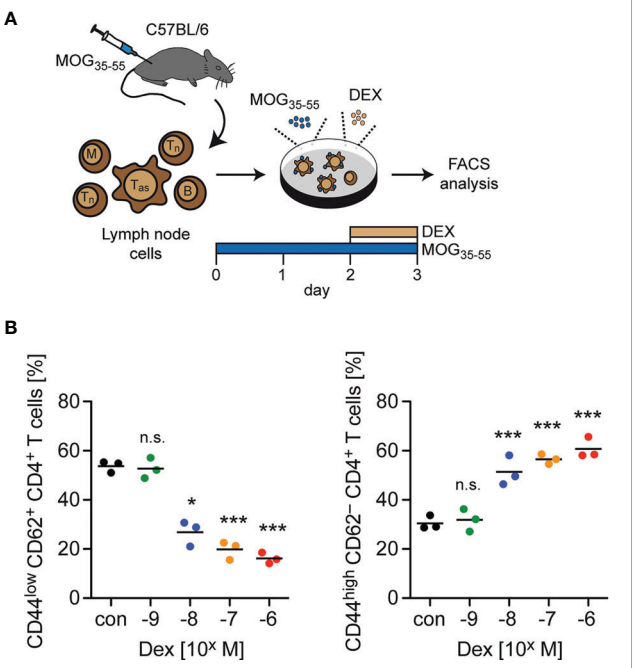


**FIGURE 4** | Enrichment of T cells with an activation/memory phenotype after GC treatment *in vitro*. **(A)** Setup of the experimental model. **(B)** Percentages of live/dead$^-$ (naïve) CD44$^{low}$CD62L$^+$ CD4$^+$ T cells (left panel) and live/dead$^-$ (activated) CD44$^{high}$CD62L$^-$ CD4$^+$ T cells (right panel) after 3 days of cell culture in the absence (con) or presence of the indicated concentrations of Dex for the last 24 hrs. Each dot corresponds to cells from one individual mouse, the horizontal line represents the mean. N=3 (biological replicates from individual mice). Statistical analysis was performed by One-way ANOVA followed by a Newman-Keuls Multiple Comparison test. Levels of significance (depicted for the comparison between control and Dex-treated cultures): n.s., not significant; *$p < 0.05$; ***$p < 0.001$. $T_{as}$, antigen-specific T cells; $T_n$, naïve T cells; B, B cells; M, myeloid cells.

## GC Treatment of Mice Preferentially Induces Apoptosis in Naïve T Cells

As a first step to confirm our results *in vivo*, lymph node cells containing antigen-primed effector T cells expressing a MOG-specific TCR were isolated from 2D2/RFP double-transgenic C57BL/6 mice previously immunized with $MOG_{35-55}$ peptide and restimulated in cell culture with the same antigen. These RFP$^+$ effector T cells were then transferred into CD45.1-congenic C57BL/6 mice together with purified GFP$^+$ naïve T cells, and 3 days later the mice were injected with 5 or 20 mg/kg Dex or PBS as a control. CD45.1-congenic mice were used as recipients to unequivocally allow to distinguish donor T cells from endogenous cells. After another 24 hours, the ratio between GFP$^+$ and RFP$^+$ T cells was determined in secondary lymphoid organs by flow cytometric analysis (**Figure 5A**). Similar to our *in vitro* findings, a dose-dependent accumulation of RFP$^+$ antigen-primed effector T cells compared to GFP$^+$ naïve T cells was observed in lymph nodes and spleen (**Figures 5B, C**). Moreover, also in this *in vivo* setting, a higher expression level of Bcl-2 was found in antigen-primed RFP$^+$CD4$^+$ T cells than in naïve GFP$^+$CD4$^+$ T cells regardless of whether or not the cells were treated with Dex (**Figure 5D**). Our data collectively provide evidence that the sensitivity of antigen-primed effector T cells to GC-induced apoptosis in mice is diminished *in vivo* as well.

## GC-Treatment of T Cells Aggravates Adoptive Transfer EAE in Mice

Finally, we asked whether the accumulation of antigen-primed effector T cells after *in vitro* treatment with Dex had any consequences in an adoptive transfer EAE model. To address this issue, C57Bl/6 wildtype mice were immunized with $MOG_{35-55}$ peptide and 12 days later the lymph nodes containing both antigen-primed effector and naïve T cells were isolated and restimulated *in vitro* with the same antigen for 3 days. During the last 24 hours, Dex was added at two different concentrations ($10^{-7}$ M and $10^{-6}$ M), or the cells were left untreated as a control. Eventually, 2.5 x $10^6$ live cells from each condition were transferred into naïve C57Bl/6 wildtype mice and the disease course was followed over 3 weeks (**Figure 6A**). Mice receiving T cells from cell cultures treated with Dex showed a significantly aggravated disease course dependent on the employed Dex

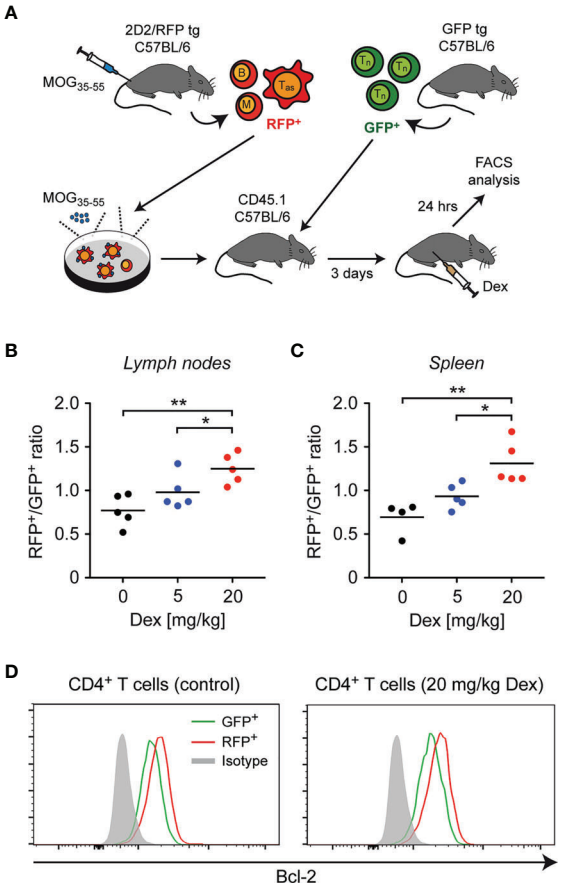


**FIGURE 5** | Enrichment of antigen-primed effector T cells after GC treatment *in vivo*. **(A)** Setup of the experimental model. **(B, C)** Weighted ratio of live/dead$^-$ CD45.2$^+$ (antigen-primed) RFP$^+$ T cells and (naïve) GFP$^+$ T cells from lymph nodes **(B)** and spleen **(C)** on day 4 after transfer and 24 hrs after application of the indicated doses of Dex (5 mg/kg or 20 mg/kg) *in vivo*. Control mice (0 mg/kg Dex) received PBS. Data are depicted as the mean +/- SEM. N=4-5 (biological replicates from individual mice). Statistical analysis was performed by One-way ANOVA followed by a Newman-Keuls Multiple Comparison test. Levels of significance (depicted for the comparison between control and Dex-treated cultures): *p < 0.05; **p < 0.01. **(D)** Intracellular stainings of Bcl-2 in (naïve) GFP$^+$ and (antigen-primed) RFP$^+$CD4$^+$ T cells isolated from mice treated with 20 mg/kg Dex or left untreated as outlined in panel **(A)**. Staining with an mIgG2b antibody served as an isotype control. One representative analysis for each condition is depicted as overlayed histograms. $T_{as}$, antigen-specific T cells; $T_n$, naïve T cells; B, B cells; M, myeloid cells.

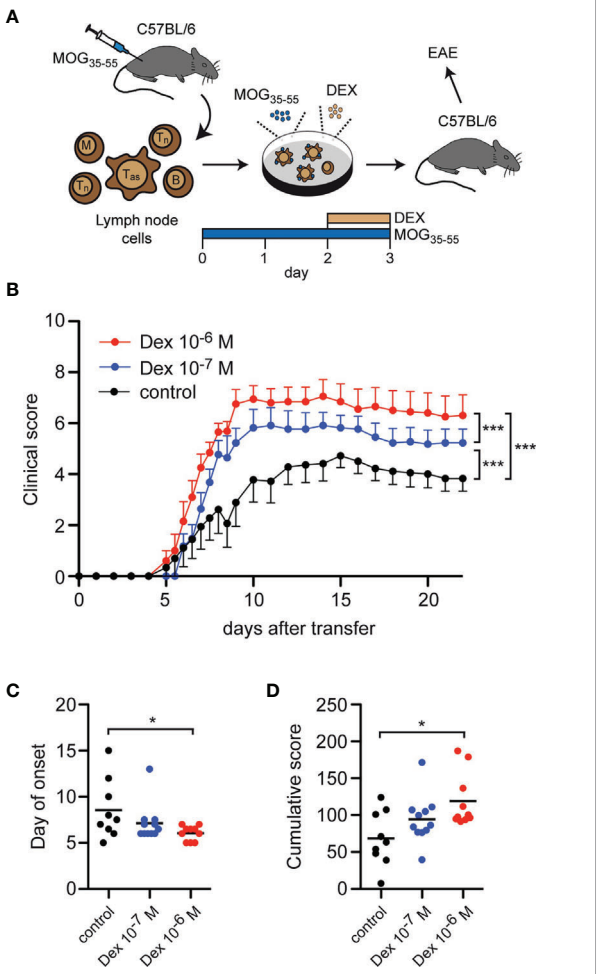


**FIGURE 6** | Enrichment of antigen-primed effector T cells after GC treatment *in vitro* aggravates clinical symptoms in an adoptive transfer EAE model. **(A)** Setup of the experimental model. **(B-D)** EAE development after transfer of 2.5 x $10^6$ T cells under control conditions (black) or after treatment with $10^{-7}$ M (blue) or $10^{-6}$ M (red) Dex for the last 24 hrs of the cell culture period *in vitro*. Clinical score of all mice that became sick, depicted as the mean +/- SEM **(B)**, the day of disease onset **(C)**, and the cumulative clinical score **(D)** are depicted. N = 11-13. Statistical analysis was performed by One-way ANOVA followed by a Newman-Keuls Multiple Comparison test. Levels of significance: *p < 0.05; ***p < 0.001.

concentration compared to mice receiving control T cells (**Figure 6B**). This negative effect was also reflected by a higher incidence of EAE [$10^{-6}$ M Dex: 91% (10/11), $10^{-7}$ M Dex: 85% (11/13), control: 75% (9/12)], an earlier onset of the disease (**Figure 6C**), and an increase of the cumulative disease score (**Figure 6D**). In summary, our findings suggest that protection of antigen-primed effector T cells from GC-induced apoptosis is relevant for the pathogenesis of T cell-dependent inflammatory diseases in mice and presumably MS in humans as well.

## DISCUSSION

Induction of T-cell apoptosis was one of the first modes of action identified for GCs and is still believed to be highly relevant for their clinical effectiveness in the treatment of various diseases. It was soon noticed that profound differences exist between individual developmental stages, cellular subsets and differentiation states

with regard to apoptosis sensitivity. DP thymocytes are presumably the most sensitive type of immune cell in this respect (16, 17), but differences were also found for mature T-cell subpopulations such as Th1, Th2 and Th17 cells (21, 32). Furthermore, it was observed that T-cell activation conferred protection against GC-induced apoptosis, which was linked to the cell cycle entry into the G1/G2/M phase (33, 34). Along these lines, T cells from burn-injured mice were shown to have a reduced sensitivity to GC-induced apoptosis, which correlated with their enhanced activation status (23). In contrast, very little is known concerning GC-induced apoptosis of antigen-primed effector T cells in the pathological setting of inflammatory diseases. It is noteworthy that a stimulation with anti-CD3/CD28 antibodies, PMA/ionomycin or ConA, as done in most published studies, mimics T cell activation only partially. In natural conditions, activation of T cells is achieved by interaction of an MHC:peptide complex on antigen-presenting cells with the TCR, and engagement of CD28 by B7 molecules in the context of an immune microenvironment composed of other leukocytes, cytokines and growth factors. Since these diverse influences not only impact the fate of effector T cells but presumably determine their sensitivity to GC-induced apoptosis, too, the latter process should be preferably investigated in more sophisticated experimental setups.

To uncover whether antigen-primed effector T cells show a different sensitivity towards GC-induced apoptosis compared to naïve T cells, we developed a co-culture system in which both cell types could be distinguished based on their expression of a red and green fluorescent protein or the expression of the congenic markers CD45.1 and 2, respectively. T cell priming *in vivo* was achieved by immunization of 2D2-transgenic mice expressing a TCR specific for the pathologically relevant myelin antigen $MOG_{35-55}$ followed by restimulation in cell culture. With this setup we ensured that T cell activation occurred in a microenvironment that mimics the situation encountered during an autoimmune response. Importantly, only antigen-specific T cells but neither B cells isolated from the same immunized mice nor naïve T cells were protected from GC-induced cell death. This effect could be assigned to a reduced induction of apoptosis based on the staining of T cell subtypes with AnnexinV and their differential loss of the mitochondrial membrane potential. While these results collectively confirmed that antigen-priming alters apoptosis induction by GCs, this effect was not only clear *in vitro* but also observed *in vivo*. In fact, we could demonstrate that the protective effect of antigen-priming with regard to GC-induced apoptosis was dose-dependent in corresponding concentration ranges *in vitro* ($10^{-8}$ and $10^{-7}$ M Dex) and *in vivo* (5 and 20 mg/kg Dex). This observation indicates that the mechanism in cell culture and living mice is very similar.

Several mechanisms are being discussed as possible explanations for the diminished sensitivity of activated T cells to GC-induced apoptosis *in vitro*, including a critical role of cytokines as part of the immune microenvironment. This concerns IL-2, IL-4, IL-10 and IL-12 acting on individual T-cell subsets (24, 35), and downstream pathways involving IκBα (36) and PI3K/AKT (37). Importantly, cytokine signaling has been shown to upregulate the anti-apoptotic proteins Bcl-2 and Bcl-$X_L$ in activated T cells, which seems to be an important mechanism of their reduced sensitivity to apoptosis induction (38). Intriguingly, Bcl-2 in T cells was found to be only induced after stimulation of total splenocyte preparations with ConA but not after activation of purified T cells with PMA/ionomycin. In contrast, Bcl-$X_L$ was induced under both conditions (39). These findings support the notion that the immune microenvironment dictates if anti-apoptotic proteins become elevated during T cell activation or not. In our study, we could show that both proteins were expressed at higher levels in antigen-primed effector T cells compared to naïve T cells, regardless of GC treatment, a finding which reflects the natural context of an autoimmune response *in vivo*. Noteworthy, the previous observation that T cells obtained from Bcl-2-transgenic mice are resistant to GC-induced apoptosis is also in line with our current finding that an increased expression of this anti-apoptotic protein in antigen-primed effector T cells is linked to a protection against cell death by GCs (8). Hence, we consider it fair to conclude that T cells, which were naturally activated in the context of an immune response, become resistant against GC-induced apoptosis through induction of Bcl-2 and Bcl-$X_L$.

