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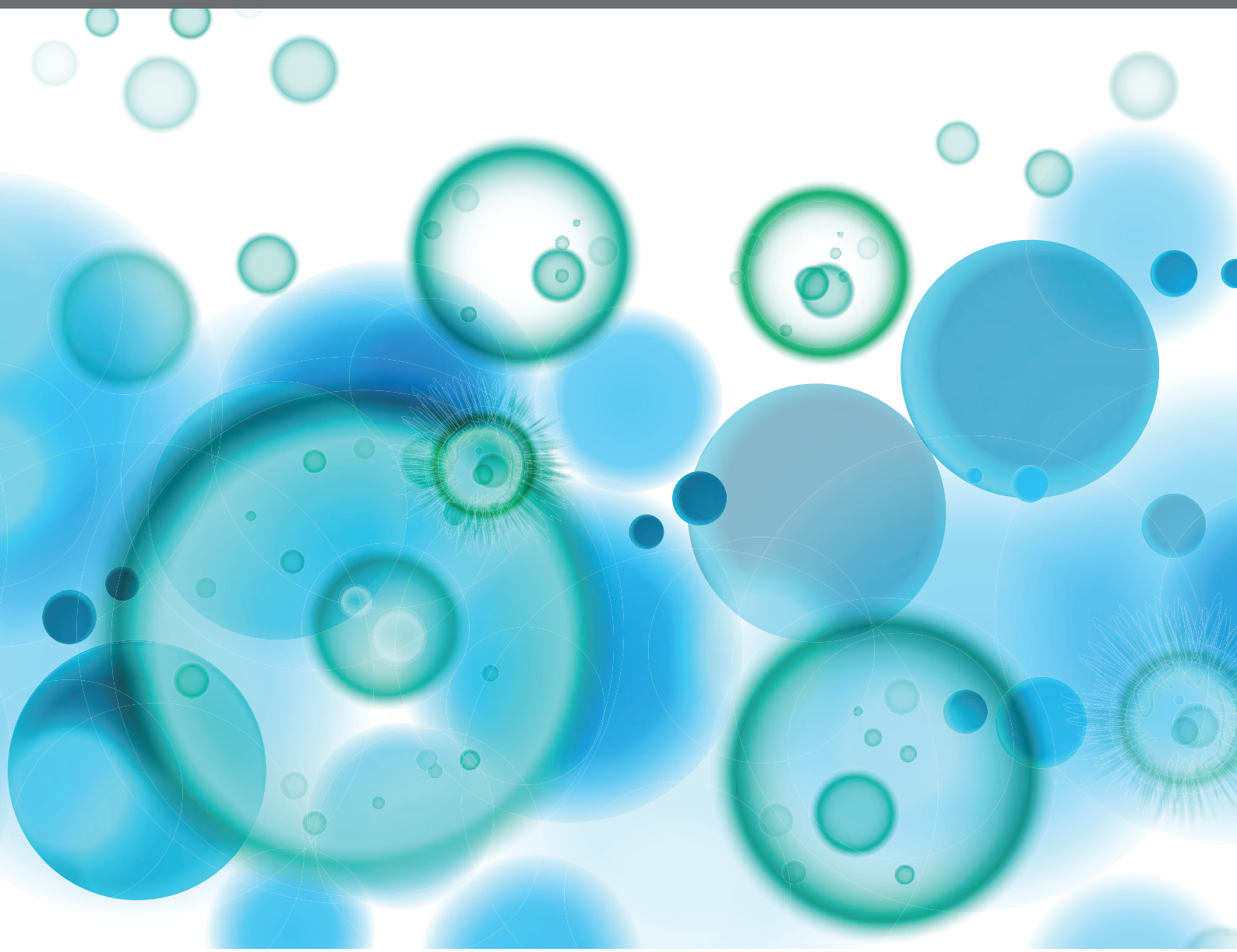

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GLUCOCORTICOIDS IN IMMUNITY AND INFLAMMATION

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ORIGINAL RESEARCH
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Glucocorticoid Receptor-Deficient Foxp3$^+$ Regulatory T Cells Fail to Control Experimental Inflammatory Bowel Disease

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Activation of the immune system increases systemic adrenal-derived glucocorticoid (GC) levels which downregulate the immune response as part of a negative feedback loop. While CD4$^+$ T cells are essential target cells affected by GC, it is not known whether these hormones exert their major effects on CD4$^+$ helper T cells, CD4$^+$Foxp3$^+$ regulatory T cells (Treg cells), or both. Here, we generated mice with a specific deletion of the glucocorticoid receptor (GR) in Foxp3$^+$ Treg cells. Remarkably, while basal Treg cell characteristics and *in vitro* suppression capacity were unchanged, Treg cells lacking the GR did not prevent the induction of inflammatory bowel disease in an *in vivo* mouse model. Under inflammatory conditions, GR-deficient Treg cells acquired Th1-like characteristics and expressed IFN-gamma, but not IL-17, and failed to inhibit pro-inflammatory CD4$^+$ T cell expansion *in situ.* These findings reveal that the GR is critical for Foxp3$^+$ Treg cell function and suggest that endogenous GC prevent Treg cell plasticity toward a Th1-like Treg cell phenotype in experimental colitis. When equally active in humans, a rationale is provided to develop GC-mimicking therapeutic strategies which specifically target Foxp3$^+$ Treg cells for the treatment of inflammatory bowel disease.

Keywords: glucocorticoid, glucocorticoid receptor, Foxp3, regulatory T cell, transfer colitis, suppression

INTRODUCTION

Regulatory T cells (Treg cells) expressing the transcription factor Foxp3 maintain immune homeostasis by limiting antigen-specific immune responses and sustaining tolerance to self-antigens (1). Most Treg cells are generated in the thymus (tTreg cells) as a separate lineage at the CD4$^+$ single-positive stage of thymocyte development. Peripheral Treg cells (pTreg cells) are induced from peripheral CD4$^+$Foxp3$^-$ T cells in the presence of TGF-beta, however, the pool size and function of these pTreg cells is not fully characterized, mainly due to the lack of useful markers to discriminate tTreg from pTreg cells (2).

Treg cell function is not mediated by one single common pathway, as many different mechanisms have been described including downregulation of costimulatory molecules (CD80/CD86) on dendritic cells, secretion of inhibitory cytokines or metabolic disruption of target cells. Beyond that, Treg cells seem to have the capacity to adjust their suppressive mechanism(s) to a particular immune or inflammatory context, although the signals driving *in vivo* Treg cell adaptation are not well-understood (3).

The original view that tTreg cells are terminally differentiated and phenotypically stable has been recently questioned. Some Treg cells may lose Foxp3 expression in autoimmune disease ("ex-Foxp3" cells), others, while maintaining Foxp3 expression, acquire a certain degree of plasticity which is illustrated by secretion of pro-inflammatory cytokines and reduced suppressive function (4). The molecular mechanisms that drive Treg cell plasticity as well as the functional consequences for autoimmune diseases are largely unknown.

Glucocorticoids (GC) are best-known for their successful clinical usage as anti-inflammatory and immunosuppressive agents, despite their high potential for serious side effects. While the potency of (synthetic) GC as negative regulators of immune and inflammatory effector molecules at higher doses is well-documented, the effects of endogenous GC on the immune response and T cells in particular are much less clear. GC suppress T cell activation, both indirectly by inhibiting dendritic cell function and directly by inhibiting TCR signaling (5). T cell-specific deletion of the glucocorticoid receptor (GR) revealed T cells as critical targets for endogenous GC to both limit clinical disease in an animal model for multiple sclerosis (6) and prevent lethal immunopathology in an animal model for toxoplasma infection (7). As both studies utilized the *lck* promoter to drive expression of Cre recombinase for conditional deletion of the GR, CD8$^+$ cytotoxic T cells, CD4$^+$ T helper cells, and Foxp3$^+$ Treg cells were GR-deficient. Treg cell development, steady-state homeostasis and function may be affected by GC, although reports are controversial. Administration of GC has been shown to increase both the proportion and number of murine CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in peripheral lymphoid organs (8). In line with this observation is the finding that Treg cells are relatively resistant to GC-induced apoptosis *in vitro* (9). In contrast, GC dose-dependently reduced both the proportion and total number of splenic Treg cells after repeated GC administration (10, 11). Likewise, therapeutic treatment of MOG-induced EAE with GC slightly reduced splenic Treg cell number and reduced Foxp3 expression levels (6). Human Treg cells accumulate relative to conventional T cells (Tcon) upon treatment of several autoimmune diseases with GC as reported for multiple sclerosis (12), systemic lupus erythematosus (13) and rheumatoid arthritis (14).

While effects of exogenous GC on Treg cells are obvious but controversial, it is not known whether endogenous GC regulate Treg cell homeostasis, both under steady state and inflammatory conditions. Lck-Cre GRfl/fl mice that lack the GR in all T cells, reportedly have reduced numbers of Treg cells in the thymus and periphery, but Treg cell function was not tested (15). Moreover, Treg cell homeostasis may be affected by GR-deficient conventional T cells that can give rise to pTreg cells.