The biological consequences of the observed GC-resistance of antigen-primed T cells have not been studied in physiologically relevant mouse models up to now. Adoptive transfer EAE mimics many hallmarks of human MS and hence makes it possible to test the *in vivo* relevance of the altered balance between naïve and antigen-primed T cells after Dex treatment *in vitro* (30). Importantly, the MOG-specific T cells analyzed in this study are able to induce clinical symptoms after transfer into C57BL/6 mice and therefore resemble pathogenic T cells found in MS patients. Our results unveiled that the enrichment of antigen-primed effector T cells after incubation with Dex enhanced disease development after adoptive transfer into mice. These data further indicate that our model is very sensitive to the input of antigen-primed effector T cells and that already a slight increase in their relative number suffices to induce a statistically significant worsening of the disease course.

In summary, our study extents previous findings concerning GC-induced apoptosis of activated T cells to the analysis of pathologically relevant antigen-primed effector T cells in a setting which is relevant for neuroinflammatory diseases. The data have several practical implications. Antigen-primed effector T cells can be enriched in cell culture with the help of GCs, which can be exploited for the analysis of autoimmune diseases but also in approaches to treat cancer where pure preparations of such cells are required. Furthermore, the observation that preventive treatment of EAE with methylprednisolone paradoxically worsened the disease outcome can now be explained by an unintended enrichment of effector T cells prior to disease induction (40). Although GCs undoubtedly remain a powerful tool to interfere with inflammatory conditions including MS, our results highlight the importance of critically considering whether their application might result in an enrichment of effector T cells during an ongoing immune response and therefore might even prove harmful.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The animal study was reviewed and approved by Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit.

## AUTHOR CONTRIBUTIONS

JB: performed and analyzed experiments. SS: performed and analyzed experiments. HR: designed the project, analyzed experiments, and wrote the manuscript. FL: designed the project, analyzed experiments, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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# Regulation of the Immune System Development by Glucocorticoids and Sex Hormones

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Through the release of hormones, the neuro-endocrine system regulates the immune system function promoting adaptation of the organism to the external environment and to intrinsic physiological changes. Glucocorticoids (GCs) and sex hormones not only regulate immune responses, but also control the hematopoietic stem cell (HSC) differentiation and subsequent maturation of immune cell subsets. During the development of an organism, this regulation has long-term consequences. Indeed, the effects of GC exposure during the perinatal period become evident in the adulthood. Analogously, in the context of HSC transplantation (HSCT), the immune system development starts *de novo* from the donor HSCs. In this review, we summarize the effects of GCs and sex hormones on the regulation of HSC, as well as of adaptive and innate immune cells. Moreover, we discuss the short and long-term implications on hematopoiesis of sex steroid ablation and synthetic GC administration upon HSCT.

**Keywords: glucocorticoids, sex hormones, hematopoietic stem cell transplantation, hematopoietic stem and progenitor cell, immune system development**

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## INTRODUCTION

Glucocorticoids (GCs) and the sex hormones estrogens, progesterone and androgens are produced under the control of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axis, respectively, through a common steroidogenic pathway from cholesterol (1) (**Figure 1**). GC synthesis occurs in the cortical part of the adrenal gland for both males and females. Androgens are produced in male gonads and, in smaller amounts, in the ovary and adrenal cortex in females. Estrogens are mainly produced from androgens precursors in ovarian granulosa cells and placenta in females, testis in males and non-glandular tissue (fat and bone) in both sexes (2). Progesterone is produced by ovarian granulosa cells, adrenal glands, corpus luteum during the menstrual cycle and placenta (**Figure 1**) in females and by adrenal glands in both males and females (3).

GCs are released into circulation in response to the circadian cycle, are essential regulators of body functions in homeostasis and allow adaptation to the environmental changes. Moreover, due to their potent immune-suppressive and anti-inflammatory effects, synthetic GCs are widely used in clinics to treat acute and chronic inflammation (4). On the other hand, besides regulating reproductive and metabolic body functions throughout the lifespan, sex hormones are also able to regulate immune response and inflammation. Indeed, a key example is given by the

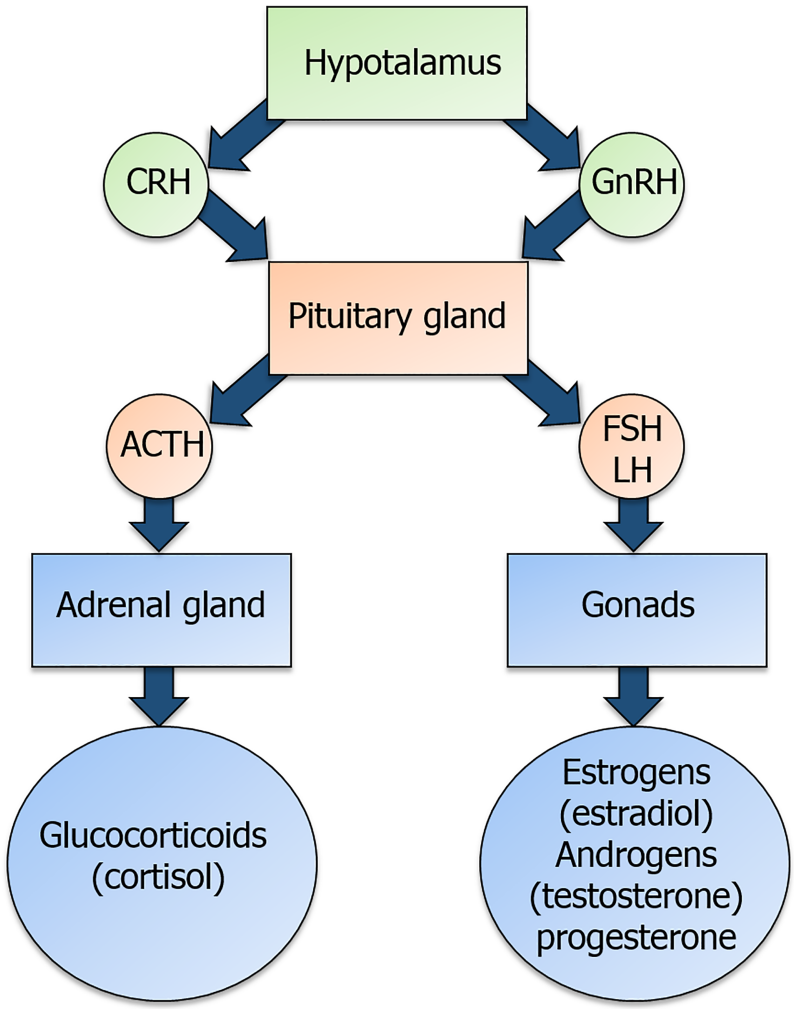


**FIGURE 1** | Overview of the hypothalamic-pituitary-adrenal (HPA) axis and of the hypothalamic-pituitary-gonadal (HPG) axis. Corticotropin-releasing hormone (CRH) and gonadotropin-releasing hormone (GnRH) are released by the hypothalamus and act on their receptors in the pituitary gland. In detail, CRH binding to its receptor triggers the production of the adrenocorticotropic-hormone (ACTH), while GnRH binding to its receptor triggers the production of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). ACTH and gonadotropins are released in the bloodstream and act on the adrenal gland and the gonads, respectively, to induce the secretion of glucocorticoids and sex hormones.

predominance of autoimmune diseases in females (5) and by sex dimorphism in antitumor immunity and response to infections (6, 7). In line with this, it was shown that estrogen-dependent protection of women from hepatitis C virus and liver cancer incidence are lost after menopause (8, 9). Age- and sex-related changes in immune response have therefore clinical consequences and should be taken into account because they may affect the efficacy of vaccination and cancer immunotherapy (10).

Because of their lipophilic nature, steroid hormones can cross cell membranes and bind to nuclear receptors belonging to the same superfamily of the ligand-regulated transcription factors (TFs) (11). In particular, they are the GC receptor (GR) and the sex hormone receptors estrogen receptor (ER) ERα and ERβ, progesterone receptor (PR), and androgen receptor (AR). These receptors are characterized by polymorphisms and multiple isoforms, responsible for variations in individual response at both molecular and clinical level. Steroid hormone receptors possess a N-terminal transactivation domain, a conserved central DNA binding domain and a C-terminal ligand-binding domain. In the cytoplasm, they are bound to chaperone proteins (e.g., HSP90). The binding of the ligand frees the receptor from the chaperone, allowing homodimerization, exposure of the nuclear localization sequence, and entry into the nucleus. Once in the nucleus, the ligand–receptor complex can directly bind to DNA sequences termed hormone response elements, and can associate with transcriptional coactivators to facilitate the regulation of target genes. It has been shown that steroid hormones can also mediate rapid "non-genomic" effects by acting directly on cytoplasmatic signal transduction pathways (12, 13). In addition, steroid hormones can alter gene expression through

their effects on epigenetic modifications (14–16). For example, GR represses IL-5 transcription by recruiting and interacting with the Histone Deacetylase 1 (HDAC1) (17), and can inhibit p65-mediated transcription by recruiting HDAC2 (18).

Thus, the steroid hormone receptor-driven transcriptional regulation in a given cellular context may be mediated by at least two non-mutually exclusive mechanisms: chromatin remodeling and partnering with a specific repertoire of TFs (19).

Immune cells express receptors for GCs and sex hormones and are therefore regulated by these steroids. However, the response depends on many factors, including the developmental stage, the activation state and the inflammatory signals the cell receives at the same time. In this review, we will summarize the effects of GCs and sex hormones on the development of the immune system and how this regulation affects the immune response in the long term (**Table 1**).

## GCs AND SEX HORMONES REGULATION OF HSC DEVELOPMENT

Hematopoietic stem cells (HSCs) are multipotent self-renewing units mediating the generation of all blood constituents. An important site for the development of HSC in the embryo is the dorsal aorta, which contains a transient population of blood-producing endothelial cells called hemogenic endothelium (40). Studies on the role of GCs on the early development of immune system in the embryo were done in a zebrafish experimental model (41). It was shown that, upon stress, the central nervous system releases serotonin that, in turn, activates the HPA axis. GC release controls HSC specification promoting hematopoietic stem and progenitor cell (HSPC) formation in the dorsal aorta of embryos (41). These findings suggest that any stress that may be experienced by the embryo during development, such as hypoxia, temperature changes, metabolic or oxidative stress, would promote blood cell formation through activation of the HPA axis.

After birth, steroid hormones control the formation of immune cells by acting on HSCs in the bone marrow (BM). Studies have been performed in mouse models and on *ex vivo* cultures of human $CD34^+$ HSCs to investigate this matter, especially with the purpose of developing therapeutic strategies to treat hematological diseases (42). For instance, it was suggested that GC treatment may be efficient to treat erythropoietin-resistant anemias because GR signaling regulates erythropoiesis. In particular, it was shown that GR synergizes with peroxisome proliferator-activated receptor α (PPAR-α) to induce self-renewal of erythroid progenitor cells *in vivo* and increase erythroid cell expansion *ex vivo* from human $CD34^+$ HSCs (20).

HSC transplantation (HSCT) represents the common therapeutic approach to treat malignant and non-malignant hematological disorders. G-CSF-mediated mobilization of HSC has emerged as the most suitable means to obtain HSCs for transplantation, complemented by plerixafor (a selective CXCR4 antagonist) treatment in "poor-mobilizers" (43, 44). In mice, a neuroendocrine pathway that promotes HSC migration from the BM to the peripheral blood has been identified. It was shown that cholinergic signals through the muscarinic receptor type-1 (Chrm1) in the brain trigger HPA axis activation and GC production. GR signaling in HSC induces an up-regulation of actin-organizing molecules, thus promoting cell migration (45). HSC homing to the BM upon intravenous injection is mediated by coordinated actions of adhesion receptors and the chemokine CXCL12 expressed in the

**TABLE 1** | Summary of the main effects of GCs and sex hormones on HSC, adaptive and innate immune cells.

| | Hormone or hormone receptor | Target cell | Effect | Reference |
|---|---|---|---|---|
| Hematopoietic stem cells | GR | erythroid progenitor | increased self-renewal | (20) |
| | GR | HSC | upregulation of CXCR4 expression and homing to the BM | (21) |
| | LH | HSC | regulation of cell expansion | (22) |
| | ERα | HSC | increased self-renewal | (23) |
| | ERα | HSC | increased telomerase activity | (24) |
| Adaptive immune cells | GC | thymocytes | apoptosis | (25) |
| | GR | CD8+ T cell | generation of memory precursor cells | (26) |
| | DT | thymocytes | TNFα-mediated apoptosis | (27) |
| | ERα | thymocytes | thymic atrophy | (28) |
| | AR | cTEC | inhibition of Dll4 expression | (29) |
| | AR | mTEC | upregulation of Aire expression | (30) |
| | ERα | mTEC | downregulation of Aire expression | (31) |
| | ERα | lymphoid precursors | decrease of B lymphopoiesis | (32) |
| | E2 | lymphoid precursors | cell depletion in the BM | (33) |
| | AR | BM stromal cell | defect in B lymphopoiesis | (34) |
| Innate immune cells | GC | HSC | induction of NK cell differentiation from myeloid precursors | (35) |
| | GC | monocytes | block of differentiation towards DCs | (36) |
| | GC | DCs | impairment of DCs terminal maturation | (37) |
| | ERα | myeloid precursors | inhibition of Flt3-induced conventional and plasmacytoid DC development | (38) |
| | ERα | myeloid precursors | increased GM-CSF-induced DC differentiation | (39) |

*GR, glucocorticoid receptor; HSC, hematopoietic stem cell; BM, bone marrow; LH, luteinizing hormone; ER, estrogen receptor; GC, glucocorticoid; DT, depo-testosterone; TNF, tumor necrosis factor; AR, androgen receptor; cTEC, cortical thymic epithelial cells; mTEC, medullary thymic epithelial cells; Dll4, Delta-like 4; E2, estradiol; NK, natural killer; DC, dendritic cell; Flt3, Fms related tyrosine kinase 3; GM-CSF, Granulocyte macrophage-colony stimulating factor.*

BM microenvironment (46). In a screen of small molecule compounds, GCs were identified as activators of CXCR4 expression in human $CD34^+$ HSC isolated from cord blood (CB). It was shown that short term GC pretreatment is able to induce GR binding to a GC-response element in the CXCR4 promoter and SRC-1-p300 complex recruitment to induce H4K5 and H4K16 histone acetylation, favoring gene transcription. GC-induced CXCR4 up-regulation mediated HSC chemotaxis in response to CXCL12 and homing in the BM. Notably, although GC pretreatment was short, long-term engraftment was observed upon HSC transplantation into primary- and secondary-recipient NSG mice (21).