We therefore generated mice with a specific deletion of the GR in Foxp3$^+$ Treg cells by crossing GRfl/fl (16) with Foxp3-Cre mice (17). Remarkably, while Treg cell number, expression of Treg cell signature molecules, and *in vitro* suppression capacity of GR-deficient Treg cells was unchanged, GR-deficient Treg cells appeared defective in suppressing T cell-driven colitis in an *in vivo* mouse model for inflammatory bowel disease (IBD). This phenotype was associated with the acquisition of Th1 cell-like features in GR-deficient Treg cells. These data suggest that endogenous GC stabilize Treg cell fate and function under inflammatory conditions and provide a rationale for the development of GC therapy for IBD that specifically targets Treg cells and expectedly reduces the strong side-effects of these hormones.

RESULTS

Verification of Specific GR Deletion in Foxp3$^+$ Treg Cells

Mice carrying a specific deletion for the GR in Foxp3$^+$ Treg cells (Foxp3-YFP-iCre x GRfl/fl mice; dubbed here: Foxp3-Cre GRfl/fl mice) developed normal and did not show any signs of disease. Lack of GR in Foxp3$^+$ Treg cells was confirmed at the protein level both in spleen (**Figure 1A**) and thymus (**Figure S1A**). Ectopic recombination by Cre-YFP expressed under the control of the FoxP3 promoter of some conditional alleles (*Cd28*), but not others (*R26-RFP*), has been reported (18). However, quantification of the GR in conventional CD4$^+$CD25$^-$ Foxp3$^-$ T cells, CD8$^+$ T cells and B cells revealed no differences between wild type (WT), Foxp3-Cre and Foxp3-Cre GRfl/fl mice (**Figure S1B**), ruling out promiscuous Foxp3-Cre expression in these lymphocyte subsets. Since endogenous GC have been shown to regulate T cell numbers (19, 20), we determined peripheral blood levels of corticosterone in our mouse strains to check for potential differences. However, this appeared not to be the case as no differences in corticosterone levels were found (**Figure 1B**). Expression levels of *Nr3c1* (encoding the GR) by CD4$^+$CD25$^-$ Tcon cells and CD4$^+$Foxp3$^+$ Treg cells were quantified by qPCR. Splenic Treg cells from heterozygous Foxp3-Cre GRwt/fl mice expressed *Nr3c1* at approximately half of control Treg cells from Foxp3-Cre mice (**Figure 1C**). Finally, Treg cells derived from Foxp3-Cre GRfl/fl mice were resistant to *in vitro* corticosterone-induced cell death, confirming the absence of the GR at the functional level (**Figure S1C**). Thus, Foxp3-Cre GRfl/fl mice lack the GR specifically in Foxp3$^+$ Treg cells with no signs of significant recombination in CD4$^+$ Tcon cells or other lymphocyte subsets.

Basic Immune Characteristics of Mice Lacking the GR in Treg Cells

Foxp3-Cre GRfl/fl mice showed normal CD4$^+$Foxp3$^+$ Treg (**Figure 2A**, left panel) and CD4$^+$ Tcon (**Figure 2A**, right panel) cell numbers in the thymus and spleen. Next, we examined steady state expression of Treg cell signature molecules such as Foxp3,

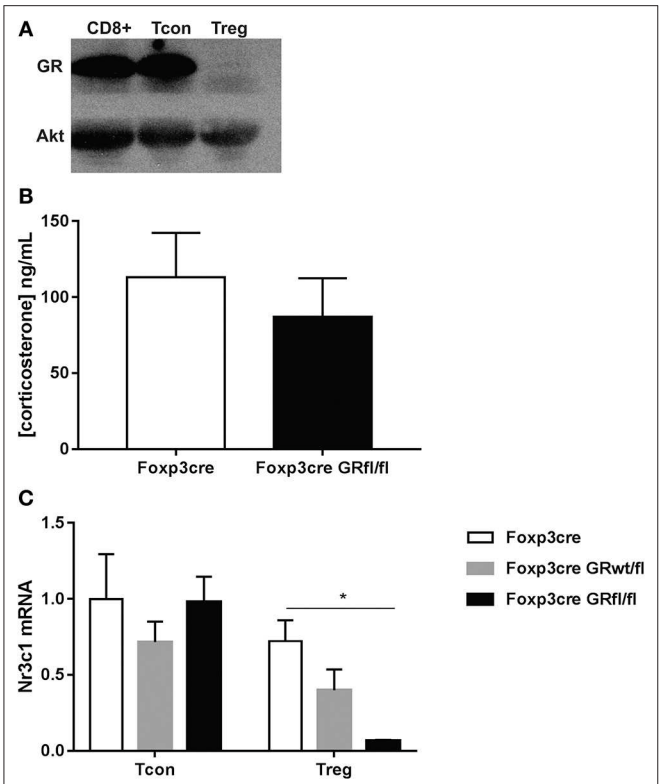

FIGURE 1 | Physical characterization of GR deletion in Foxp3$^+$ Treg cells. **(A)** Immunoblotting shows GR protein expression in purified CD8$^+$, CD4$^+$CD25$^-$ Tcon, and CD4$^+$Foxp3$^+$ Treg cells from Foxp3-Cre GRfl/fl splenocytes. **(B)** Serum samples from Foxp3-Cre and Foxp3-Cre GRfl/fl mice were analyzed for corticosterone content by ELISA. **(C)** Real time qPCR analysis of *Nr3c1* (GR) mRNA expression in CD4$^+$CD25$^-$ Tcon and CD4$^+$Foxp3$^+$ Treg cells from Foxp3-Cre, Foxp3-Cre GRwt/fl and Foxp3-Cre GRfl/fl mice. Nr3c1 mRNA expression levels are referred to mRNA levels of CD4$^+$ Tcon cells from Foxp3-Cre mice according to the $\Delta\Delta$Ct relative quantification method. Data are shown as mean ± SEM ($n \geq 3$).

CD25, GITR, and CTLA-4 by Treg cells of Foxp3-Cre and Foxp3-Cre GRfl/fl mice. In both thymus and spleen, expression levels of these markers were comparable between GR-expressing and GR-deficient Treg cells, except for thymic GITR that showed significantly higher expression in Treg cells from Foxp3-Cre GRfl/fl mice (**Figure 2B**, left panel). Since GITR expression levels are critical for Treg cell maturation (21), we further analyzed CD4$^+$Foxp3$^+$ thymocytes for GITR^{int} and GITR^{high} expressing subsets. A moderately enhanced frequency of Foxp3$^+$GITR^{high} and a reduction of Foxp3$^+$GITR^{low} cells in Foxp3-Cre GRfl/fl mice was observed, as compared to control Foxp3-Cre mice (**Figure 2C**). The functional relevance of this observation is presently unclear, yet this suggests that basal GITR expression in splenic Treg cells is not dependent on a functional GR. Treg cells consist of both naïve CD44^{low}CD62L^{high} and CD44^{high}CD62L^{low} "effector-like" subpopulations, the latter exerting suppressor activity (22). We therefore analyzed the fractions of both Treg cell subsets in the context of GR-deficiency and found equal amounts in both Foxp3-Cre and Foxp3-Cre GRfl/fl mice (**Figure 2D**). The transcription factor Helios has been proposed as a marker to discriminate tTreg from pTreg cells (3). Examination of both fractions and Helios expression levels, however, revealed no changes between Treg cells from Foxp3-Cre and Foxp3-Cre GRfl/fl mice (**Figure S2A**). Finally, activation of CD4$^+$ Tcon and CD8$^+$ T cells by anti-CD3/anti-CD28 antibodies was comparable in Foxp3-Cre and Foxp3-Cre GRfl/fl mice regarding induction of CD44 and production of IFN-gamma (**Figure S2B**). In summary, deletion of the GR in Treg cells does not modify their basal cell number, phenotype or activation competence.

In vivo Survival of Treg Cells Does Not Depend on GR Expression

To directly assess the impact of GR deletion in Treg cells on their survival in a competitive setting, we generated heterozygous female Foxp3-Cre/wt GRfl/fl mice. As *Foxp3* is located on the X chromosome, random inactivation of one allele in these mice is predicted to produce 50% of Treg cells that use the WT allele (i.e., GR-sufficient) and 50% of Treg cells that use the Foxp3-Cre allele (i.e., GR-deficient). In spleen, but not in thymus, we observed a moderate competitive disadvantage of Treg cells expressing the Foxp3-Cre allele (**Figure 3A**), a finding previously reported by others (23). However, in both thymus and spleen, equal proportions of WT and GR-deficient Treg cells were generated and/or survived, suggesting that the GR does not influence survival of Treg cells in a physiologically normal setting (**Figure 3A**). The observation that the Foxp3-Cre allele may affect peripheral Treg cell survival, together with the finding that the Foxp3-Cre allele is mildly hypomorph as reported by others (18), prompted us to determine Foxp3 expression levels in WT and Foxp3-Cre-expressing mouse strains. In agreement with Franckaert et al. (18) we found a ∼30% reduction of Foxp3 protein expression in mice expressing the Foxp3-Cre allele as compared to WT mice (**Figure 3B**, left panel), whereas all mouse strains expressing the Foxp3-Cre allele displayed equal amounts of Foxp3 (**Figure 3B**, right panel). Since we did not find deviations produced by the Foxp3-Cre allele other than those shown in **Figure 3**, mice expressing this allele were used as controls in our experiments (Foxp3-Cre mice). Finally, to test for potential epigenetic changes in critical regions of the *Foxp3* locus we analyzed the methylation status as described previously (24). However, the degree of methylation of CpG islands within the *Foxp3* locus appeared comparable in WT, Foxp3-Cre, and Foxp3-Cre GRfl/fl mice (**Figure S3**), supporting the finding that Foxp3 expression is unchanged in the absence of the GR (**Figure 3B**). Hence, *in vivo* survival of Treg cells and expression of their lineage specification factor Foxp3 is, at least under basal conditions, not dependent on expression of the GR by these cells.