The regulation of HSC homeostasis by the sex hormones takes place after birth and affects their number and self-renewal capacity at different developmental stages. Luteinizing hormone (LH) is secreted by the pituitary gland at the onset of puberty, promoting the maturation of the reproductive system in both males and females. In post-natal BM, HSCs are a direct target of LH, whose signaling acts like a brake of cell overexpansion, ensuring a proper HSC count for normal hematopoiesis in adulthood (22). HSCs from mice treated with the estrogen estradiol (E2) have increased regenerative capacity after transplantation or irradiation, thus explaining why female immunodeficient recipient mice support reconstitution of the blood system by transplanted human HSCs more efficiently than the male counterpart (47). It was suggested that E2-ERα signaling in HSCs induces the expression of Ern1, which encodes Ire1a, thus activating the Ire1a-Xbp1 pathway of the unfolded protein response and promoting HSC resistance against proteotoxic stress (48). It was shown that Tamoxifen (a selective ER modulator) by signaling through ERα has a minor impact on primitive normal HSCs, but induces apoptosis in malignant HSCs enhancing chemotherapy response in a mouse model of acute myeloid leukemia (49). During pregnancy, ERα signaling in HSCs increases cell division, frequency, and erythropoiesis in the spleen of female mice (23). In particular, E2 promotes increased HSC self-renewal in the bone marrow, while another endogenous ligand for ERα (27-Hydroxycholesterol-27HC) promotes HSC mobilization to the spleen (50). Thus, these two ERα agonists collaborate to favor extramedullary hematopoiesis, required during pregnancy to maintain red blood cell counts despite a rapidly increasing blood volume.

Impaired DNA repair, altered DNA methylation patterns, aberrant metabolism and skewed upregulation of myeloid- (at the expense of lymphoid-) associated genes contribute to altering HSC functions with age (51). Both intrinsic functional changes in the earliest HSCs and extrinsic alterations of the HSC niche contribute to this degeneration (52). Evidence suggests that sex steroids play at least some roles in age-related decline of the immune functions (53) which, indeed, becomes more evident from the onset of puberty, when the levels of these hormones increase (54, 55). A comparison of healthy men and women's PBMCs revealed a shared epigenomic signature of aging, including declining naïve T cell and increasing monocyte and cytotoxic cell functions. Age-related epigenomic changes first spike around late-thirties with similar timing and magnitude between sexes, whereas the second spike occurs earlier and stronger in men (56). Consistent with a role for sex steroids in the decline of the immune functions, sex steroid ablation (SSA) today represents an attractive therapeutic approach to restore immune competence in immunodeficient individuals (57). It has been shown that SSA has the potential to accelerate the immune recovery in many clinical conditions such as upon autologous and allogeneic HSCT, or after damaging cytoablative treatments like chemotherapy. SSA consists in therapeutically targeting the upstream signaling events such as LH releasing hormone (LHRH) or directly blocking sex steroid receptors (58, 59). This has been achieved clinically for over 35 years because, in addition to its well established role in improving thymopoiesis (see following section), several studies have found that SSA contrasts the age-related decline of early lymphoid progenitors in the BM. SSA does not enhance HSC homing to the BM, but increases their self-renewal and lymphoid differentiation capacities (60). These effects are due to intrinsic changes in HSCs, as well as to an extrinsic effect on the stromal microenvironment. In particular, SSA induces the upregulation of genes implicated in the protection from aging such as Foxo1 in the hematopoietic niche thus increasing its ability to support hematopoiesis (60). Interestingly, while SSA through direct and indirect mechanisms contrasts aging of early lymphoid progenitors, androgens have been shown to increase telomerase activity in primary HSCs (24). For its therapeutic effect on erythropoiesis, the androgen derivative Danazol is now used in the treatment of acquired severe aplastic anemia (61).

## GCs AND SEX HORMONES CONTROL THE DEVELOPMENT OF THE ADAPTIVE IMMUNE SYSTEM

The thymus is a critical site for generating a diverse T cell repertoire while maintaining self-tolerance, and both GCs and sex hormones play a critical role in shaping thymic functions. In particular, GCs are crucial in the selection of the appropriate T cell receptor (TCR) self-affinities repertoire in the thymus (62). By dampening the strength of TCR signals, GCs prevent negative selection of T cells that have high affinity for self MHC (63). On the other hand, double positive thymocytes are the most sensitive to GC-induced apoptosis, and indeed stress response inducing GC production (including psychological stress, fasting, injury and infection) causes an acute reduction in thymus size (25, 64, 65). GCs also regulate the subsequent thymus-independent steps of T cell development. A characterization of the epigenetic landscapes of naïve, terminal-effector, memory precursor and memory $CD8^+$ T cells revealed that the expression and binding of specific TFs contribute to the establishment of subset-specific enhancers during differentiation, which control the *in vivo* response to bacterial infections (26). One of these key TF is Nr3c1, encoding for GR, constitutively expressed during $CD8^+$ T cell differentiation and involved in the generation of memory precursor cells (26). GR has an important role in T cell differentiation not only in physiological, but also in pathological conditions. Indeed, in tumor infiltrating lymphocytes, an increasing gradient of GR

expression and signaling from naïve to dysfunctional $CD8^+$ T cells was found, suggesting that endogenous GCs also promote T cell dysfunction and tumor growth (66).

Testosterone can directly induce apoptosis of double positive thymocytes through the upregulation of TNF-α, while estrogens can induce thymic atrophy by eliminating early thymic progenitors and inhibiting the proliferation of β-selected thymocytes (27, 28). They have also an indirect effect on thymopoiesis since the expression of sex steroid receptor is significantly higher on thymic epithelia cells than on thymocytes. Indeed, sex steroids inhibit in cortical thymic epithelial cells (cTECs) the expression of Delta-like 4 (Dll4), a Notch ligand crucial for the commitment and differentiation of T cell progenitors in a dose-dependent manner (29). It was demonstrated that androgen-response elements are present in the promoter of the Dll4 gene and androgen/AR complexes were localized to the Dll4 promoter by chromatin immunoprecipitation (29). Sex hormones also control the expression of Aire (Autoimmune Regulator), the transcription factor responsible for the expression of thousands of tissue-restricted proteins in cells deputed to the presentation of self-antigens to the maturing T lymphocytes. By upregulating Aire expression in medullary TEC (mTEC) by directly binding its promoter, androgens enforce self-tolerance and lead to a more efficient negative selection of self-reactive T cells (30). At the same time, estrogens can decrease Aire expression by epigenetically regulating its methylation (31). Indeed, Aire expression in mice and human thymus is higher in males compared to females, suggesting that increased levels of androgens and decreased levels of estrogens protect males from autoimmunity by promoting Aire expression. All of these molecular mechanisms identified for sex hormones explain why SSA increases thymic cellularity, restores thymic architecture and organization, and enhances thymopoiesis in young and adult animals (58).

Age-related immune system decline is not only restricted to thymic atrophy, but also to an intrinsic defect in the HSC ability to commit to the lymphoid lineage. Therefore, with age there is a significant decline in B lymphopoiesis and humoral immunity, accompanied by a reduced peripheral immune repertoire, leading to increased opportunistic infections and limited recovery following cytoablation after chemo or radiotherapy (67). Estrogens reduce responsiveness to IL-7 causing decreased B lymphopoiesis (32) and deplete lymphoid committed precursors in the BM (33). Androgens affect B lymphopoiesis indirectly, by acting on BM stromal cells (34). Following surgical SSA in mice displaying age- or chemotherapy-induced immunodepletion, a rapid and sustained increase in developing B cells and their upstream lymphoid progenitors was observed. SSA not only increased B cell number, but also enhanced humoral response to challenge by hepatitis B vaccine (68).

## GCs AND SEX HORMONES REGULATION OF INNATE IMMUNE CELLS

Innate lymphoid cells (ILCs) differentiate from HSC in the BM, and common ILC precursors continue their development in secondary lymphoid tissues (69). GC treatment of human CB $CD34^+$ HSCs accelerated Natural Killer (NK) cell differentiation, promoted a switch of myeloid precursors towards immature NK cells and induced NK cell cytolytic activity (35). Although a role for the neuroendocrine system in the regulation of helper ILC functions has been shown (70), its contribution to their development has not been investigated so far.

In mice, GC exposure favors myelopoiesis in the bone marrow, suggesting that preservation of granulocytes and their progenitors may be a mechanism to ensure rapid protection of the organism upon stress (71). Neutrophil development and function are modulated also by sex hormones. In particular, healthy young adult women display an activated/mature neutrophil profile characterized by enhanced type I IFN pathway activity, enhanced proinflammatory responses, and distinct immunometabolism compared to young men (72).

Blood monocyte precursors originate in the BM, enter the circulation and are present in the blood until they migrate into tissues where they can differentiate into macrophages and dendritic cells (DC) (73). GCs are long known to act on monocytes influencing their short time mediator release, but there is growing evidence that they are also involved in differentiation processes skewing towards an anti-inflammatory phenotype (74). GCs block the generation of immature DCs from monocytes (36) and impair terminal maturation of already differentiated DCs (37). Similarly, ER signaling inhibits Flt3-induced conventional and plasmacytoid DC development from myeloid precursors (38). On the contrary, it was shown that GM-CSF-induced DC differentiation as Langerhans cells from myeloid progenitors was promoted by adding E2 *in vitro* and inhibited by ER antagonists and ERα-deficiency (39). Indeed, ERα signaling during this process targets IRF4, a TF critical for Langerhans cell development (75).

Interestingly, mast cell cytokine production seems to be sex dependent. Indeed, in females they mainly produce proinflammatory cytokines, such as TNF and IL-1β, while their activation in males results in a predominant production of IL-33 (76). Although both male and female derived mast cells express AR, testosterone induces IL-33 production only in male-derived cells *in vitro* (77). These data suggest that, in addition to a direct effect, testosterone may have other effects during development that poise mast cells for IL-33 expression potential, perhaps at an epigenetic level. Therefore, the ability to express a particular array of cytokines may be programmed by prolonged exposure to sex hormones starting early in life and is not solely due to acute sex hormone-receptor interactions.

## CONCLUSIONS AND FUTURE PERSPECTIVES: A ROLE FOR GCs AND SEX HORMONES IN THE DEVELOPMENTAL PROGRAMMING OF IMMUNE SYSTEM?

Early life exposure to environmental cues, particularly during the perinatal period, can have a life-long impact on the organism

development and physiology. The biological rationale for this phenomenon is to promote physiologic adaptation to the anticipated environment based on early life experience. A role in this "developmental programming" for early life stress or prenatal treatment with synthetic GCs during sensitive windows of development has been established (78). Excessive or premature exposure to GCs has been associated to long term effects on tissues and organs (hypertension, hyperglycemia, anxiety), as well as to higher risks for atopic diseases, asthma, autoimmune type I diabetes, infectious diseases and decreased adaptive and anti-tumor immune response (79–83). GC-induced programming of the immune system is mediated by effects on HSCs or other persistent progenitors, which endure through the individual's lifespan (84, 85), rather than upon the short-lived fully differentiated immune cell populations. The long-term consequences of perinatal GC exposure were recently evaluated in mice in a study by putting the synthetic GC dexamethasone in the drinking water from mid-pregnancy to early post-natal period, when hematological and lymphoid organogenesis takes place (86). As a result, the authors observed a diminished $CD8^+$ T cell response in adulthood and impaired control of tumor growth and bacterial infections. Perinatal GC exposure led to HPA axis reprogramming with alterations in its threshold, decreased systemic levels of GCs and persistent changes in the chromatin state of naïve T cells (86). The gene expression program was "imprinted", as the changes were maintained also upon cell adoptive transfer to new environments and, importantly, the stem cell compartment contributed to compromised T cell responses, as the defect could be observed also in irradiated mice transplanted with the BM of GC-treated mice (86).

These findings suggest that GCs, as well as the other hormones whose receptors function as TFs, may be able to induce an "imprinting" in HSC gene expression program, most likely at the epigenetic level, thus stably affecting HSC developmental trajectories. This kind of regulation that occurs during organism development may be recapitulated upon HSCT when the immune system development from donor-derived HSCs starts *de novo*. Since SSA and GCs are widely used to improve HSCT outcome and prevent complications such as graft *vs* host disease, respectively, it would be interesting to gain further insights into the short and long term consequences of these treatments on hematopoiesis. A better understanding of HSC regulation by GCs and sex hormones is extremely important to predict their effects on the immune system development and design therapeutic approaches to improve the long-term reconstitution in HSCT recipients.

## AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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# 20S-Hydroxyvitamin D3, a Secosteroid Produced in Humans, Is Anti-Inflammatory and Inhibits Murine Autoimmune Arthritis

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The ability to use large doses of vitamin D3 (D3) to chronically treat autoimmune diseases such as rheumatoid arthritis (RA) is prohibitive due to its calcemic effect which can damage vital organs. Cytochrome P450scc (CYP11A1) is able to convert D3 into the noncalcemic analog 20*S*-hydroxyvitamin D3 [20*S*(OH)D3]. We demonstrate that 20*S*(OH)D3 markedly suppresses clinical signs of arthritis and joint damage in a mouse model of RA. Furthermore, treatment with 20*S*(OH)D3 reduces lymphocyte subsets such as $CD4^+$ T cells and $CD19^+$ B cells leading to a significant reduction in inflammatory cytokines. The ratio of T reg cells (CD4+CD25+Foxp3+ T cells) to CD3+CD4+ T cells is increased while there is a decrease in critical complement-fixing anti-CII antibodies. Since pro-inflammatory cytokines and antibodies against type II collagen ordinarily lead to destruction of cartilage and bone, their decline explains why arthritis is attenuated by 20(OH) D3. These results provide a basis for further consideration of 20*S*(OH)D3 as a potential treatment for RA and other autoimmune disorders.

**Keywords: vitamin D, arthritis, RA, 20S(OH)$D_3$, mouse, collagen**

## INTRODUCTION

Rheumatoid arthritis (RA) is one of the more common autoimmune diseases (affecting approximately 0.5-1% of the world's population (1, 2). Although, multiple diathroidial joints are the main targets of autoimmune attack, other organ systems are also involved, reducing life expectancy by about 10 years in patients with RA (3, 4). Standard disease modifying anti-rheumatic drugs, (DMARDs), such as methotrexate (MTX) and "biologics" that target specific cytokines (e.g., TNFα) or surface molecules on immune cells (e.g., CTLA-4 on T cells), do reduce both joint damage and certain systemic complications of RA. However, their use occasionally triggers adverse events such as increased infections and development of certain neoplasms, other autoimmune diseases, or interstitial lung disease (5–8). Clearly, safer and effective therapeutic agents for RA are needed.

In humans, vitamin D3 (D3 aka cholecalciferol) is synthesized in the skin *via* ultraviolet B (UVB) induced photoconversion of 7-dehydrocholestrol (7-DHC), a precursor to cholesterol (9, 10). D3, which is inert as a pro-hormone, undergoes two hydroxylation steps: first in the liver (or skin itself)

by a 25-hydroxylase (CYP2R1 or CYP27A1) to form 25(OH)D3, and then in the kidney and other tissues by 25-hydroxyvitamin D-1 alpha hydroxylase (CYP27B1) to form 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D3$) (11–15). $1,25(OH)_2D3$ exerts positive or negative influences on D3-dependent gene expression by binding to the ligand binding domain of the vitamin D receptor (VDR) (16). A heterodimer is then formed within the retinoic acid X receptor (RXR), which, (with the contributions of basal transcriptional machinery plus coactivators and corepressors) forms transcription complexes at vitamin D response elements (VDRE) of target genes (17).

$1,25(OH)_2D3$ is the most extensively characterized active naturally occurring D3 metabolite that, not only systematically regulates calcium homeostasis and bone metabolism, but also possesses immunomodulatory properties. Clinically, normal D3 level is associated with better outcomes in patients with a variety of autoimmune diseases (18–21). In RA, disease activity, C reactive protein and disability scores are inversely related to serum levels of 25(OH)D, and anticyclic citrullinated peptide antibody positivity in RA patients is correlated with D3 insufficiency [25(OH)D, 21-29 ng/ml] and deficiency [25(OH)D <20 ng/ml] (18, 22–25). Furthermore, the VDR Fok1 polymorphism may confer susceptibility to RA in Europeans and Native Americans (26, 27). These observations suggest D3 may have salutary effects in RA. Earlier studies demonstrated $1,25(OH)_2D3$ inhibited arthritis in the type II collagen (CII)-induced arthritis (CIA) model of RA in mice fed a low calcium diet to protect against development of hypercalcemia (28). Unfortunately, $1,25(OH)_2D3$ or its precursors, 25(OH)D3 or D3 (cholecalciferol), induce hypercalcemic toxicity when given chronically at the pharmacological doses needed to maximally suppress arthritis and autoimmunity, limiting the amounts that can be given chronically to patients to treat autoimmune diseases such as RA.

We have discovered a novel pathway of D3 metabolism operative in humans, mediated by cytochrome P450scc (CYP11A1), which is modified by CYP27B1, that generates additional biologically active products (29–31). These are at least as potent as classical $1,25(OH)_2D3$ when tested *in vitro* and *in vivo* in several model systems and, like $1,25(OH)_2D3$, bind to the VDR (32–37). The main and first product of the pathway, 20*S*(OH)D3, in contrast to 25(OH)D3 or $1,25(OH)_2D3$, is nontoxic (i.e., noncalcemic and nontoxic to the hematopoietic system, liver, kidney, and heart) at doses as high as 60 μg/kg, while 25(OH)D3 or $1,25(OH)_2D3$ induce hypercalcemia at doses ≤2 μg/kg (38–41). Finally, 20*S*(OH)D3 is produced *in vivo*, being present in human serum at $1/20^{th}$ the concentration of classical 25(OH)D3 (31) and has also been detected in honey (42). The cytochrome SCC enzymes are unusual in that they hydroxylate vitamin D3 to produce 20S-hydroxyvitamin D3 [20(OH)D3] rather than cleaving the side chains of D3 (43). These properties define 20*S*(OH)D3 as a hither to unrecognized novel endogenous regulator/natural product.

In the present study, CIA in DBA/1 mice (the most widely studied animal model of RA) was used to collect preclinical data on 20*S*(OH)D3 as a potential treatment for human RA. When immunized with bovine CII, DBA/1 mice rapidly develop an anti-CII Th1 and Th17 T cell (IFNγ, IL-2, GM-CSF, TNFα, and IL-17) and later a B cell response characterized by production of IgG1 and complement fixing IgG2a antibodies to CII that triggers inflammation (44–49). At about 3 weeks post immunization, arthritis (which histologically resembles RA) begins to develop in peripheral joints (44, 46, 50–52). As time passes arthritis develops in more joints and with greater degrees of inflammation and damage. We demonstrated that treatment of mice with CIA with 20*S*(OH)D3 reduces the severity of clinical arthritis accompanied by reduction in joint destruction, in serum anti-CII antibodies, in lymphoid organ $CD4^+$ T and $CD19^+$ B cells, and production by cultured draining lymph node cells of TH1, TH17, and inflammatory cytokines and chemokines.

# MATERIALS AND METHODS

## Production and Purification of 20(OH)D3

20*S*(OH)D3 was generated by enzymatic hydroxylation of D3 catalyzed by CYP11A1 as previously described by our group (29, 53). Briefly, 10 mM D3 in 45% 2-hydroxpropyl-β-cyclodextrin was prepared. Buffer comprising 20 mM HEPES (pH 7.4), 100 mM NaCl, 0.1mM dithiothreitol, 2 μM CYP11A1, 0.1 mM EDTA, 0.3 μM adrenodoxin reductase, 10 μM adrendoxin, 2 mM glucose 6-phosphate, 2U/ml glucose 6-phosphate dehydrogenase, and 50 μM NADPH was mixed with the D3 stock solution rendering a final D3 concentration of 200 μM and 0.9% concentration of 2-hydroxypropyl-β-cyclodexrtin. Following 8 min pre-incubation, the reaction was initiated by adding NADPH, after which the samples were incubated for 3 h at 37°C with gentle shaking. Ice cold dichloromethane (20 ml) was then added to stop the reactions after which the reaction products were extracted with dichloromethane as previously described (53–55). The 20(OH)D3 product was purified by preparative thin-layer chromatography, followed by reverse phase high performance liquid chromatography, as previously described (55). Routinely 0.3 mg of purified 20*S*(OH)D3 was recovered from 12.5 ml of the starting incubation mixture. Aliquots of the purified 20*S*(OH)D3 were dried under nitrogen and stored at -80°C until used.

### Mice

Female DBA/1 Lac J mice, age 6 weeks old (Jackson Laboratories, Bar Harbour, ME) were housed in a pathogen-free AAALAC-approved animal care facility at the University of Tennessee Health Science Center (UTHSC) and Department of Veterans Affairs Medical Center (VAMC), Memphis, TN. Mice were fed regular laboratory chow and water ad libitam, and housed under a 12 h light and 12 h darkness cycle. Animal protocols for the study were approved by the Institutional Animal Care and Use Committees at UTHSC and VAMC Memphis.

## Induction and Assessment of CIA

Mice were immunized with native bovine CII prepared (as previously described from fetal calf articular cartilage) (47). Groups of 12 mice (for assessment of arthritis development) or

groups of 5 or 6 mice (for other studies) were administered different amounts of 20*S*(OH)D3 dissolved in sterile sesame oil (S.O) or propylene glycol [(PG) (Sigma-Aldrich, St. Louis, MO)]. The S.O. or PG, with-or-without 20*S*(OH)D3, was administered daily intraperitoneally (i.p.) or *via* daily oral gavage in volumes of 50 µl and 100 µl, respectively. For studies using PG to solubilize 20*S*(OH)D3, the PG, with-or-without 20*S*(OH)D3, was diluted 1:5 by volume with sterile normal saline, and 100 µl was administered daily by oral gavage. Rheumatrex tablets (DAVA Pharmaceuticals, Inc., Fort Lee, NJ) were used as a source of methotrexate sodium. Methotrexate 2.5 mg/kg was administered in 100 µl normal saline weekly by oral gavage. The tablets were crushed and solubilized in sterile normal saline.

Arthritis severity was assessed in each paw every other day by two observers (one of whom was blinded to treatment) using the following scale: 0=no swelling or redness, 1=slight swelling and redness, 2=moderate swelling or redness, 3=marked swelling and redness, and 4=marked swelling and redness with some deformity (47). For histological assessment of joint tissue, mice were euthanized, all paws were removed, decalcified, processed and scored histologically, as previously described (56). Evaluation of each joint was done in a blinded manner using 4 parameters (0–3 scale for each parameter): synovial inflammation and thickness, synovial leukocyte invasion into the joint, cartilage unevenness caused by inflammation related cartilage damage, and subchondral bone erosion. The total histologic score represented the sum of the 4 parameters. The maximal histologic score per mouse paw was 12 and 48 per mouse.

## Quantitation of Anti-CII Antibodies in Sera

Specific murine IgG1, IgG2a, and Ig2b anti-CII antibodies were quantitated in sera using a commercial ELISA, according to the manufacturer's instructions (Chondrex, Redwood, WA).

## Flow Cytometric Assessment of Lymphoid Cells

Isolated cells from spleen or draining para aortic, popliteal or inguinal lymph nodes were labeled as follows: Alexa-700 labeled rat anti-mouse CD3, PE-Cy7-labeled rat anti-mouse CD25, Per CP-Cy5.5-labeled rat anti-mouse CD4, FoxP3 Tregs were detected by FoxP3 staining kit, and FITC-labeled rat anti-mouse CD-19 (BD Bioscience, San Diego, CA). Flow cytometry was performed on a SORP BD LSRII instrument and results analyzed by FlowJo.

## Cytokine Quantitation

Draining lymph node cells were isolated and cultured at $2x10^6$ cells/ml in RPMI 1640 medium containing 9% fetal calf serum, penicillin 100 u/ml, streptomycin 100 µg/ml, 1% glutamax, 1% pyruvate, and 1% 2-mercaptoethanol. Supernatants were collected after 48 h or 120 h of culture and analyzed by multiplex sandwich immunoassay (Bio-plex mouse cytokine/chemokine kits, Bio Rad) for levels of different cytokines using a Luminex instrument according to the manufacturer's protocol.

## Quantitation of Total Serum Calcium

Levels of calcium in mice serum were quantitated by atomic absorption spectroscopy as previously described (57).

## Statistical Analyses

Differences between groups were analyzed using 2 way RM ANOVA when multiple comparisons were made, Student's 2-sample *t*-test was used for single comparisons between groups with normally distributed data, or by Mann-Whitney rank sum test for data not normally distributed. The level of significance was set at $P < 0.05$.

# RESULTS

## 20S(OH)D3 Treatment Suppresses Development of CIA and Associated Joint Damage

To determine whether 20*S*(OH)D3 treatment started day 14 post CII immunization, after T cell priming to CII is firmly established, would suppress the development of CIA, two groups of mice (12 per group) were immunized with CII and from days 14-to-40 post immunization treated daily with i.p. injections of sterile 50 µl S.O. or 50 µl S.O. containing 20*S*(OH)D3 at a dose of 2.4 µg/kg/day. The 20*S*(OH)D3 treatment markedly reduced the mean arthritis severity score (**Figure 1A**) and arthritis incidence (**Figure 1B**). The curves comparing drug to vehicle are roughly parallel, suggesting that the greatest effect is on the magnitude of the immune response rather than elicitation of the response. The histologic scores reflecting inflammation, cartilage damage and subchondral bone erosion in joints harvested at sacrifice on Day 40 post CII immunization were significantly reduced in 20*S*(OH)D3 treated versus S.O. treated CIA mice (**Figure 1E**), reflecting the ability of 20(OH)D3 to protect joints from damage in this model.

Representative images of hematoxylin and eosin-stained sections of hind paws harvested on Day 40 post CII immunization show marked destruction of joint structures in S.O. treated CIA mice (**Figure 1D**), while joint structure was maintained in mice with CIA treated with *20S(OH)D3* (**Figure 1C**). Aliquots of sera were subjected to analysis of calcium content by atomic absorption spectroscopy. There was no difference in levels of serum calcium between 20*S*(OH)D3- and S.O.-treated mice with CIA (S.O. = 9.50 ± 0.50 mg/dL, 20*S*(OH)D3 = 9.57± 0.50 mg/dL, p = NS). This is compatible with our earlier report that 20*S*(OH)D3 does not induce hypercalcemia at doses up to 60 µg/kg when administered to C57BL/6 daily by i.p. injections for 21 days (39).

## 20S(OH)D3 Treatment Reduces Levels of Serum Antibodies to CII

Earlier studies demonstrated that the generation of complement fixing anti-CII antibodies is essential for development of CIA (58, 59). The major complement fixing anti-CII antibodies generated in DBA/1 mice immunized with CII are of the IgG2a subclass (59). However, the less potent complement fixing anti CII IgG1 and anti CII Ig G2b antibodies are also generated (59). Therefore, it was essential to assess whether 20*S*(OH)D3 versus S.O. vehicle treatment of mice immunized with CII developed less IgG2a and IgG1 anti-CII antibodies. On Day 40, sera from mice in **Figure 1A** were analyzed for anti-CII specific antibodies by a commercial ELISA kit from Chondrex specific for IgG1, IgG2a and Ig2b anti-CII antibodies. Significant