Antinuclear Antibody Prevalence Is Increased in Foxp3-Cre GRfl/fl Mice

Since both Treg cell number and function change with age, we analyzed 13 months old Foxp3-Cre and Foxp3-Cre GRfl/fl mice for splenic Treg cell number and found no major changes between these genotypes (**Figure 4A**; **Figure S4**). Scurfy mice, who are deficient for regulatory T cells, develop antinuclear

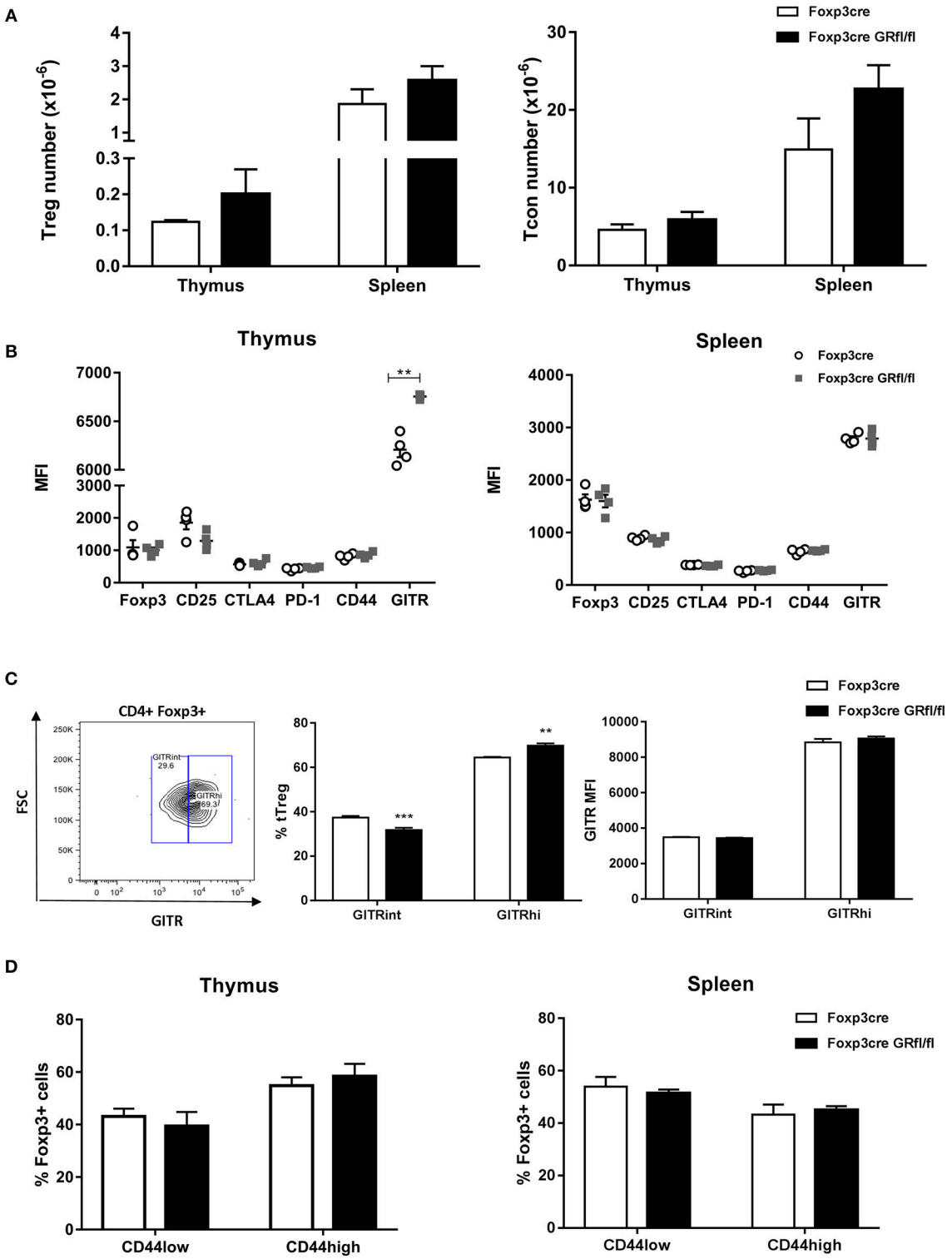

FIGURE 2 | Immune characteristics of Foxp3-Cre GRfl/fl mice. **(A)** Thymi and spleens from Foxp3-Cre and Foxp3-Cre GRfl/fl mice were analyzed for cellularity of $CD4^+Foxp3^+$ Treg (left panel) and $CD4^+CD25^-$ Tcon cells (right panel). **(B)** Treg cell signature marker expression by thymic (left panel) and splenic (right panel) $CD4^+Foxp3^+$ Treg cells. Data shown are median immunofluorescence intensity values from individual Foxp3-Cre or Foxp3-Cre GRfl/fl mice. **(C)** Thymic Treg cells were divided into subsets according to their GITR expression levels ($GITR^{int}$ or $GITR^{high}$; left panel: gating; middle panel: frequency; right panel: MFI). **(D)** CD44 expression level of thymic (left panel) or splenic (right panel) $CD4^+Foxp3^+$ Treg cells from Foxp3-Cre and Foxp3-Cre GRfl/fl mice. Data are shown as mean ± SEM ($n = 4$).

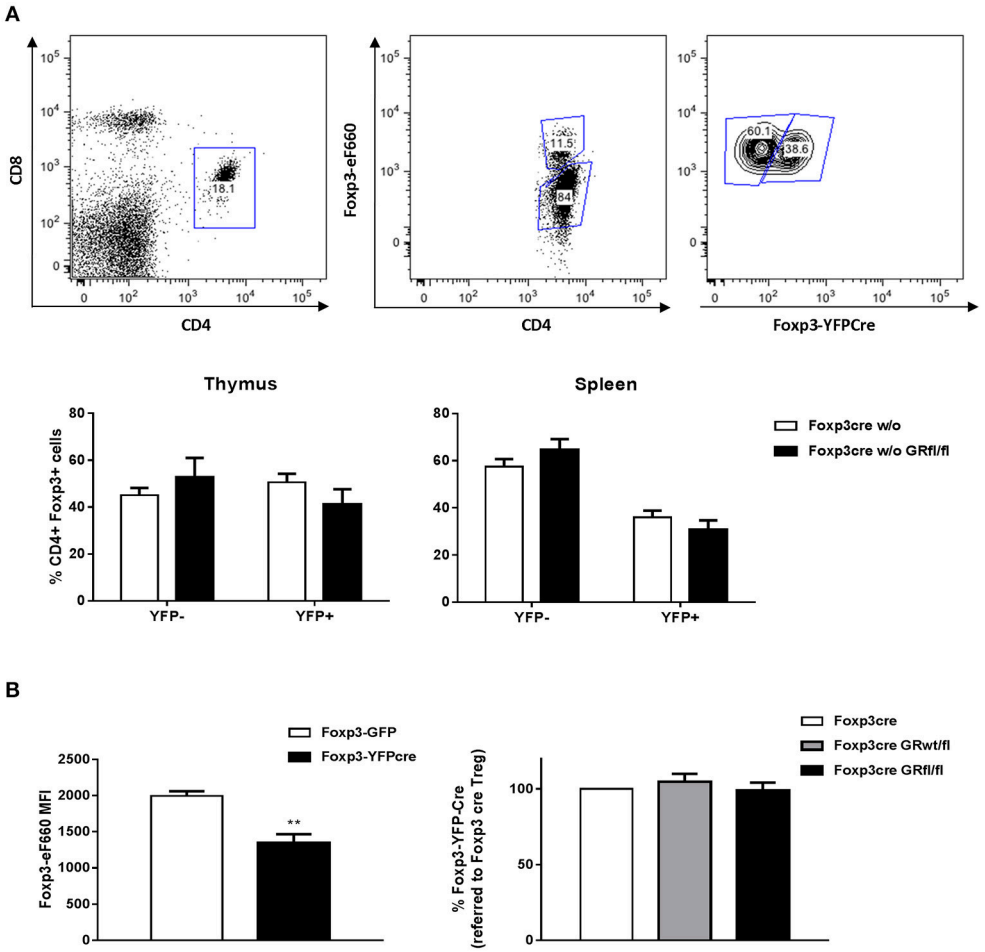

FIGURE 3 | Treg cell survival does not depend on GR expression. **(A)** $CD4^+Foxp3^+$ Treg cells from heterozygous female Foxp3-Cre/wt and Foxp3-Cre/wt GRfl/fl mice were divided into YFP^+ (Cre^+) and YFP^- (Cre^-) cells according to the gating strategy shown in the upper panels. Lower panels show YFP^+ vs. YFP^- fractions in the thymus (left panel) or spleen (right panel). $CD4^+Foxp3^+YFP^+$ Treg cells from heterozygous female Foxp3-Cre/wt GRfl/fl mice are GR-deficient whereas $CD4^+Foxp3^+YFP^-$ Treg cells are GR-sufficient. **(B)** Foxp3 expression levels of Treg cells from WT (Foxp3-GFP reporter) and Foxp3-YFP-Cre mice (left panel). Data are shown as mean ± SEM ($n = 3$). The right panel shows Foxp3 expression in Treg cells from Foxp3-Cre, Foxp3-Cre GRwt/fl and Foxp3-Cre GRfl/fl mice. Data are shown as mean ± SEM ($n = 5$).

antibodies (ANA) and lupus-like disease (25, 26). When sera of GR-deficient Treg cell mice were investigated for the presence of ANA, it appeared that higher fractions were positive as compared to Foxp3-Cre mice (**Figures 4B,C**). This observation appeared to be sex independent. Accordingly, Treg cell-intrinsic expression of the GR seems to prevent loss of tolerance to these autoantigens with age.