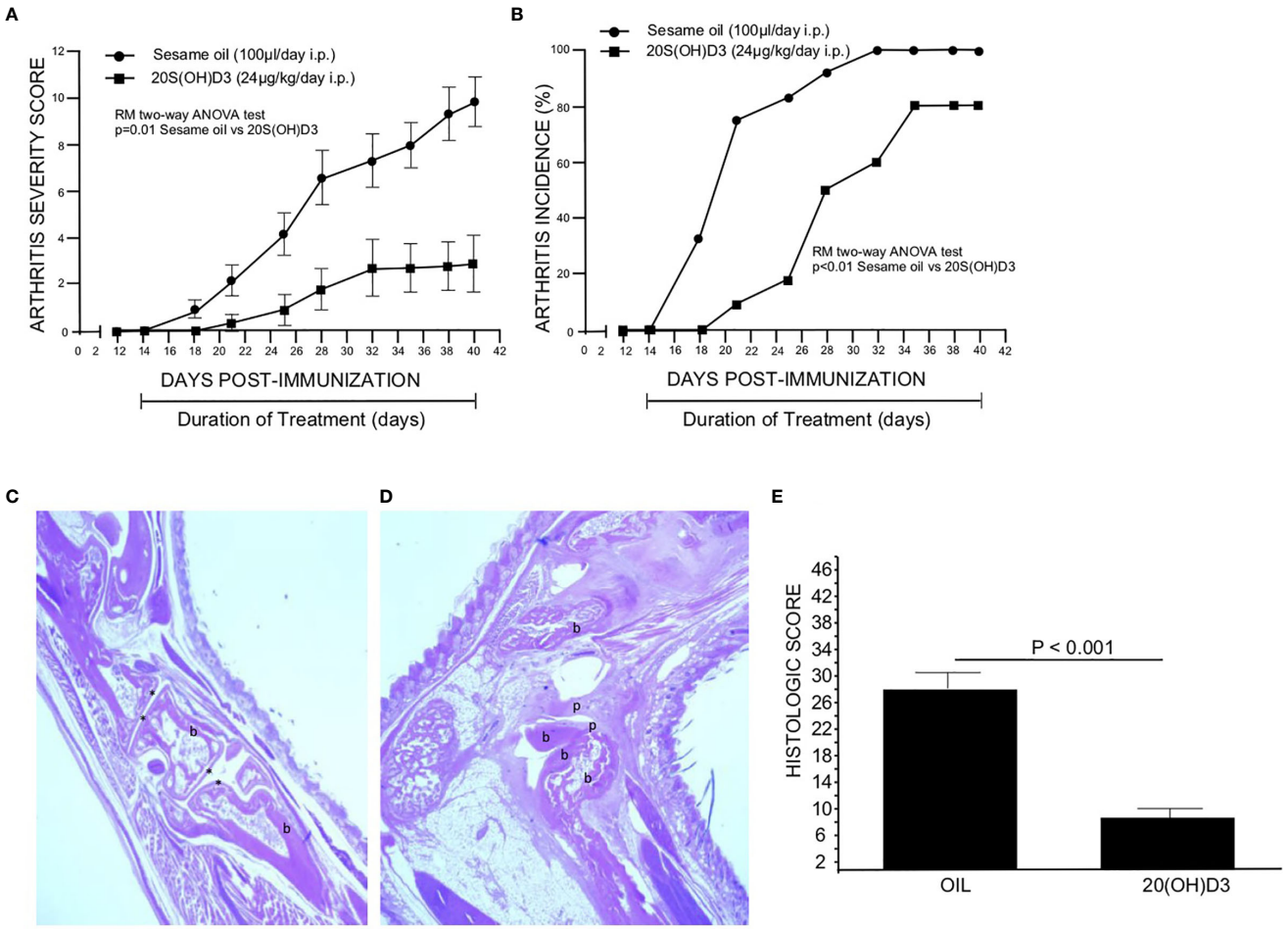


**FIGURE 1** | 20*S*(OH)D3 suppresses CIA: 24 DBA/1 Lac J female mice at 6 wks of age were immunized with bovine CII and 14 days later 12 mice were treated daily with intraperitoneal (i.p.) 50 µl S.O. and 12 mice with 50 µl S.O. containing 2.4 µg/kg 20*S*(OH)D3. Arthritis severity **(A)** and incidence **(B)** were assessed by a blinded observer and each paw was scored on a scale of 0 to 4 with 16 being the maximal score per mouse. All mice survived to day 40. On Day 40, mice were euthanized, sera obtained, and paws processed for histology and scored histologically as described in Methods. An H&E stained section of the decalcified hind paw of a mouse treated with 20*S*(OH)$D_3$ shows preservation of joint structures with minimal inflammation (10X magnification, b, bone, * identifies joint space) **(C)** while S.O.-treated mouse hind paws showed marked joint destruction with extensive loss of cartilage and bone with invasion of pannus (20X magnification, b, bone; p, pannus) **(D)**. 20*S*(OH)D3- treated CIA mice had a lower histologic score than S.O. treated CIA mice **(E)**. Photographs of typical paws for the scoring system we used are available online at the Hooke Laboratories Web Site (https://hookelabs.com/services/cro/cia/MouseCIAscoring.html).

reductions in serum levels of anti-CII antibodies of the IgG1 (**Figure 2A**) and IgG2a (**Figure 2B**) subclasses ($P$=0.017 and $P$=0.03), respectively occurred in 20*S*(OH)D3 treated mice compared to S.O. treated mice with CIA. However, IgG2b anti-CII antibody levels were not significantly different between 20*S* (OH)D3- and S.O.- treated cohort with CIA (data not shown). These reduced complement fixing anti-CII antibodies likely resulted in reduced arthritis severity observed in mice treated with CIA treated with 20*S*(OH)D3.

## 20S(OH)D3 Treatment Modulates Production of Cytokines and Chemokines by Cultured Draining LN Cells

Popliteal and inguinal lymph nodes contain immune cells that traffic to and from the joints in the hind limbs of mice with CIA (50, 60). Cytokine and chemokine production by draining LN cells from mice with CIA, in part, reflect production of cytokines and chemokines by immune cells in arthritic joints (61). To assess whether cytokine and/or chemokine production are changed by treatment of mice with CIA with 20*S*(OH)D3, groups of 9 mice immunized with CII were treated beginning on the day of CII immunization with i.p. administration of 2.4 µg/kg 20*S*(OH)D3 or S.O. daily for 14 days. The mice were then euthanized and isolated popliteal and inguinal LN cells were cultured for 48 h to allow cytokines and chemokines to be released into the culture medium. Levels of Th1, Th2, Th17, and inflammatory cytokines were significantly reduced in the supernatants of the draining LN from mice treated with 20*S*(OH) D3 *vs* S.O. vehicle (**Table 1**). Similar reductions in production of these types of cytokines were observed when we cultured spleen

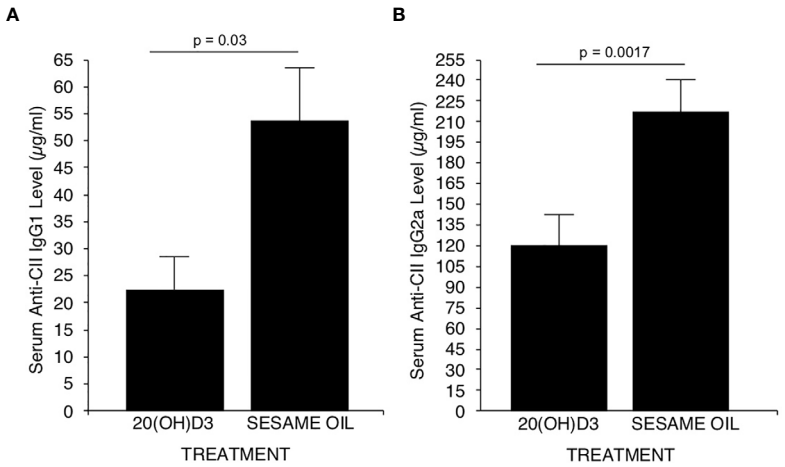


**FIGURE 2** | 20(OH)D3 reduces serum levels of anti-CII antibodies in CIA mice: Aliquots of sera harvested at day 40 post-immunization with CII from mice described in **Figure 1A** were analyzed for levels of CII specific murine IgG1, IgG2a and IgG2b antibodies as described in Methods. 20*S*(OH)D3 treatment significantly reduced levels of anti CII IgG1 and IgG2a **(A, B)** but not of anti-CII IgG2b antibodies (data not shown).

cells from a similarly treated different group of CIA mice (data not shown).

## Treatment of CIA Mice With 20S(OH)D3 Reduces Percentages of CD4$^+$ T Cells and CD19$^+$ B Cells From Draining LN Cells and Increases T Regulatory (Treg) CD4$^+$T Cell Ratio

Since arthritis in mice with CIA results from a contribution by CD4$^+$ T cells and B cells which may be suppressed by CD4$^+$CD25$^+$Foxp3$^+$ Tregs (62), it was important to determine whether percentages of these cell populations were changed by 20*S*(OH)D3 treatment. Groups of DBA/1 mice were immunized with CII and treated with 20*S*(OH)D3 (N=12) or S.O. (N=12) following the same protocol as in the experiment depicted in **Figure 1A**, except mice were euthanized on Day 14 post CII immunization. Draining LN cells were isolated and subjected to analysis by flow cytometry as described under Materials and Methods. The mice treated with 20*S*(OH)D3 had reduced percentages of CD3$^+$ CD4$^+$ T cells and CD19$^+$ B cells ($P$=0.004 and $P$=0.004, respectively) (**Table 2**). We also assessed percentages of CD4$^+$CD25+ Foxp3$^+$ Tregs in this experiment and found no absolute change in the percentage of these Tregs in the draining LN population (data not shown). However, the ratio of CD4$^+$CD25$^+$ Foxp3$^+$ Tregs to CD3$^+$ CD4$^+$ T cells in mice with CIA treated with 20*S*(OH)D3 increased, indicating the equilibrium was shifted in favor of less CD3$^+$ CD4$^+$ T cells compared to this type of Treg (**Table 2**).

## 20S(OH)D3 Administered by Oral Gavage Suppresses Development of CIA

Since 20*S*(OH)D3 would be given *via* the oral route to humans with RA, if eventually approved to treat this disease, we evaluated whether CIA would be suppressed if 20*S*(OH)D3 were administered *via* the oral route using gavage and how it compared to methotrexate in its ability to suppress CIA. Groups of DBA/1 mice (N=10-12 per group) were immunized with CII and were assigned to different treatments as follows: daily oral gavage 100 µl 1:5 diluted PG containing 15 µg/kg 20*S*(OH)D3 and 100 µl normal saline by oral gavage every 7 days daily oral gavage 100 µl 1:5 diluted PG and 100 µl normal saline by oral gavage every 7 days; and daily oral gavage 100 µl 1:5 diluted PG and every 7 days by oral gavage methotrexate (MTX) 2.5 mg/kg dissolved in 100 µl normal saline. Treatments began at Day 13 Post CII immunization of the mice and continued through 48 days post CII immunization.

**TABLE 1** | Treatment of Mice with 20*S*(OH)D3 Modulates Cytokine Production by Lymph node cells.

| Treatment of mice | IFNγ Pg/ml | GCSF Pg/ml | GMCSF Pg/ml | IL-17 Pg/ml | IL-1α Pg/ml | TNFα Pg/ml |
|---|---|---|---|---|---|---|
| VEHICLE | 6008 ± 174 | 1859 ± 870 | 711 ± 277 | 339 ± 10 | 33 ± 10 | 65 ± 13 |
| 20*S*(OH)D3 | 36 ± 20 | 50 ± 23 | 23 ± 19 | 2 ± 1 | 4 ± 1 | 3 ≤ 1 |
| p-Value | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |

*Female DBA/1 Lac J mice (N=6 per group) were treated daily after immunization with CII for 14 days with i.p. 20S(OH)D3 (2.5 µg/kg) in 50 µl sesame oil (S.O.) vehicle or with 50 µl of S.O. vehicle daily. Mice were euthanized and lymph node cells were isolated and cultured as described in Methods for 72 h with 1 µg/ml anti CD3/CD28 monoclonal antibodies for 72 h after which the culture supernatants were harvested and analyzed by multiplex cytokine assay and a Luminex instrument. Differences between groups of vehicle-and 20S(OH)D3-treated mice were analyzed by repeated measures ANOVA.*

**TABLE 2** | Treatment of CIA mice with 20*S*(OH)$D_3$ reduces percentages of CD $4^+$ T cells and B cells in draining lymph nodes while elevating FoxP3 Treg/$CD3^+$ $CD4^+$ ratio*.

| *In Vivo* | Treatment Percentage | | | |
|---|---|---|---|---|
| | $CD3^+$ $CD4^+$ T cells | $CD4^+$ $CD25^+$ FoxP3 Tregs | $CD19^+$ B cells | $CD4^+$ CD25 $FoxP3^+$ Tregs/$CD3^+$ $CD4^+$ T cells |
| Sesame oil (100 µl/day | 12.1 ± 0.54 | 8.8 ± 1.9 | 17.7 ± 3.5 | 1.1 ± 0.11 |
| 20S(OH)D3 (2.4 µg/kg/day | 9.1 ± 0.5 | 7.0 ± 1.04 | 11.4 ± 0.7 | 1.6 ± 0.14 |
| pValue | 0.004 | NS | 0.004 | 0.023 |

**Lymph nodes were harvested from DB A/1 LacJ mice immunized with CII and treated daily for 14 days with i.p. S.O. (n=5) or 20S(OH)D3 (2.4 µg/kg) (n=5) dissolved in S.O. Percentage of CD3+ T cells and B cells were determined by flow cytometry after staining with fluorochrome tagged rat anti-mouse CD3, anti-mouse CD19. FoxP3 Tregs were detected by FoxP3 staining kit (eBioscience, San Diego, CA).*
*NS, not significant.*

Arthritis Severity Scores were significantly lower in 20*S*(OH)D3- and MTX-treated mice than in PG saline vehicle–treated mice (**Figure 3A**). The incidence of arthritis (percentage of mice with one or more arthritic joints) was also significantly lower in 20*S*(OH)D3–treated mice, but not in MTX-treated mice (**Figure 3B**). This experiment demonstrates that, like MTX (a commonly used medication to treat RA), 20*S*(OH)D3 can suppress CIA when given *via* the oral route.

## 20S(OH)D3 Administered by Gavage Suppresses Established Arthritis in CIA Mice

To determine whether 20*S*(OH)D3 would suppress arthritis severity when started later after CII immunization when arthritis is firmly established, we immunized 24 mice with CII and waited until arthritis was present to begin treatment with 20*S*(OH)D3 and S.O. Beginning Day 21, after CII immunization when the mean arthritis severity score was 2.4, we treated 12 of the mice with S.O. administered by daily gavage and 12 with S.O. containing 20*S*(OH)D3 administered by daily gavage at a dose of 30 µg/kg/day. After 46 days treatment, mice were euthanized. As shown in **Figure 4**, mice treated with 20*S*(OH)D3 had less severe arthritis, demonstrating 20*S*(OH)D3 can suppress CIA during the inflammatory phase when anti-CII antibodies play a major role in mediating inflammation in the joints (59, 63). On Day 21 under the conditions we employ, arthritis is highly inflammatory and driven by complement fixing anti-CII antibodies. This suggests 20*S*(OH)D3 also has anti-inflammatory effects.

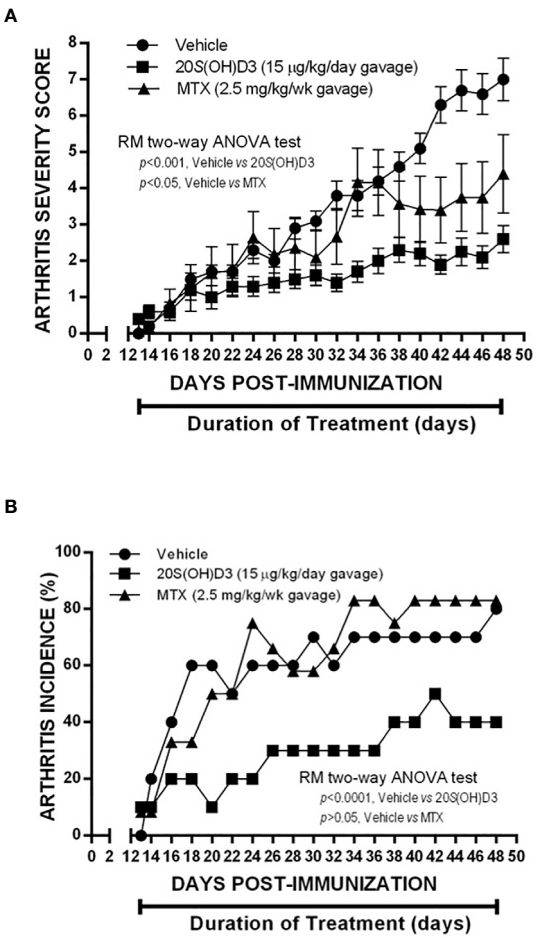


**FIGURE 3** | Suppression of CIA by 20*S*(OH)D3 given by gavage: 36 DBA/Lac J female mice 6 weeks of age were immunized with bovine CII (Day 0) and groups of 12 were gavaged orally beginning on day 13 post CII immunization with daily 15 µg/kg 20*S*(OH)$D_{3\ in}$ 50 µl 1:5 diluted PG and 50 µl normal saline by oral gavage every 7 days; oral gavage daily with 50 µl 1:5 diluted PG and every 7 days by oral gavage with MTX 2.5 mg/kg dissolved in 50 µl normal saline; or oral gavage daily with 50 µl 1:5 diluted PG and 50 µl normal saline by oral gavage every 7 days. Treatment began at Day 13 after CII immunization and continued through 48 days post CII immunization. Arthritis severity **(A)** and arthritis incidence **(B)** were assessed every 2 days. All mice survived until termination of the experiment.

## DISCUSSION

This is the first demonstration that a natural noncalcemic D3 analog, 20*S*(OH)D3, which is normally produced in humans, suppresses the CIA model of human RA, both clinical arthritis and joint destruction, providing a rationale for further consideration of 20*S*(OH)D3 as a potential mono or adjunctive therapy for RA and other autoimmune diseases. It must be noted that CYP11A1 is also expressed outside the classical steroidogenic organs including skin (64, 65), brain, gastrointestinal tract (66, 67), and immune system (68–71). This suggests that 20*S*(OH)D3

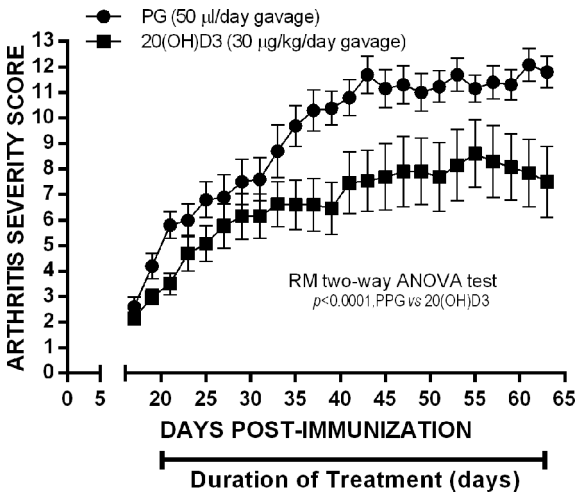


**FIGURE 4** | 20*S*(OH)D3 given by oral gavage Suppresses established arthritis: 24 6 wk old DBA/1 Lac J female mice were immunized on day 0 with CII and scored for arthritis by a blinded observer every other day for 64 days post CII immunization. Treatment was started day 20 post CII immunization by daily oral gavage with (N=12) 100 µl/day PPG 1:5 dilution in sterile saline (N=12) or 100µl of the PPG 1:5 dilution containing 30 µg/kg 20*S*(OH)D3.

may also be produced by the immune cells. Furthermore, 20*S*(OH)D3 has been detected in honey identifying it as a natural product (42). The mechanisms by which 20*S*(OH)D3 downregulates arthritis severity in the CIA model is likely related to reduction in $CD4^+$ T cells, $CD19^+$ B cells, anti-CII antibodies, and maintenance of $CD4^+CD24^+FoxP3^+$Tregs. Treatment with 20(OH) D3 leads to a significant reduction in inflammatory cytokines, likely caused by reduction in the numbers of $CD4^+$ T cells together with an increase in the ratio of T reg cells (CD4+CD25+Foxp3+ T cells) to CD3+CD4+ T cells. The decrease in arthritis was also accentuated by a decrease in critical complement-fixing anti-CII antibodies together with a reduction in the number of $CD19^+$ B cells. Since pro-inflammatory mediators interact to produce an inflammatory cascade and antibodies against type II collagen lead to destruction of cartilage and bone, these data explain why arthritis is attenuated by 20(OH) D3. These results have some similarities to results obtained using a natural plant product (72). The currently used and/or FDA approved therapeutics to treat RA have the potential to cause mild-to-severe life-threatening adverse events such as bacterial, fungal, or mycobacterium tuberculosis infections, neoplasms such as skin cancers and lymphoma, vasculitis, SLE, MS, and interstitial lung disease, etc (73–75).

20*S*(OH)D3 is produced *in vivo* by the hydroxylation of D3 by CYP11A1 and is non-calcemic in rats and mice (38–41). The serum levels in normal humans of 20*S*(OH)D3 is approximately 5% of *25*(OH)D3. In preclinical studies on C57BL6 mice treated with 20*S*(OH)D3 up to 60 µg/kg given i.p. daily for 3 weeks, there was no evidence of hematologic, renal, or liver toxicity (39). In addition, 20*S*(OH)D3 *in vitro* exhibited anti-inflammatory and pro-differentiatory effects on epidermal cells (32, 34, 76, 77). In contrast, C57B/L6 mice given either 2 µ/kg $1,25(OH)_2D3$ or 25 (OH)D3 i.p. daily for 3 weeks displayed hypercalcemia (41). This hypercalcemic property of $1,25(OH)_2D3$ and 25(OH)D3 markedly limits the dosages that can be safely administered to humans on a chronic basis that would be required to treat autoimmune diseases such as RA (78). $20S(OH)D_3$ in addition to inhibiting CIA, shares some other biological properties with $1,25(OH)_2D3$ (32). 20S(OH)D3 like $1,25(OH)_2D_3$ inhibits collagen synthesis by dermal fibroblasts *in vitro*, and at a dose of 3 µ/kg *in vivo* inhibits fibrosis induced by repeated subcutaneous injection of bleomycin into mice (36). 20*S*(OH) D3 also like $1,25(OH)_2D3$ inhibits growth of melanoma cells *in vitro* (37, 40, 79), and it inhibits growth of melanoma at a dose of 30 µg/kg applied daily *in vivo* (37). This is in addition to the aforementioned anti-cancer, pro-differentiation and photoprotective activities of 20*S*(OH)D3 in cells of different origins (80–82). Of significance are anti-inflammatory and immunomodulatory (downregulation of T-cell responses) properties of 20*S*(OH)D3 in conjunction with its ability to decrease NF-kB activity by increasing IkBα levels and inhibiting translocation of NF-kB to the nucleus (76, 81) and inhibit production of IL-17, interferon-γ, TNF-α, and IL-2 (32, 83) and inverse agonism on RORγ (77). These immunomodulatory properties are consistent with the beneficial effect of 20*S*(OH)D3 in the CII-induced arthritis model of RA reported in this paper.

In addition, 20*S*(OH)D3, in contrast to the classical 1,25 $(OH)_2D3$, is non-calcemic and acts as a biased agonist on the VDR having different interactions with the ligand binding domain in comparison to $1,25(OH)_2D3$ or $1,20(OH)_2D3$ (83, 84). Furthermore, 20*S*(OH)D3 acts as an inverse agonist on RORα and γ (77, 84) and acts as an agonist on the aryl hydrocarbon receptor (AhR) (85). Defining the relative contribution of these nuclear receptors (80, 86) to the reported attenuation of the RA will be addressed in the future studies using transgenic mice with silenced VDR, RORs and AhR receptors.

In summary, we provided for the first time preclinical evidence that 20*S*(OH)D3 can significantly attenuate the progression of arthritis in a murine model of RA *in vivo* through suppression of immune responses by T and B-cells. Thus, we synthesized a novel non-calcemic and nontoxic vitamin D3 hydroxyderivative and demonstrated it to be an excellent candidate for clinical trials in RA and other autoimmune diseases.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by University of Tennessee IACUC Committee.

## AUTHOR CONTRIBUTIONS

AP and AS conceived and designed the study. RT, TK, WL, SB, LM, and DB contributed data. All authors contributed to the article and approved the submitted version.

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## IN MEMORIAM

This article is dedicated to our distinguished colleague, the late Professor Arnold E. Postlethwaite, in loving memory of his humanity, humility,integrity and seminal contributions to the broad field of Rheumatology, spanning over five decades.

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# Pleiotropic Effects of Glucocorticoids on the Immune System in Circadian Rhythm and Stress

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Glucocorticoids (GCs) are a class of steroid hormones secreted from the adrenal cortex. Their production is controlled by circadian rhythm and stress, the latter of which includes physical restraint, hunger, and inflammation. Importantly, GCs have various effects on immunity, metabolism, and cognition, including pleiotropic effects on the immune system. In general, GCs have strong anti-inflammatory and immunosuppressive effects. Indeed, they suppress inflammatory cytokine expression and cell-mediated immunity, leading to increased risks of some infections. However, recent studies have shown that endogenous GCs induced by the diurnal cycle and dietary restriction enhance immune responses against some infections by promoting the survival, redistribution, and response of T and B cells *via* cytokine and chemokine receptors. Furthermore, although GCs are reported to reduce expression of Th2 cytokines, GCs enhance type 2 immunity and IL-17-associated immunity in some stress conditions. Taken together, GCs have both immunoenhancing and immunosuppressive effects on the immune system.

**Keywords: glucocorticoids, circadian rhythm, stress, cell-mediated immunity, IL-7 receptor**

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## INTRODUCTION

Glucocorticoids (GCs) are a class of steroid hormones with multiple functions. GCs not only regulate functions of the brain, liver, muscle, and bone, they also exert immunoregulatory effects (1). In general, they have strong anti-inflammatory and immunosuppressive effects and are commonly used to treat allergies, autoimmunity conditions, and inflammation by suppressing the expression of inflammatory cytokines, increasing immunosuppressive proteins, and inducing the apoptosis of lymphocytes (1–3). Especially, GCs strongly inhibit cell-mediated immune responses against cancer and infection (4–6). However, recent studies have reported that GCs, when driven by the diurnal cycle or dietary restriction (DR), enhance immune responses by inducing lymphocyte homing to the lymphoid organs (7, 8). Furthermore, stress-induced GCs have the potential to aggravate inflammation by promoting the differentiation and function of Th17 cells (9, 10). Thus, GCs play important roles both in immune responses against infection and cancer and in triggering inflammation. In this review, we will discuss the immunoenhancing and immunosuppressive functions of GCs, which depend on the immune microenvironment.

## PRODUCTION AND ACTION OF GCs

In steady state, GC production is controlled by circadian rhythm through multiple steps (1). First, the suprachiasmatic nucleus (SCN) stimulate the paraventricular nucleus (PVN) of the hypothalamus, which secretes corticotropin-releasing hormone (CRH). The circadian rhythm of the SCN is regulated by transcriptional-translational feedback loop (TTFL) of the molecular circadian clock comprising positive, negative, and accessory loops (11). In addition, light input from retina controls the TTFL in the SCN to synchronize the intrinsic circadian rhythm with environmental light/dark cycle. Next, CRH goes on to stimulate the anterior pituitary to produce adrenocorticotropic hormone (ACTH) into the blood. ACTH induces the expression of the enzyme 11β-hydroxylase, which catalyzes the synthesis of corticosterone and cortisol in the adrenal cortex. Lastly, secreted GCs suppress CRH production from PVN by negative feedback (12). Due to the induction and suppression of GC production, serum GC levels exhibit diurnal oscillation, with a peak at early morning and a nadir at night in diurnal animals like humans, but the opposite in nocturnal animals like rodents. Moreover, psychological, physical, and nutritional stresses induce high levels of GCs. Adrenergic neurons in the locus coeruleus (LC) of the brain stem sense the stress and produce noradrenaline to stimulate CRH-releasing neurons (13, 14). In addition to neuronal signals, inflammatory cytokines such as IL-1, IL-2, IL6, IL-12, TNF-α, and IFN-γ stimulate the hypothalamus-PVN axis and induce GC production (15).

After the production of GCs from the adrenal cortex, bioavailability of GCs is regulated *via* corticosteroid binding globulin (CBG) and corticosteroid 11β-dehydrogenase (11β-HSD) in peripheral organs (16). Because GCs are hydrophobic molecules, GCs require a transporter and CBG acts as a buffer and carrier. Neutrophil elastase induces cleavage of CBG to lead the delivery of GCs to cells. As the expression level of CBG also follow the circadian rhythm (17), this might contribute to the circadian oscillation of bioavailability of GCs. After delivery of GCs, active cortisol and inactive cortisone are interconverted by two isozymes of 11β-HSD, 11β-HSD1 and 11β-HSD2, in each organ (16). The 11β-HSD1 mainly metabolizes cortisone to cortisol, while the 11β-HSD2 converts cortisol to cortisone. Several papers reported that inflammation induced 11β-HSD1 expression in tissues *via* TNF-α in rheumatoid arthritis (RA), colitis, and chronic kidney disease, suggesting that inflammation augments effects of GCs by induction of 11β-HSD1 (18–21). Taken together, not only the hypothalamus-PVN axis but also CBG and 11β-HSD control the effects of GC *via* circadian rhythm and inflammation.

GCs exert their effects through complicated mechanisms (22). In general, GCs bind to glucocorticoid receptor (GR) in the cytoplasm (23), which induces the dimerization of GR and its translocation into the nucleus. There, GR acts as a transcription factor that promotes or suppresses the transcription of target genes by binding to specific DNA sequences known as glucocorticoid-response elements (GREs). In some cases, GR represses transcription by binding to negative GREs (nGREs). Binding of GR monomer to nGRE recruits transcriptional co-repressors, which suppresses transcriptional activation by NF-κB nearby, without direct interaction to NF-κB (22). In other case, GR monomer directly interacts with DNA-bound NF-κB and AP-1 and tethers transcriptional corepressors, without DNA binding of GR (24, 25). Transcriptional induction by GR, however, might be more important than transcriptional repression *via* nGREs and tethering. First, GCs induce the transcription of IκBα, A20, DUSP1, and GILZ, negative regulators of NF-κB and AP-1, which inhibits macrophage activation by LPS (26). Second, GC-induced transcription might snatch transcriptional coactivators and chromatin remodeling factors from the enhancers in target genes of inflammatory cytokines and reduce their transcription (2).