Defective Function of GR-Deficient Treg Cells *in vivo* but not *in vitro*

The increased presence of ANA in our GR-deficient Treg cell mice prompted us to study the suppressive capacity of their Treg cells, first tested in an *in vitro* assay. Naïve $CD4^+$ Tcon cells from Foxp3-Cre control mice were stimulated with anti-CD3 mAb in the presence of irradiated antigen presenting cells (APCs) and co-cultured with different Treg cell numbers derived from Foxp3-Cre or Foxp3-Cre GRfl/fl mice. Proliferation of Tcon cells, assessed after 3 days, was potently suppressed by Treg cells, however, Foxp3-Cre and Foxp3-Cre GRfl/fl Treg cells exhibited an equal inhibitory capacity (**Figure 5A**). Similar results were obtained when Treg cells from Foxp3-Cre or Foxp3-Cre GRfl/fl mice were compared for their ability to inhibit proliferation of $CD4^+CD44^+CD62L^-$ memory T cells (**Figure S5A**).

Since many autoimmune-prone mouse strains carrying Treg cell specific mutations have normal Treg cell suppressor function *in vitro* (3), we set out for *in vivo* functional testing of GR-deficient Treg cells in a mouse model for inflammatory bowel disease, i.e., T cell transfer colitis in $RAG1^{-/-}$ mice (27). These mice produce no mature T cells or B cells and develop colitis upon transfer of Treg cell-depleted $CD4^+Foxp3^-CD25^-CD45RB^{high}$ Tcon cells (WT-Tcon only; **Figure 5B**). Co-transfer of $CD4^+Foxp3^+CD25^+CD45RB^{low}$ Treg cells from Foxp3-Cre mice (WT-Tcon + Foxp3-Cre Treg) prevented, as expected, the development of disease. Strikingly, Treg cells derived

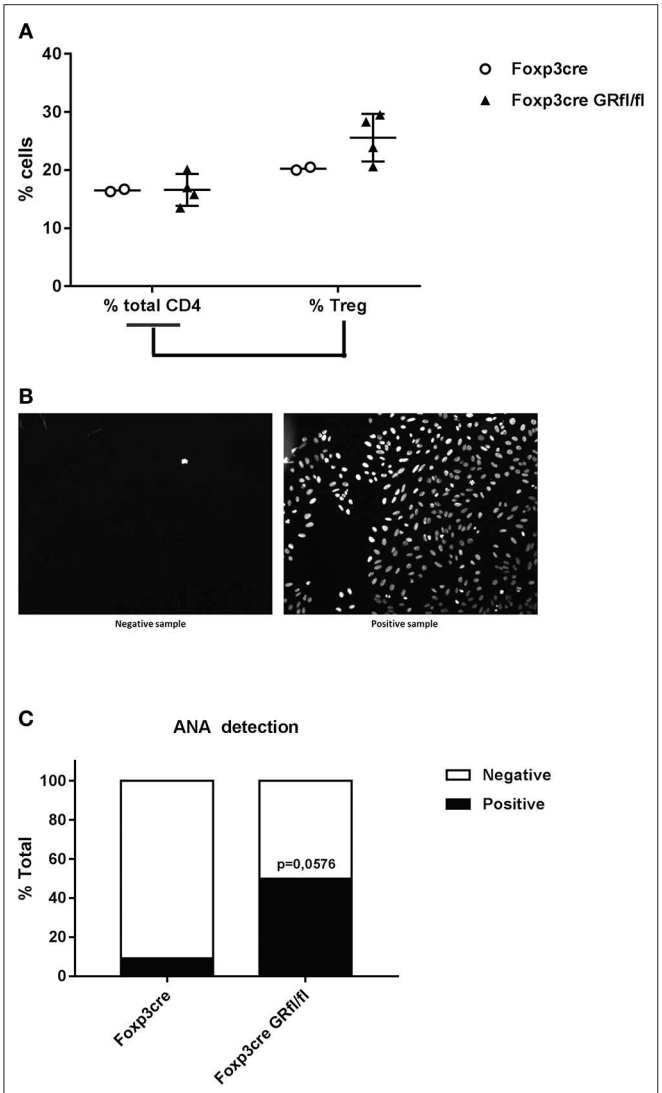

FIGURE 4 | Increased frequency of antinuclear antibodies (ANA) in Foxp3-Cre GRfl/fl mice. **(A)** Flow cytometry analysis of splenic total $CD4^+$ and $CD4^+Foxp3^+$ Treg cells (expressed as a percentage of total $CD4^+$ cells) from Foxp3-Cre and Foxp3-Cre GRfl/fl mice. **(B)** Example of ANA determination by immunofluorescence. Mouse sera were incubated with HEp-2 cells and the presence of ANA was determined by indirect immunofluorescence microscopy. The sample on the left is ANA negative, while the sample on the right is considered ANA positive. **(C)** Presence of ANA in sera from 8 to 13 months old Foxp3-Cre and Foxp3-Cre GRfl/fl mice. Data are shown as mean ± SEM ($n \geq 7$).

from Foxp3-Cre GRfl/fl mice were largely ineffective under these experimental conditions (WT-Tcon + Foxp3-Cre GRfl/fl Treg; **Figure 5B**).

The failure of GR-deficient Treg cells to protect against colitis was also evident from histological assessment of intestinal inflammation (**Figure S5B**). Blinded grading of colonic inflammation revealed complete protection in mice treated with control Treg cells whereas suppression of intestinal inflammation in mice treated with GR-deficient Treg cells was incomplete.

Analysis of the Tcon:Treg cell ratio in spleens of $RAG1^{-/-}$ mice sacrificed after 4 weeks revealed a striking 8-fold higher ratio in mice receiving Treg cells from Foxp3-Cre GRfl/fl, as compared to Foxp3-Cre mice (**Figure 5C**, left panel), indicating strong relative expansion of Tcon cells in the presence of GR-deficient Treg cells (**Figure 5C**, right panel).

Since expansion of Tcon cells is consistent with a pro-inflammatory phenotype, we next studied pro-inflammatory cytokine expression by splenic Tcon and Treg cells. The fraction of Tcon cells producing IFN-gamma after 4 weeks was highest in mice with the strongest disease symptoms, i.e., those receiving either Tcon cells (WT-Tcon only) or those co-injected with Tcon cells plus Treg cells from GR-deficient Treg cell mice (WT-Tcon + Foxp3-Cre GRfl/fl Treg; **Figure 5D**). Remarkably, significantly more Treg cells producing IFN-gamma (**Figure 5D**), but not IL-17 (**Figure 5E**), were present in mice that were treated with GR-deficient Treg cells than in mice receiving control Treg cells. Treg cell signature marker expression levels were similar between control and GR-deficient Treg cells (Foxp3, GITR and CD25), with the exception of CTLA-4 which was significantly elevated on GR-deficient Treg cells (**Figure 5F** and **Figure S5C**). Analysis of IFN-gamma producing Treg cells for Foxp3 expression levels revealed no significant differences between GR-deficient and GR-proficient Treg cells (**Figure S5D**).

Further in depth analysis of Treg cell markers and subsets was performed on splenic Treg cells that were used in the transfer colitis experiments, i.e., $CD4^+Foxp3^+CD45RB^{low}$ cells (for gating, see **Figure S6A**) derived from Foxp3-Cre and Foxp3-Cre GRfl/fl mice, revealing no differences regarding expression levels of Foxp3, CD25, Latency Associated Peptide (LAP), Lymphocyte-activation gene 3 (LAG-3), PD-1 and GITR (**Figure S6A**, right panel). Fractions of $CD4^+Foxp3^+CD45RB^{low}$ cells expressing these markers were also similar in both mouse strains with the exception of a reduction in PD-1 expressing cells (**Figure S6A**, middle panel). We next analyzed the presence of two recently described Treg cell subsets, i.e., $GITR^{high}PD\text{-}1^{high}CD25^{high}$ ($Triple^{high}$) Treg cells, which reportedly control *in vivo* lymphocyte proliferation, and $GITR^{low}PD\text{-}1^{low}CD25^{low}$ ($Triple^{low}$) Treg cells, which have been shown to limit colitis (28). Interestingly, while the fraction of $Triple^{high}$ Treg cells appeared reduced in Foxp3-Cre GRfl/fl mice, $Triple^{low}$ Treg cells were not significantly changed as compared to Foxp3-Cre mice (**Figure S6B**, lower left panel). In addition, mean expression levels of GITR, PD-1, and CD25 were similar between both mouse strains (**Figure S6B**, lower right panel).

A different Treg cell subset which may suppress colitis has the phenotype $Foxp3^{low}CD25^-GITR^+$, designated GITR single-positive cells (29). A comparison of this subset in spleens from Foxp3-Cre and Foxp3-Cre GRfl/fl mice revealed, however, no significant differences (**Figure S6C**).