## REGULATION OF IMMUNE-RELATED GENE EXPRESSION BY GR

GR represses the production of inflammatory cytokines and proteins such as IL-6, C3, and TSLP by binding to nGREs and recruiting the corepressors NCOR2 and HDAC2 (27). In addition, GR induces the expression of immunosuppressive molecules such as IκBα, A20 (TNFAIP3), DUSP1, and GILZ. IκBα binds to NF-κB and blocks the activation of NF-κB (28). The A20/TAX1BP1 deubiquitinase complex inhibits the ubiquitination and degradation of RIP1 and enhances the degradation of E2 enzyme Ubc13, which suppresses NF-κB activation (28). The phosphatase DUSP1 suppresses the MAPK pathway including p38, JNK, and ERK (29, 30). DUSP1 dephosphorylates ERK and inhibits the activation of p38 and JNK, which reduces the expression of inflammatory cytokines and chemokines such as IL-1β, IL-6, GM-CSF, CCL2, CXCL1, and CXCL2. GILZ suppresses NF-κB by preventing nuclear transport of NF-κB p65 subunit and inhibits MAPK signaling by directly binding to Ras and Raf-1 (31–33). Thus, these molecules induced by GCs attenuate inflammation by inhibiting NF-κB and MAPK cascades.

Furthermore, NF-κB and GR might cooperatively control gene expression. Vollmer et al. reported that induction of DUSP-1 by GR agonist was enhanced by LPS stimulation in macrophages (34). In addition, Kadiyala et al. reported that GR and NF-κB cooperatively bound to the enhancer of the A20 locus and induced A20 expression (35). However, Rao et al. reported that increase of GR-binding sites in HeLa cells after stimulation with TNF-α and a GR agonist (~1,000 sites) was much smaller than all GR-binding sites after GR agonist stimulation (~8,700 sites) and NF-κB binding sites after TNF-α stimulation (~12,000 sites), suggesting that GR-NF-κB interaction might be partial in GR repression mechanisms (36).

On the other hand, Oh and colleagues suggested that inflammation is not critical for immunosuppressive functions of GR. They analyzed the transcription and chromatinscape of macrophages with dexamethasone (DEX) treatment before and after LPS-stimulation (26). They found that DEX treatment after LPS stimulation showed similar gene expression profile to DEX

treatment before LPS stimulation. DEX treatment before and after LPS stimulation showed the upregulation of NF-κB and AP-1 inhibitors such as IκBα, A20, DUSP1, GILZ, which were critical for extinguishing inflammation. Thus, these results suggest that the induction of immunoregulatory factors by GR is important for immune regulation, independently of nGREs and tethering.

To induce transcription, GR binding to palindromic GRE might not be necessary. Schiller et al. performed ChIP-seq analysis with a human osteosarcoma cell line expressing GR with a mutation in dimerization domain and found that DNA-binding of the mutant GR was mostly overlapped with that of wild-type GR (37). It suggests that GR monomer and widespread GRE half-sites are enough for effects of GR. Sasse et al. performed global run-on sequencing (GRO-seq) in human airway epithelial cells to detect nascent RNA and found that GR repressed transcription within 10 minutes (38). By integrating the results of ChIP-seq and GRO-seq, they found that rapid repression of TNF-induced genes by GR did not require local GR-binding to canonical GREs in TNF-induced gene loci. Moreover, GR rapidly changed the accessibility of TNF-induced gene enhancers. Based on these observations, Gerber et al. proposed the squelching model that GR binding to GREs might snatch transcriptional coactivators and chromatin remodeling factors from the enhancers of TNF-induced genes and reduce their transcription of the TNF-induced genes (2). Taken together, transcriptional regulation mediated by GR is highly diversified.

## IMMUNOSUPPRESSIVE EFFECTS OF GCs

Endogenous GCs suppress inflammatory responses *via* innate immune cells and stromal cells in mouse disease models. As for the innate immune cells, GR-deficient macrophages express higher levels of inflammatory molecules, such as IL-6, TNF-α, and COX-2, through the overactivation of p38 MAPK after stimulation with LPS, leading to higher mortality (39). In contrast to the suppression of inflammatory macrophages, GCs enhance the differentiation of tissue-repair macrophages (40, 41). Galuppo et al. reported that LysM-Cre GR-deficient mice exhibited higher mortality and impaired tissue repair in a myocardial infarction model (42). In addition, Ly6C$^{low}$ monocyte-derived macrophages in the infarcted myocardium of GR-deficient mice were reduced in number and expressed lower levels of genes related with neovascularization, collagen degradation, and scar formation. At the same time, the expression of the inflammatory chemokine CCL5 was upregulated. Thus, endogenous GCs suppress inflammatory macrophages but enhance suppressive macrophages.

Endogenous GCs suppress the maturation and function of dendritic cells (DCs). Li et al. reported that GR-deficient DCs secrete large amounts of inflammatory cytokines, such as IL-1β, IL-12, and TNF-α, which increased IFN-γ production in NK cells and caused higher mortality of GR-deficient mice (43). Elftman et al. showed that the expression of B7 and MHC class II, the maturation markers of DCs, was attenuated by GC treatment *in vitro* and *in vivo*, suggesting that GCs suppress DC maturation (44). Thus, GC-treated DCs failed to activate CD8 T cells in herpes simplex virus infection. On the other hand, DEX promotes the differentiation of IL-10-producing tolerogenic DCs (45). Hodrea et al. reported that DEX enhances phagocytosis of human DCs by inducing the expression of the apoptophagocytic genes, ADORA3 (adenosine receptor guiding macrophages to apoptotic cells), CD14, and MERTK (phagocytic receptor for apoptotic cells) (46). Therefore, endogenous and exogenous GCs suppress immunoenhancing function but enhance immunosuppressive function of DCs.

Like innate immune cells, GCs suppress cytokine production in stromal cells and alleviate colitis and asthma. Aranda et al. showed that intestinal epithelial cell-specific villin-Cre GR-deficient mice exacerbated DSS-induced colitis by increase of the neutrophil-recruiting chemokines, CXCL1, CXCL5, and CCL5 (47). Klassen et al. reported that OVA-induced allergic asthma in the lung was alleviated by DEX but that GR deficiency in the airway epithelium (SPC-Cre GR-deficient mice), but not in T cells nor DCs, canceled the suppressive effect of DEX (48). Gibbs et al. reported that GR represses the expression of the neutrophil-recruiting chemokine CXCL5 *via* nGRE in the CXCL5 promoter in an LPS-induced lung inflammation model (49). Indeed, adrenalectomized mice exhibited higher levels of CXCL5 at night, when the GC concentration is high, with a loss of circadian changes in the neutrophil accumulation in the lung. By contrast, Ince et al. reported that airway club cell-specific GR-deficient mice showed normal oscillations of LPS-induced neutrophil homing to the lung despite the loss of the circadian rhythm of CXCL5 and IL-6 (50). In addition, macrophage-specific LysM-Cre GR-deficient mice showed no diurnal change of CXCL5 and TNF-α but normal change in neutrophil count in the lung. These findings demonstrate that GCs at physiological concentrations suppress inflammation by affecting both immune cells and stromal cells. However, further studies are needed to understand how GCs control neutrophil homing to the lung. In contrast to suppression by GCs, it was also reported that DEX promoted the expression of TLR2 and soluble leukocyte protease inhibitor (SLPI), an antimicrobial molecule, in airway epithelial cells, suggesting that exogenous GCs contribute to immune response against bacterial infection (51–53). Interestingly, DEX-induced DUSP1 promoted IL-1β-driven TLR2 induction by inhibiting p38- and JNK-mediated negative feedback of TLR2 expression (52, 54, 55). Furthermore, DEX-induced DUSP1 maintained IL-1β-induced IRF1 upregulation and IRF-dependent CXCL10 expression. Thus, endogenous and exogenous GCs might support the first defense of lung epithelium but suppress excessive inflammation after infection.

GCs strongly impair the cell-mediated immunity mediated by IFN-γ-producing type-1 helper T (Th1) cells, CD8 T cells, and NK cells. Blotta et al. reported that DEX suppressed IL-12 production in human monocytes and impair IFN-γ production in NK cells (56). Moreover, Quatrini et al. reported that endogenous GCs directly suppressed the immune response of NK cells (57). GR-deficient NK cells from NK cell-specific Ncr1-

Cre GR-deficient mice produced higher amounts of IFN-γ after stimulation with IL-12 and IL-18 *in vitro*. In addition, these mice exhibited increased IFN-γ production and higher lethality after the administration of LPS without any change in IL-6 or TNF-α. Furthermore, GR-deficient NK cells induced excessive inflammation and the lethality of mice with mouse cytomegalovirus (MCMV) infection, because GR deficiency decreased PD-1 expression and elevated IFN-γ production (4).

Like NK cells, endogenous GCs suppress the differentiation and IFN-γ expression of T cells. They also reduce the expression of IL-12R in T cells (58, 59). In addition, GR interacts with T-bet to inhibit its DNA-binding activity (59). Kugler et al. reported that, in a Toxoplasma infection model, T cell-specific Lck-Cre GR-deficient mice exhibited Th1 cells that had normal differentiation but also produced abnormally high levels of IFN-γ and TNF-α, leading to higher mortality (5). Thus, GCs suppress IFN-γ production by Th1 cells and thereby prevent excessive inflammation. GCs also cause dysfunctional CD8 T cells. Acharya et al. found that monocyte-macrophage lineage cells in tumors expressed the enzyme for steroidogenesis, Cyp11a1. and produced local GCs (60). In addition, exhausted CD8 T cells in tumors showed higher expressions of GR. In a MC38-OVA tumor model, GR-deficient CD8 T cells expressed higher IL-2, TNF-α, and IFN-γ; blocked tumor-growth and were less exhausted. These data suggest that GCs produced by monocyte-macrophage lineage cells promote the exhaustion of CD8 T cells and impair the immune surveillance against tumors. Taken together, endogenous GCs suppress excessive inflammation and cell-mediated immunity *via* innate and cytotoxic immune cells.

## THE CIRCADIAN RHYTHM OF T CELL IMMUNITY IS CONTROLLED BY ENDOGENOUS OSCILLATING GCs

Although GCs strongly repress inflammatory cytokine production, past studies have reported that GCs induced the expression of receptors for IL-2, IL-6, IFN-γ, GM-CSF, and TNF-α, implicating positive effects by GCs on immunity (61). In addition, Franchimont et al. reported that human blood T cells stimulated with DEX increased the transcription of genes related with cell proliferation (Mt1l, Mt1e, and Mt1b), metabolism (Sat1, Vdr), oxidation damage (Ido1), and cell surface receptors (Il1r2, Il7r) (62). IL-7 is a member of the common γ-chain cytokine family and binds to the complex of the IL-7Rα-chain and common γ-chain. IL-7 supports the development, survival, and proliferation of T cells, B cells, and innate lymphoid cells (ILCs) (63–65). In addition, IL-7R signaling protects T cells from the apoptosis induced by GCs (62). Indeed, DEX administration increased IL-7Rα expression in human T-acute lymphoblastic leukemia cells and augmented Bcl-2 expression by IL-7, which protected leukemia cells from apoptosis (66). Like IL-7Rα, GCs also induce CXCR4 expression in immune and stromal cells. GCs elevated the CXCR4 expression in T and B lymphocytes, granulocytes, and monocytes in mouse and human (67–71). Interestingly, Leigh et al. reported that GCs enhanced CXCR4 expression in human bronchial cells, whereas Carolina et al. showed that GCs reduced CXCR4 expression in lung endothelial progenitors, implying a complicated regulation of CXCR4 expression by GCs (72, 73). Thus, GC may enhance T cell immunity by inducing IL-7Rα and CXCR4.

In general, how GCs induce IL-7Rα expression on T cells is well studied. There exists a non-coding conserved sequence 1 (CNS-1) 3.6 kb upstream of the IL-7Rα promoter, the deletion of which prevented the IL-7Rα induction by GCs in mice (74, 75). The CNS-1 region contains two GRE motifs conserved between human and mouse. To investigate the biological significance of the IL-7R induction by GCs, mice harboring point mutations in the two GREs of CNS-1 (GREm mice) as well as T cell-specific CD4-Cre GR-deficient mice have been generated (7). The IL-7R expression on T cells was elevated at night and reduced at daytime in control mice, consistent with the diurnal fluctuation of GCs. Moreover, T cells in the control mice accumulated in the spleen, lymph nodes, and Peyer's patches at nighttime, but circulated more in peripheral blood at daytime. However, the diurnal fluctuation of IL-7R expression and T cell numbers in the blood and lymphoid organs was abolished in CD4-Cre GR-deficient mice and GREm mice. This oscillation seems to be regulated partly by CXCR4, because CXCR4 expression was also induced by GCs and IL-7R. Therefore, the GC-IL-7R axis controls the diurnal oscillation of T cell distribution between the blood and lymphoid organs by regulating CXCR4 expression (**Figure 1**).

The accumulation of T cells in the lymphoid organs by GCs and IL-7R may enhance immune responses. The infection of control mice with *Listeria monocytogenes* at nighttime induced antigen-specific effector CD8 T cells more efficiently than at daytime (7). By contrast, the increase of effector CD8 T cells with nighttime infection was not observed in CD4-Cre GR-deficient or GREm mice, suggesting that the diurnal surge of GCs at night enhances the CD8 T cell response against bacterial infection. Similarly, immunization with soluble antigens at night enhanced the generation of follicular helper T (Tfh) cells, germinal center B cells, and class-switched B cells, an effect lost in the mutant mice. Furthermore, previous studies reported that GR affects the differentiation and function of helper T cell subsets (76). GCs strongly suppress the function of Th1 cells but promote the function of Th2 cells. Ramirez et al. found that primed CD4 T cells pretreated with DEX *in vitro* produced a large amount of IL-4 and IL-13 (77). Consistently, GR-deficient CD4 T cells produced lower levels of IL-4 and IL-13 in Th2-skewed culture (7). Thus, endogenous GCs promote the differentiation and function of Th2 cells. Taken together, oscillating GCs induced by the circadian rhythm have immunoenhancing effects on immunity.