Taken together, our findings during experimental intestinal inflammation indicate that GR-deficient Treg cells, while retaining expression of Treg cell markers, acquired an increased plasticity toward a Th1-like Treg cell phenotype that was accompanied by a reduction in the suppressive capacity of these cells.

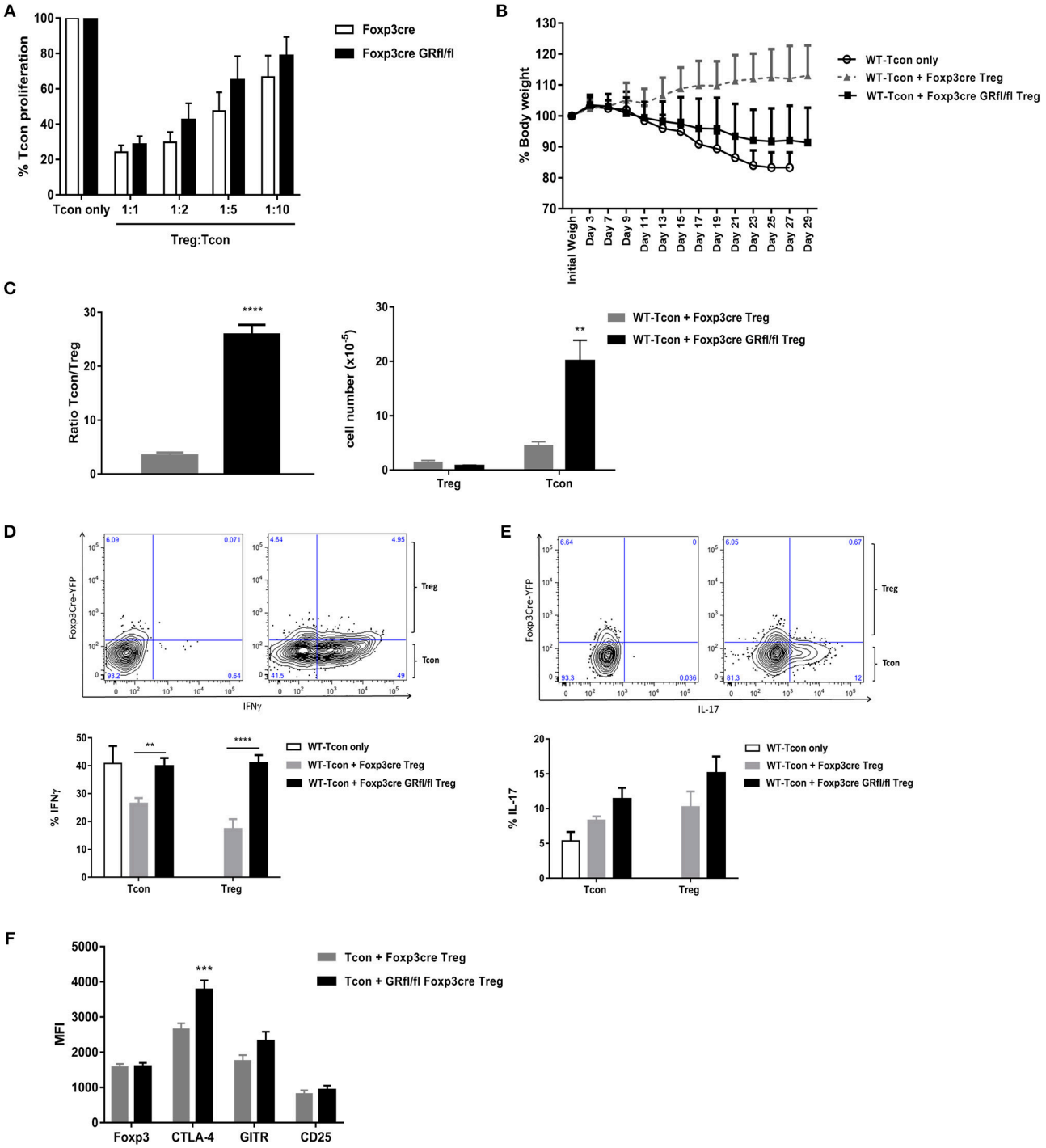

FIGURE 5 | Suppression capacity of GR-deficient Treg cells is defective *in vivo* but not *in vitro*. **(A)** *in vitro* T cell suppression assay: WT $CD4^+Foxp3^-CD25^-CD45RB^{high}$ Tcon cells were cultured either alone or co-cultured at different ratios with $CD4^+Foxp3^+CD25^+CD45RB^{low}$ Treg cells derived from Foxp3-Cre or Foxp3-Cre GRfl/fl mice. Data are shown as mean ± SEM ($n = 5$). **(B)** T cell transfer model of colitis in $RAG1^{-/-}$ mice. WT $CD4^+Foxp3^-CD25^-CD45RB^{high}$ Tcon cells were either transferred alone (WT-Tcon only) or co-transferred with $CD4^+Foxp3^+CD25^+CD45RB^{low}$ Treg cells from Foxp3-Cre or Foxp3-Cre GRfl/fl mice. Body weight was assessed over time and animals were sacrificed either when weight loss exceeded 15% or 4 weeks after cell transfer (day 29) **(C)** Splenic $CD4^+CD25^-$ Tcon and $CD4^+Foxp3^+$ Treg cells were enumerated (right panel) and the ratio between these subsets calculated (left panel). *In vitro* cytokine production of IFN-gamma **(D)** and IL-17 **(E)** by either splenic $CD4^+CD25^-$ Tcon or $CD4^+Foxp3^+$ Treg cells obtained from $RAG1^{-/-}$ mice treated and sacrificed as described in **(B)**. Representative gating strategy for IFN-gamma [**(D)**, upper panels] and IL-17 [**(E)**, upper panels] shows unstained samples (upper left panels) and cytokine-stained samples (upper right panels), derived from a mouse receiving WT-Tcon + GR-deficient Treg cells. **(F)** Treg cell signature marker expression by splenic Treg cells taken from mice described in **(B)**. Data are shown as mean ± SEM ($n \geq 7$).

DISCUSSION

Here, we show that GR-deficient Treg cells, while displaying no changes in function under basal conditions, are defective under inflammatory conditions and gain the ability to produce effector cytokines that are characteristic for Th1-like Treg cells. In line with their general inhibitory properties during inflammation, endogenous GC are apparently required to prevent Treg cell plasticity that is associated with reduced suppressive function.

The finding that GR-deficient Treg cell mice have normal numbers of Treg cells in both thymus and spleen differs from a report by Mittelstadt et al. (15) showing reduced Treg cell numbers in both organs in mice deficient for the GR in all T cells (Lck-Cre GRfl/fl mice). However, the lack of the GR in non-Treg T cells in Lck-Cre GRfl/fl mice may account for this difference. Supporting the view that GC, at least under basal conditions, do not influence Treg cell homeostasis are the results of the competitive experiments in heterozygous female Foxp3-Cre/wt GRfl/fl mice, clearly showing that in a physiologically normal environment no differences were observed in development and/or survival between WT and GR-deficient Treg cells.

GC may regulate T cell number in both thymus and peripheral lymphoid organs but data from previous studies are conflicting and Foxp3$^+$Treg cells were not specifically analyzed. Thymocyte number and subset distribution in different mouse strains targeting exon 2 has been shown to be unchanged (30, 31). Studies conditionally targeting exon 3 of the GR revealed either no changes in thymocyte numbers (32) or a clear reduction (15) without a change in major subset composition. Similar results were found for peripheral T cell numbers in both mouse strains. It is currently unclear why both mouse models targeting exon 3 display substantial differences with respect to the size of their T cell pools. Conversely, transgenic mice overexpressing the GR 2-fold selectively in T cells showed a reduction in both thymocyte and peripheral T cell numbers (19). Moreover, transfer of bone marrow cells from mice expressing a gain of function GR knock-in into irradiated WT mice revealed a strong reduction of T-cell numbers analyzed ten weeks later, as compared to irradiated mice that received bone marrow from WT mice (33). Collectively, these findings suggest that endogenous GC regulate T cell homeostasis to some extent and such control may be more pronounced once peripheral GC concentrations are elevated which reportedly occurs upon activation of the immune system (34).

Expression of Treg cell signature molecules by Treg cells were similar between Foxp3-cre and Foxp3-Cre GRfl/fl mice, with the exception of a small, but significant increase in frequency of GR-deficient thymic Foxp3$^+$ cells expressing the TNF receptor superfamily member GITR at high levels. Since on the one hand GITR regulates Treg cell development and correlates with TCR signal strength (21, 35) and, on the other hand, GC reportedly induce GITR in T cell hybridoma cells (36), this observation seems counterintuitive at first sight. However, GC were shown to induce very limited upregulation of GITR in primary CD4$^+$ T cells, whereas TCR signaling appears to be a much stronger inducer of GITR than GC (37). Moreover, TCR signaling in the presence of GC seems to reduce GITR expression in these cells as compared to TCR signaling alone (37). Our observation that GITR expression is increased in GR-deficient Treg cells suggests that endogenous GC may inhibit TCR-induced GITR in Foxp3$^+$ Treg cells as well. According to the "mutual antagonism" hypothesis, crosstalk between GR signaling and TCR signaling leads to survival of conventional T cells bearing TCRs that build up a repertoire that is required for a robust adaptive immune response (15, 38). Whether GC also change the TCR repertoire of Treg cells and, by this means, affect their functional competence, remains to be established. Our finding that splenic GITR^{high}PD-1^{high}CD25^{high}Foxp3$^+$CD45RB^{low} Treg cells were reduced in Foxp3cre GR/fl/fl mice does not point to an increased TCR affinity for self-antigens of these GR-deficient Treg cells, at least in the periphery.

While Treg cell signature molecule expression and the suppressive capacity of GR-deficient Treg cells on *in vitro* Tcon cell proliferation appeared unaffected, the increased presence of ANA in Foxp3-Cre GRfl/fl mice at older age provided the first indication for a potentiating role of the GR in Treg cell function. Since we did not observe significant changes in both the percentage and the absolute Treg cell number in our mouse strains, we assume that the functional competence of Treg cells decreases with age in the absence of cell-intrinsic GR expression. Alternatively, in the GR-deficient Treg cell population, we detected a reduced fraction of GITR^{high}PD-1^{high}CD25^{high} Treg cells, which reportedly inhibit *in vivo* lymphocyte proliferation (28). However, whether this observation contributes to the increased presence of ANA in Foxp3-Cre GRfl/fl mice at older age is currently not known. The second observation that the GR is required for full Treg cell function was made in the transfer colitis model. Our findings suggest that under inflammatory conditions Treg cells that lack the GR may become unstable regarding their suppressive regulatory T cell function. Moreover, it seems that GR-deficient Treg cells gained the ability to produce effector cytokines that are characteristic for Th1 cells. Such Th1-like Treg cells producing IFN-gamma (but maintaining Foxp3 expression) have been reported to be present at an increased frequency in both mouse models (39) and patients with autoimmune diseases such as type 1 diabetes (40) or multiple sclerosis (41). The physiological relevance of the plasticity and instability of helper T cell-like Treg cells (Th1-, Th2-, and Th17-like Tregs) and their role in the development of autoimmune diseases has yet to be clarified. Moreover, the molecular mechanisms and the environmental signals that trigger the development of helper T cell-like Treg cells in general and Th1-like Treg cells in particular are largely unknown (4). Our data suggest that endogenous GC act as an environmental signal to prevent Treg cell differentiation into Th1-like Treg cells and maintain Treg cell function in a T cell transfer model of colitis. The failure of GR-deficient Treg cells to respond to GC that are produced at increased levels during immune system activation (34), notably not only by the adrenals but also locally by the intestine itself (42), most likely leads to dysfunctional Treg cells in this disease model. Indeed, in another model of experimental colitis, dextran sodium sulfate (DSS)-induced colitis, endogenous circulating corticosterone levels were increased (43).

The proportion of functionally impaired GR-deficient Treg cells producing IFN-gamma more than doubled compared to control Treg cells but retained Foxp3, GITR and CD25 expression, whereas CTLA-4 was higher. IFN-gamma was shown by others to be involved in a functional defect of IFN-gamma$^+$Foxp3$^+$ Treg cells lacking Foxo1 (44). Foxo1-deficient Treg cells, which display a Th1-like phenotype, did not prevent disease in the T cell transfer colitis model. However, these Treg cells were partially protective when *Ifng* was deleted in addition to *Foxo1*. Phenotypically, Foxo1-deficient Treg cells expressed similar Foxp3, increased CD25 and marginally reduced CTLA-4 levels, compared to WT control Treg cells (44). In contrast, antigen (flagellin)-specific IFN-gamma$^+$Foxp3$^+$ Treg cells tested for suppression capacity in the same model of chronic colitis were found to maintain their regulatory function without reporting on Treg cell marker expression (45). Hence, it remains to be clarified on the one hand whether IFN-gamma$^+$Foxp3$^+$ Treg cells in general play a pathogenic or protective role in this setting and on the other hand which environmental signals and signaling pathways are responsible for driving the induction of IFN-gamma$^+$Foxp3$^+$ Treg cells. With respect to GR-deficient Treg cells, the generation of an animal model where IFN-gamma would be deleted together with the GR in Foxp3$^+$ Treg cells (double-deficient Treg cells) would clarify whether Treg cell-derived IFN-gamma is causal for the dysfunction of GR-deficient Treg cells in experimental colitis.

The molecular mechanisms of how GR signaling prevents Treg cell plasticity and functional instability in transfer colitis are presently unknown. GC have been shown to upregulate *Foxp3* mRNA in CD4$^+$ T cells of asthmatic patients (46) and in murine splenic CD4$^+$CD25^{high} cells (11). Furthermore, the GR has been shown to interact with Foxp3 at the protein level as part of large multiprotein complexes (47). Conversely, Foxp3 binds the *Nr3c1* locus (47) and increases *Nr3c1* mRNA expression in thymic Treg cells (48), suggesting that both Foxp3 and GR are able to mutually regulate each other's expression levels and likely also their downstream targets. Supporting the view that the GR enhances Treg cell function is the observation that GC treatment of patients suffering from myasthenia gravis or multiple sclerosis not only improved clinical disease symptoms but also enhanced Treg cell function (12, 49) and inhibitory cytokine production (12), as compared to untreated patients.

Despite being defective under inflammatory conditions *in vivo*, the inhibitory potency of GR-deficient Treg cells in the *in vitro* suppression assays was not affected, irrespective of whether naïve Tcon or Tmem cells were used as target cells. The apparently contrasting results between *in vitro* and *in vivo* Treg cell functional assays have been previously reported in several other mouse models carrying a Treg cell specific deletion or mutation of a given gene (3). To explain this discrepancy, the current view is that Treg cells do not use one particular mechanism by which they exert their suppressor function, but rather use several pathways simultaneously, especially *in vivo* (3).

Collectively, our findings demonstrate that the GR is critical for Treg cell function under inflammatory conditions. Endogenous GC levels are typically increased in the course of immune and inflammatory responses and may, by GR signaling, counterregulate the acquisition of Th1 cell-like characteristics by Treg cells, such as the production of IFN-gamma, that would reduce their potency to suppress inflammation. Future studies will determine whether the loss of GR in Treg cells also accounts for increased Treg cell plasticity in other inflammatory and autoimmune disease models. If that would be the case, it may be justified to develop GC therapies for autoimmune and inflammatory disorders that specifically target Treg cells in order to reduce the strong side-effects of these hormones.

MATERIALS AND METHODS

Mice

GRfl/fl mice (16) were bred on a C57BL/6 background to mice expressing Foxp3-YFP/Cre as a knocked-in YFP/iCre-recombinase fusion protein from the Foxp3 locus (17) to generate mice with GR-deficient Treg cells (Foxp3-YFP-Cre GRfl/fl mice). Foxp3-YFP-Cre mice were used as littermate controls for Foxp3-YFP-Cre GRfl/fl mice. Animals were housed in the Central Laboratory Animal Facilities of the Medical University of Innsbruck under standard light cycles and temperatures, and food and tap water were available *ad libitum*. C57BL/6 Foxp3-GFP reporter mice (50) were purchased from Jackson Labs (Bar Harbor, ME, USA) and served as CD4$^+$ Tcon cell donors for the T cell transfer colitis experiments. RAG1$^{-/-}$ mice were a kind gift from A. Moschen, Department of Internal Medicine II, Medical University Innsbruck. All animal experiments were performed in accordance with the Austrian "Tierversuchsgesetz" (BGBl. Nr. 501/1988 i.d.F. 162/2005) and have been granted by the Bundesministerium für Bildung, Wissenschaft und Kultur (bm:bwk).

Flow Cytometry

Cell suspensions were prepared in KDS-BSS buffer containing 10% FCS. Cells were stained with combinations of the following antibodies for 20 min at 4°C: anti-CD4-PerCP/Cy5.5 (clone RM4-5) and anti-PD-1-PE (anti-CD279, clone J43) (both from eBiosciences, CA, USA); anti-CD8-PECy7 or anti-CD8-AF647 (clone 53-6.7), anti-B220-APC/Cy7 (clone RA3-6B2), anti-CD25-PE (clone 3C7) or CD25-BV421 (clone PC61), GITR-PE/Cy7 (clone YGITR765), CD45Rb-AF647 (clone C363-16A), LAP-PE (clone TW7-16B4), and CD223(LAG-3)-BV421 (clone C9B7W) (all from Biolegend, CA, USA); CD62L-PE (clone MEL-14) and CD44-BV510 (clone IM-7) (both from BD Biosciences; San Jose, CA). DAPI and Annexin-V (eBiosciences, CA, USA) were used to quantify or gate out apoptotic or dead cells.

For Foxp3 intracellular staining Foxp3/Transcription Factor Buffer set and anti-Foxp3-eF660 (clone FJK-16s) (both from eBiosciences) were used according to the manufacturer's instructions. For GR intracellular staining we used BD Cytofix and BD Cytoperm reagents (BD Pharmingen, CA, USA) and stained with anti-GR (clone D6H2L) (Cell Signaling, MA, USA), followed by a secondary antibody (goat anti-rabbit IgG AF647 (Invitrogen, OR, USA)). The same buffer

set was used for cytokine staining using anti-IFN-gamma-PE (clone XMG1.2), IL-17-AF647 (clone TC11-18H10.1), anti-CTLA-4-PE (clone UC10-4B9; all from BioLegend), anti-IL-10-PE (clone JESS-16E3) and anti-Helios-APC (clone 22F6; both from eBiosciences).