Like T cells, GCs induced by the circadian rhythm enhance B cell responses. Cain et al. reported that the diurnal induction of CXCR4 in B cells was impaired in B cell-specific mb1-Cre GR-deficient mice, which attenuated B cell homing into the bone marrow (69). Additionally, B cell numbers in the blood of GR-deficient mice lost their normal diurnal change, indicating that GCs control the diurnal change of B cell recirculation between the bone marrow and blood *via* CXCR4 induction. IgG

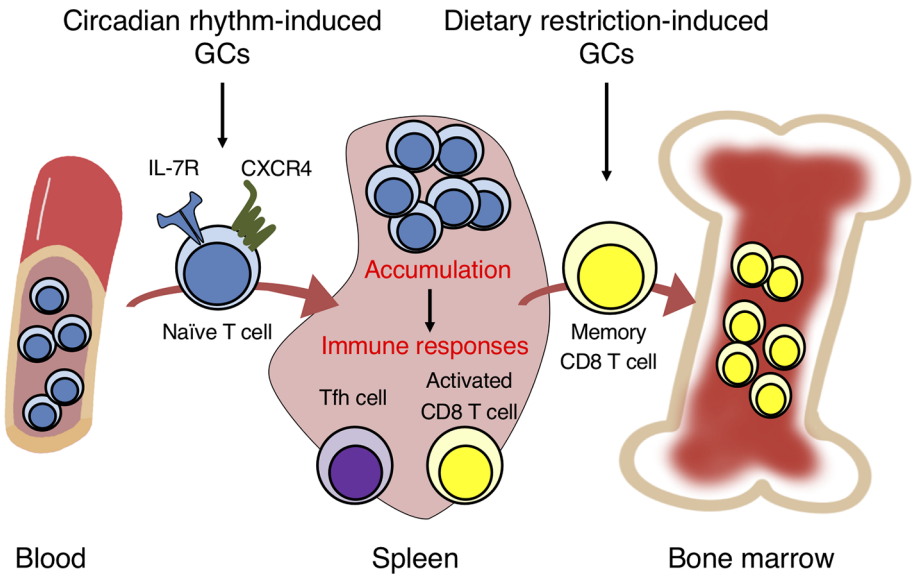


**FIGURE 1** | Glucocorticoids in circadian rhythm and dietary restriction induce the migration of naïve and memory T cells into the spleen and bone marrow. GCs induced by circadian rhythm promote the homing of naïve T cells into secondary lymphoid organs from peripheral blood by inducing the expression of IL-7R and CXCR4. The T cell accumulation induces strong immune responses by activated CD8 T and follicular helper T (Tfh) cells against bacterial infection and soluble antigens. GCs induced by dietary restriction trigger the egress of memory CD8 T cells from secondary lymphoid organs and homing into the bone marrow. The accumulation in the bone marrow enhances the survival and response of memory CD8 T cells.

production after immunization with the T-independent antigen NP-Ficoll was impaired in GR-deficient mice, suggesting that endogenous GCs per se might promote the activation of B cells. Furthermore, it is reported that adrenergic signaling and the clock gene Bmal1 control the diurnal change of B cell recirculation between the blood and lymph nodes and that B cell retention in the lymph nodes enhances B cell responses (78, 79). Therefore, CXCR4 induction by GCs might trigger B cell homing to the lymph nodes and bone marrow and elevate the responses of B cells. Taken together, GCs induced by the circadian rhythm enhance B cell responses through the accumulation of lymphocytes in lymphoid organs and the induction of Th2 and Tfh cell differentiation (**Table 1**).

## GCs UNDER DIETARY RESTRICTION (DR) PROMOTE MEMORY CD8 T CELL RESPONSE

DR contributes to longevity and reduces inflammation and cancer (80, 87). Because immune cells consume large amount

**TABLE 1** | A summary of the pleiotropic effects of glucocorticoids in different conditions.

| | Suppression of immune responses | Enhancement of immune responses |
|---|---|---|
| **Exogeneous glucocorticoids** | • Suppression of inflammatory cytokine production (1–3)<br>• Induction of lymphocyte apoptosis (3)<br>• Suppression of function and development of Th1, NK, and CD8 T cells (4, 5, 56–60) | • Promotion of differentiation of Th2 and Th17 cells (7, 9, 77, 85, 86) |
| **Circadian rhythminduced glucocorticoids** | • Suppression of CXCL5 production and neutrophil recruitment in lung inflammation (49, 50) | • Induction of IL-7R and CXCR4 (7, 61, 74, 75)<br>• Homing of T cells to lymphoid organs (7)<br>• Enhancement of Immune response of CD8 T and Tfh cells (7) |
| **Dietary restrictioninduced glucocorticoids** | • Suppression of inflammatory cytokine level in serum (80) | • Migration of memory CD8 T cells into bone marrow (8)<br>• Induction of Bcl2 expression to enhance the survival of memory CD8 T cells (8)<br>• Enhancement of anti-cancer response by memory CD8 T cells (8) |
| **Stressinduced glucocorticoids** | • Suppression of IFN-γ production in Th1 and CD8 T cells (81–84)<br>• Inhibition of CD8 T cell response against cancer and viral infection (81–84) | • Increase of IL-17 and neutrophil recruitment in sickle cell disease model (10) |

*References are shown in parentheses.*

of energy in inflammation, M1 macrophages and effector T cells activate glycolytic and lipogenic metabolic pathways for rapid ATP synthesis (88). Thus, the undernutrition might impair the maintenance and response of leukocytes. DR reduced the mass of thymus and spleen and induced reversible lymphopenia of T, B, and NK cells (89, 90). DR also suppressed the PI3K/Akt/mTOR signaling *via* activation of AMPK and sirtuin, which impaired the function of effector T cells and M1 macrophages (91). In addition, Yang et al. reported that serum TNF-α levels decreased with elevated cortisol under calorie restriction (CR), suggesting that CR suppresses inflammation by inducing GC production (80). On the other hand, DR enhances the cytotoxicity of CD8 T cells. Di Biase et al. reported that a fasting-mimicking diet increased common lymphoid progenitors in the bone marrow and tumor-infiltrating CD8 T cells in a breast cancer model (92). Therefore, DR promotes lymphopoiesis and cell-mediated immunity. In line with that study, it is reported that diet affects cell-mediated immune responses by inducing GCs. Collins et al. reported that GCs induced by DR enhanced the maintenance and function of memory CD8 T cells (8). DR triggered the migration of memory CD8 T cells from secondary lymphoid organs into the bone marrow, but this effect was abolished in adrenalectomized mice and T cell-specific Lck-Cre GR-deficient mice. In addition, the accumulation of memory CD8 T cells in the bone marrow by DR was impaired in CXCR4- and S1PR1-deficient mice, suggesting that GCs might induce the egress of memory CD8 T cells from secondary lymphoid organs *via* S1PR1 and drive homing to the bone marrow *via* CXCR4 (**Figure 1**). Interestingly, DR reduced mTOR signaling in memory CD8 T cells and changed gene expressions associated with heat-shock protein chaperone binding and the regulation of protein folding. It also induced amino-acid deprivation and the cellular response to rapamycin, suggesting that DR changes the metabolic state of memory CD8 T cells to quiescent. In addition, DR and DEX increased Bcl-2 expression in memory CD8 T cells. Finally, memory CD8 T cells efficiently responded to bacterial infection and tumors during DR. Together, these reports demonstrate that GCs induced by DR enhance the long-term maintenance of memory CD8 T cells, which promotes cytotoxic responses against infection and tumors.

As described above, GCs produced by monocyte-macrophage lineage cells in the tumor microenvironment suppress the function of effector CD8 T cells, whereas DR-induced GCs enhance the maintenance and response of memory CD8 T cells (8, 60). Thus, GCs seem to exert different effects on immune cells, probably by factors supplied from the microenvironment. Circadian rhythm- and DR-induced GCs trigger the homing of IL-7R-positive CD8 T cells into lymphoid organs, which supply pro-survival factors and enhance the maintenance and response of T cells. By contrast, GCs produced in the tumor microenvironment accelerate the dysfunction of IL-7R-negative effector CD8 T cells. Therefore, the supply of IL-7 from the microenvironment may determine whether the GC effects are positive or negative.

## STRESS SUPPRESSES CELL-MEDIATED IMMUNITY BUT ENHANCES INFLAMMATORY DISEASES

Because stress-induced GCs suppress cell-mediated immunity, stress might exacerbate viral infection and tumor growth. Steelman et al. reported that restraint stress reduced IFN-γ-expressing CD4 T and CD8 T cells and their T-bet expression following Theiler's murine encephalomyelitis virus (TMEV) infection (81). In addition, treatment with a GC antagonist alleviated the clinical manifestations induced by the restraint stress. Elftman et al. found that restraint stress impaired the expression of granzyme B and IFN-γ in CD8 T cells following herpes simplex virus (HSV) infection (82). However, this effect was alleviated if the infection was in T cell-specific Lck-Cre GR-deficient mice, suggesting that stress-induced GCs suppress the production of granzyme B and IFN-γ by CD8 T cells *via* GR. These findings indicate that stress exacerbates the viral infection by suppressing cell-mediated immunity *via* GCs. Like viral infection, stress caused by chronic sleep restriction, forced swimming, and abdominal surgery promoted the progression of cancers (83, 84). Hong et al. reported the relationship between perinatal stress and cell-mediated immunity (6). The perinatal exposure of fetal mice to DEX as a stress model diminished CD8 T cell response against tumors in adulthood. Thus, exposure to stress-induced GCs during pregnancy appear to cause dysfunction of anti-tumor response by CD8 T cells in offspring after birth. Overall, stress-induced GCs might impair cell-mediated immunity over the lifetime.

Although stress suppresses cell-mediated immunity, it also triggers chronic inflammation and autoimmunity. Qiu et al. reported that restraint stress enhanced tissue destruction in a DNBS-induced colitis model (93). The colitis was ameliorated in CD4-deficient mice, suggesting that helper T cell function is critical for the stress-induced tissue destruction. Arima et al. reported that wet bedding and restraint stress triggered upper gastrointestinal bleeding in mice transferred with myelin oligodendrocyte glycoprotein (MOG) peptide-primed pathogenic T cells (94). Because Th17 cells play a critical role in experimental autoimmune encephalomyelitis (EAE), the stress might enhance Th17 cell function. de Castro Kroner et al. reported that GCs elevated RAR-related orphan receptor C (RORC) expression by inhibiting IL-2 secretion in human T cells (85). In addition, GCs induced the expression of defensins and CCL20. In mouse T cells, GCs promoted the IL-23-dependent differentiation of Th17 cells *in vitro* but not if IL-6 and TGF-β were also present (9). In addition, Marchetti et al. reported that transgenic (Tg) mice expressing GR antisense RNA exhibited less severe neurological inflammation (86). Thus, GCs have the potential to enhance the function of Th17 cells. Furthermore, Xu et al. showed that restraint stress increased serum IL-17 and aged neutrophils, which exacerbated vaso-occlusive episodes *via* microbiota in a sickle cell disease model (10). Treatment with metyrapone, an inhibitor of GC production, alleviated the leukocyte recruitment and

inflammation under stress. Overall, stress-induced GCs aggravate inflammation by promoting the differentiation and function of Th17 cells *via* microbiota.

Although GCs exert some anti-inflammatory effects in neutrophils, such as reduction in COX-2 and iNOS expression and superoxide release (95–97), GCs also promote the development and function of neutrophils. First, GCs enhanced the development and proliferation of neutrophils in bone marrow and induced neutrophilia in peripheral blood (98, 99). Second, GCs promote survival of neutrophils. GCs inhibited spontaneous apoptosis of human neutrophils *in vitro* (100, 101). Chang et al. reported that DEX treatment reduced the expression of Fas and caspase-8 in neutrophils (102). Furthermore, Bouterse et al. found that the expression of a pro-apoptotic factor Bak was reduced in DEX-treated neutrophils (103). Third, GCs elevated the expression of the IL-1R in neutrophils and enhanced IL-1β-triggered inflammation (104). Thus, GCs might augment tissue inflammation by enhancing the functions of neutrophils.

Stress-induced GCs control inflammation also *via* Treg cells. Harpaz et al. found that chronic variable stress reduced the number of Treg cells and increased the susceptibility to EAE (105). In addition, the administration of mifepristone, an antagonist of GCs, blocked the stress-induced exacerbation of EAE. On the other hand, it is reported that GCs also promote Treg cell function. GC stimulation upregulates the expression of Foxp3, IL-10, TGF-β, and CTLA4 (106–108). Rocamora-Reverte et al. reported that GR-deficient Treg cells failed to suppress tissue damage in a colitis model in Treg-specific Foxp3-Cre GR-deficient mice (109). Furthermore, Engler et al. reported that the onset of EAE was delayed in pregnant mice, because progesterone binding to GR increased the number of Treg cells (110). Treatment with a GC antagonist and T cell-specific Lck-Cre GR-deletion blocked the activation of Treg cells. However, it remains unknown whether stress enhances inflammation by blocking Treg cells even though GCs enhance Treg function. One possibility is that an unknown factor might block the GC-enhanced Treg function to promote inflammation under stress. Thus, because stress-induced GCs may affect both Th17 and Treg cells, it remains unclear whether GCs enhance inflammation *via* Th17 cells or suppress it *via* Treg cells. Taken together, stress-induced GCs seem to have pleiotropic effects either to attenuate cell-mediated immunity or to aggravate inflammation, depending on the cell types and the disease models (**Table 1**).

## GC RESISTANCE IN INFLAMMATORY DISEASES

GCs are well used for therapy of allergic and autoimmune diseases. GCs mitigate the symptoms in psoriasis and multiple sclerosis, whereas some patients with ulcerative colitis (UC) and asthma are refractory to treatment with GCs (111, 112). However, the mechanism of this GC resistance is unclear. One explanation is that GCs might promote survival of immune cells and enhance immune responses. As explained above, GCs have the potential to augment immune responses of Th2 cells, Th17 cells, and neutrophils, which exacerbates tissue damage. Second, some immune cells escape from the suppressive effects of GCs by reducing the amounts of GCs and GR in the cytoplasm. Ramesh et al. reported that human Th17 cells expressed multiple drug resistance 1 (MDR1), a membrane efflux pump with broad substrate specificity, which reduces the sensitivity to GCs (113). Paugh et al. found that caspase-1 activated by NLRP3 inflammasome cleaved GR (114). Third, some inflammatory cytokines such as IFN-γ, IL-17, IL-4, and TNF-α canceled the suppressive effects of GCs (115–119). These cytokines diminish GC-induced apoptosis of immune cells and blunt the repression of inflammatory cytokine production by GCs. Thus, both the enhancing effects of GCs and the cancelation of GC effects might contribute to the GC resistance in the treatment of inflammatory diseases. Manipulation of these mechanisms will facilitate to overcome the GC resistance and contribute to cure allergic and autoimmune diseases.

## CONCLUSIONS

This review summarizes the immunoenhancing and immunosuppressive effects of GCs (**Table 1**). Exogeneous and stress-induced GCs suppress the IFN-γ production and exhaustion of CD8 T cells, whereas GCs under circadian rhythm or DR enhance the maintenance and activation of naïve and memory CD8 T cells. In addition, GCs strongly suppress the function of Th1 cells and enhance the differentiation of Th2 and Th17 cells. Thus, the effects of GCs on immunity can be positive or negative depending on the tissue and cell type. The immunoenhancing effects of GCs possibly depend on the microenvironment because GCs trigger T cell homing to lymphoid organs, which supply pro-survival cytokines. Further studies are required to understand how GCs control the interaction between immune cells and the microenvironment. Moreover, the effects of GCs might depend on different GR-target genes in different cell types. To address this question, it is necessary to investigate GR-induced changes in gene transcription, DNA binding, and chromatin accessibility in immune and stromal cells. Revealing the pleiotropic effects of GCs will help understand how GCs trigger immune dysfunction and chronic inflammation and maximize the therapeutic effects of GCs in refractory allergies and autoimmune diseases.

## AUTHOR CONTRIBUTIONS

AS wrote the first draft of the manuscript. AE and KI modified, revised, and approved the submitted version. All authors contributed to the article andapproved the submitted version.

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