Cell Sorting

To obtain naïve conventional T cells (Tcon), $CD4^+Foxp3^-(GFP^-)CD25^-CD45RB^{high}$ cells were sorted from spleen and/or mesenteric lymph nodes from Foxp3-GFP reporter mice. Memory T (Tmem) cells ($CD4^+Foxp3^-(YFP^-)CD44^+CD62L^-CD45RB^{high}$) were sorted from splenocytes from Foxp3-YFP-Cre mice. $CD4^+Foxp3^+(YFP^+)CD25^+CD45RB^{low}$ Treg cells were isolated from Foxp3-YFP-Cre and Foxp3-YFP-Cre GRfl/fl mice. Cell sorting was performed using a FACSAria III cell sorter (Becton Dickinson) and purity of isolated cell populations was routinely at least 98%.

Cell Culture

For the GC sensitivity test, single cell suspension of splenocytes (1×10^6cells/mL) was incubated in flat bottom 96-well plates with corticosterone (Sigma, MO, USA) at 125 or 625 nM for 48 h and then analyzed for cell death as decribed (51). For TCR activation experiments, T cells (enriched by MACS) were seeded in anti-CD3 coated (5 μg/mL) 96-well round bottom plates and treated with soluble anti-CD28 (1 μg/mL; both antibodies from Biolegend) in the presence of 100 U/ml IL-2 (PreproTech, USA) in RPMI medium (supplemented with 50 uM beta-Mercaptoethanol, 100 U/mL Penicillin/Streptomycin, 2 mM L-Glutamine, 1 mM Na-Pyruvate, and non-essential amino acids), cultured for 48 h and then analyzed for activation status and cytokine production. For the *in vitro* T cell suppression assay, single cell suspensions were prepared from spleens and mesenteric lymph nodes from Foxp3-YFP-Cre and Foxp3-YFP-Cre GRfl/fl mice. 2×10^5/mL Tcon cells or Tmem were stained with a cell proliferation dye (CPD-eF450) (eBioscience) and cultured together with irradiated (30 Gy) splenocytes (8×10^5 cells/mL) in RPMI complete medium in 96-well round bottom plates. To induce cell proliferation, anti-CD3 (Biolegend, CA, USA) 0.5 μg/mL was added to the medium. In the indicated cases, different Treg cell concentrations were added to obtain Treg:Tcon ratios of 1:1, 1:2, 1:5, and 1:10. Cells were incubated for 72 h at 37°C and 5% CO_2 and then analyzed for cell proliferation by Flow cytometry. For cytokine staining experiments, splenocytes were stimulated with 50 ng/mL PMA (Fluka Biochemika) and 1 mg/mL Ionomycin (Sigma) for 4 h. During the last 3 h of cell culture Monensin (Biolegend) was added.

RNA Isolation and Quantitative RT-PCR (qPCR)

Total RNA was isolated from 1×10^5 sorted cells using Quick-RNA MicroPrep kit (Zymo Research, CA, USA) and cDNA was synthesized using iScript cDNA Synthesis Kit (BioRad, CA, USA), according to the manufacturer's instructions. Real time PCR was performed using the following TaqMan Gene Expression Assays: GR (*Nr3c1*; Mm00433833_mH) and Actin-beta (*Actb;* Mm00607939_s1) and Luminaris Color Probe Master Mix (all from Thermo Fischer Scientific, MA, USA). Quantitative RT-PCR was analyzed using the StepOnePlus system (Applied Biosystems, Thermo Fischer Scientific, MA, USA) according to the manufacturer's instructions. The results were normalized to *Actb* expression and evaluated using the ΔΔCt relative quantification method.

Antinuclear Antibodies (ANA) Detection

For detection of ANA we used Kallestad HEp-2 cell line 12-well slides from Bio-Rad (Hercules, California). Serum samples were diluted 1:50 and 1:100 and incubated on the slides according to the manufacturer's instructions. Fluorescence-labeled antibody AF488 donkey anti-mouse (Jackson ImmunoResearch Inc., West Baltimore Pike, West Grove, PA, USA) was used as a secondary antibody and slides were analyzed using a fluorescence microscope. Serum samples from MRL/lpr mice were used as positive controls for ANA detection.

T Cell Transfer Model of Colitis

$CD4^+Foxp3^-CD25^-CD45RB^{high}$ Tcon cells were sorted from congenic C57BL/6 Foxp3-GFP mice and injected i.p. into 6 to 15-weeks-old C57BL/6 $RAG1^{-/-}$ immunodeficient recipients (3×10^5 cells/mouse). 1.5×10^5 Foxp3-YFP-Cre or Foxp3-YFP-Cre GRfl/fl Treg cells ($CD4^+Foxp3^+CD25^+$ $CD45RB^{low}$) were co-injected i.p. where indicated. Mice were monitored every second day for wasting disease. Mice were sacrificed either when having lost >15% of their initial body weight or 4 weeks after cell transfer.

Histology of Intestinal Inflammation

Samples of mid-colon were fixed in buffered 4% formalin solution. Three millimeter paraffin-embedded sections were cut and stained with hematoxylin and eosin. Tissues were evaluated semi-quantitatively and assigned a grade of 0 to 4 in a blinded fashion. Grade 0: no changes observed, grade 1: discrete increased inflammatory cells in the lamina propria with granulocytes in the lamina epithelialis, grade 2: as grade 1 with scattered erosions of the mucosa, grade 3: increased inflammatory cells in the lamina propria and scattered crypt abscesses, grade 4: all signs of grade 3 plus more than 3 crypt abscesses per colon circumference in the scanning magnification.

Western Blot

Cellular subsets (3.5×10^5 cells/subset) were resuspended in Laemmli sample buffer and heated in boiling water for 5 min. Total proteins were loaded on 10% Bis-Tris acryl-amide gels and blotted on AmershamTM HybondTM-ECL nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). Rabbit anti-mouse GR (clone D6H2L) (Cell Signaling, MA, USA) and rabbit anti-mouse AKT (Cell signaling Technology, Danvers, MA) were used for protein detection. All primary antibodies were diluted in 5% BSA in PBST and blots were incubated overnight at 4°C.

ELISA for Serum Corticosterone

Serum samples from Foxp3-YFP-Cre and Foxp3-YFP-Cre GRfl/fl mice were collected between 9 and 10 a.m. and analyzed for corticosterone content by ELISA (Enzo Life Sciences, CH) according to the manufacturer's instructions.

DNA Methylation Analysis

Genomic DNA was isolated from sorted $CD4^+CD25^-CD45RB^{high}$ conventional T cells as well as $CD4^+Foxp3^+CD25^+$ Treg cells sorted from WT Foxp3-GFP reporter, Foxp3-YFP-Cre mice and Foxp3-YFP-Cre GRfl/fl mice using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Bisulfite modification was performed using the EZ DNA Methylation-Gold Kit (ZymoResearch). MethyLight PCR analysis and the calculation of the percentage of methylated reference (PMR) were done as described previously (52, 53). Two Foxp3 assays (one reaction for DNA methylation analysis and one for internal reference, with a mean distance of −2.226 base pairs, or −3.866 base pairs respectively, to the transcription start site) were determined with the assistance of the computer program Primer Express version 2.0.0 (Applied Biosystems, Foster City, CA, USA). Primers used have been described previously (53).

Statistics

Estimation of statistical differences between groups was carried out using the unpaired Student's *t*-test or two-way ANOVA test, where appropriate. A chi-square test was used to test for differences between groups regarding prevalence of ANA. $P \leq 0.05$ were considered to indicate statistically significant differences. $^{ns}p \geq 0.05$; $^{*}p < 0.05$; $^{**}p \leq 0.01$; $^{***}p < 0.001$; and $^{****}p < 0.0001$.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

LR-R designed and performed most experiments, statistical analysis, prepared figures, and wrote paper. ST designed and performed experiments. LvR, MT, and HF performed experiments. MD performed histopathological assessment. HR interpreted data and wrote sections of the paper. AV designed research, interpreted data and wrote sections of the paper. DT and GW designed research, interpreted data, wrote paper, and conceived study.

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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.2019.00472/full#supplementary-material

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Dynamics of the Type I Interferon Response During Immunosuppressive Therapy in Rheumatoid Arthritis

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Objective: The type I interferon (IFN) response in rheumatoid arthritis (RA) has been extensively studied in relation to therapy with biological DMARDs (bDMARDs). However, the effect of conventional synthetic (cs)DMARDs and glucocorticoids (GCs) on IFN response gene (IRG) expression remains largely unknown, even though csDMARDS are used throughout all disease phases, including simultaneously with biologic therapy. This study was aimed to determine the dynamics of IFN response upon immunosuppressive treatment.

Methods: Whole blood was collected in PAXgene tubes from 35 RA patients who received either COBRA therapy (combination of prednisone, initially 60 mg, methotrexate and sulfasalazine) ($n = 14$) or COBRA-light therapy (prednisone, initially 30 mg, and methotrexate) ($n = 21$). Expression of 10 IRGs was determined by real-time PCR at baseline (T0), after 4 weeks (T4), and 13 weeks (T13) of treatment. IRG selection was based on the differential presence of transcription factor binding sites (TFBS), in order to study the therapy effect on different pathway components involved in IFN signaling.

Results: Seven of the 10 IRGs displayed significant changes during treatment ($p \leq 0.016$). These 7 IRGs all displayed a particularly pronounced decrease between T0 and T4 ($\geq$1.6-fold, $p \leq 0.0059$). The differences between IRG sensitivity to the treatment appeared related to the presence of TFBS for STAT1 and IRF proteins within the genes. The extent of the decreases between T0 and T4 was similar for the COBRA- and COBRA-light-treated group, despite the differences in drug combination and doses in those groups. Between T4 and T13, however, IRG expression in the COBRA-light-treated group displayed a significant increase, whereas it remained stable or decreased even further in most COBRA-treated patients (comparison of mean fold changes, $p = 0.011$). A significant association between IRG dynamics and clinical response to therapy was not detected.

Conclusions: Immunosuppressive treatment with csDMARDs, in this case a combination of prednisolone, methotrexate and sulfasalazine, substantially

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downregulates the IFN response in RA patients. The dynamics of this downregulation were partly dependent on the presence of TFBS within the IRGs and the combination and dosages of agents, but they were irrespective of the clinical response to therapy.

Keywords: rheumatoid arthritis, interferon, interferon response, biomarker, immunosuppression

INTRODUCTION

Early treatment of rheumatoid arthritis (RA) has proven effective in decreasing disease activity and limiting joint damage (1, 2). One treatment strategy which has shown effectiveness in early RA is COBRA (Dutch acronym for COmbinatietherapy Bij Reumatoïde Arthritis), which is a step-down strategy consisting of initial high dose prednisolone (60 mg per day), methotrexate (MTX) and sulfasalazine (SSZ). Due to rheumatologists' concerns with respect to the high initial prednisolone dose and the complexity of the drug schedule, COBRA-light strategy was introduced, which consists of a lower initial prednisolone dose (30 mg/day), combined with increasing doses of MTX (10–25 mg in 9 weeks) and no SSZ. The two strategies have shown to be similarly effective (3–5).

The use of glucocorticoids (GCs) such as prednisolone and conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs) such as MTX and SSZ is not restricted to early disease. In fact, these therapies are used throughout all phases of the disease, either as monotherapy or in combination, including simultaneously with biologic therapy (6).

With regard to biologic therapy, we have previously demonstrated that the predictive performance of the type I interferon (IFN) response gene set for non-response to rituximab was impaired when patients were using prednisolone at the moment of blood collection (7). Besides rituximab, IFN response gene (IRG) expression has also been described as a predictor for other bDMARDS such as anti-TNF agents and tocilizumab, and RA onset (8–11).

However, studies on the potential influence of csDMARD and GC (co-)medication yet remain scarce. Insight into the effect of these therapies on the IFN response, as well as the potential relation between IRG expression and the clinical response to csDMARD and GC therapy, are highly relevant in order to further understand the role of the IFN response in RA.

In vitro studies have shown that GC signaling could inhibit type I IFN signaling by competition for the same intracellular signaling components, i.e., the IFN regulatory factors (IRFs) (12, 13) and by inhibition of the transcription factor STAT1 (14). Accordingly, we have observed that RA patients who were treated with the GC prednisolone indeed displayed lower IRG expression compared to patients who had not received this treatment (7, 15). Although this decrease was not observed with methotrexate (MTX) use and appeared dependent on prednisolone dose, a causal relation could not be established due to the cross-sectional nature of the study. Moreover, since the study was performed in patients who were about to start on biologic therapy, hence who no longer benefitted from the csDMARD and GC therapies, an analysis in relation to clinical response to these therapies could not be made. The present study was focused on exploration of the IFN response during COBRA and COBRA-LIGHT therapy in RA. The sample collection within the COBRA and COBRA-light cohorts enabled us to investigate this in a longitudinal manner and additionally examine the potential relation with clinical response.

METHODS

Patients and Treatment

All patients in the current study participated in the COBRA-light study, a randomized, open, multicenter trial comparing two treatment schedules for the treatment of early RA (http://www.controlled-trials.com; ISRCTN55552928). Details of that study have been reported previously (3). In short, DMARD-naïve Dutch patients with recent-onset RA according to the 1987 revised American College of Rheumatology criteria (16) were included and randomized to the COBRA-light or COBRA strategy. Whereas, COBRA therapy consists of initially high-dose prednisolone (60 mg/day) combined with sulfasalazine (SSZ) and low-dose methotrexate (MTX) (7.5 mg/week), COBRA-light consists of a lower initial prednisolone dose (30 mg/day) but a higher starting dose of MTX (10 mg/week) and no SSZ.

For this study, 36 patients were selected based on availability of PAXgene tubes at baseline (T0), after 4 weeks (T4) and 13 weeks (T13) at the Amsterdam Rheumatology and Immunology Center, location Reade, Amsterdam, The Netherlands. Fifteen patients received COBRA therapy and 21 patients received COBRA-light therapy. Therapy response was defined as a Disease Activity Score in 44 joints (DAS) ≤ 2.4 after 26 weeks of treatment. Additionally, the change in DAS (ΔDAS) after 13 weeks and 26 weeks was also assessed.

This study was approved by the medical ethics committee of VU University Medical Center and Reade, Amsterdam, The Netherlands, and informed consent was obtained from all donors.

RNA Isolation and cDNA Synthesis

From each donor, blood was collected into a PAXgene tube (PreAnalytiX GmbH) at baseline and after 4 weeks and 13 weeks of treatment. The PAXgene tubes were stored at -20°C until further processing. After overnight thawing at room temperature, total RNA was isolated using the PAXgene Blood RNA kit (PreAnalytiX GmbH) according to the manufacturer's instructions. Total RNA concentration was measured using the Nanodrop spectrophotometer (ThermoFisher Scientific Inc.). From each sample, 250 ng RNA was reverse-transcribed into cDNA using a Revertaid H-minus cDNA synthesis kit (ThermoFisher Scientific Inc.).

TABLE 1 | IFN response gene selection.

Genes	Transcription factor binding sites				Reason for selection
	IRF proteins	STAT1	STAT3	NFκB	
INITIAL GENE SELECTION					
IFI44L	IRF7, IRF8	X	X	–	Technical control
IFI6	IRF7, IRF8, ISRE	–	–	–	IRF-specific
IFITM1	–	–	–	X	NFκB-specific
IL1RN	–	–	X	–	STAT-specific
MX1	IRF7, ISRE	X	X	–	Technical control
RSAD2	IRF7	–	X	–	Technical control
ADDITIONAL SELECTION					
HERC5	IRF7, ISRE	–	–	–	IRF-specific
IFITM2	–	–	X	X	IRF- and STAT1-lacking
LY6E	–	X	X	X	IRF-lacking
SERPING1	–	X	X	X	IRF-lacking

IRGs that contained a binding site for only one type of transcription factor were selected. Additionally, three other genes were included as technical controls.
"X" indicates that the gene contains a binding site for that transcription factor, whereas "–" indicates absence of the TFBS in that gene. ISRE; IFN stimulated response element, binding site of the ISGF3 complex which consists of STAT1, STAT2, and IRF9. Binding is IRF9-dependent, hence this is considered an IRF-specific binding site.

TABLE 2 | Cohort characteristics of the COBRA and COBRA-light groups.

	All patients	COBRA group	COBRA-light group
N	35	14	21
Age, years, median (IQR)	54 (45–60)	56 (44–61)	54 (45–59)
Female gender, *n* (%)	25 (71)	9 (64)	16 (76)
DAS at baseline, median (IQR)	4.0 (3.3–4.6)	4.0 (3.7–4.6)	4.0 (3.3–4.5)
DAS at T26, median (IQR)	1.7 (0.8–2.1)	1.2 (0.4–2.0)	1.8 (1.0–2.4)
DAS at T26 ≤ 2.4, *n* (%)	28 (80)	12 (86)	16 (76)

IQR, interquartile range.

Interferon Response Gene Selection and Real-Time PCR

Because GCs have been demonstrated to inhibit the IFN response *in vitro* via interaction with specific signaling components such as IFN regulatory factors (IRFs) (12, 13) and STAT1 protein (14), three IFN response genes (IRGs) were selected for the presence of specific transcription factor binding sites (TFBS). Thereto, all 45 IRGs that were previously described to be part of the IFN signature in RA (17), were submitted to the Transfac algorithm available from Interferome (http://interferome.its.monash.edu.au), an online database of IRGs (18). As shown in **Table 1**, *IL1RN* only contained a binding site for the transcription factors STAT3, *IFITM1* only for NFκB and *IFI6* only for IRF-proteins, such as IRF7, IRF8, and IRF9, which binds the IFN responsive element (ISRE). In addition, *RSAD2, MX1,* and *IFI44L* were taken along as positive controls because of their known well-detectability (9, 15). To confirm our initial observations, four additional genes were included based on the presence of certain TFBS (see **Table 1**). Real-time PCR was performed using Taqman gene expression assays and ABI Prism 7500 HT Sequence Detection System (Thermo Fisher Scientific Inc.), according to the manufacturer's protocols. Gene expression values were calculated relative to a standard curve and normalized to the average expression of two housekeeping genes: *18S rRNA* and *HPRT*.

Statistical Analysis

One patient was not included in the analyses as the RNA yield of its T4 sample was not sufficient for further measurements. Statistical analyses were performed using IBM SPSS Statistics 22. Data normality was checked according to Shapiro-Wilk test, with a normal distribution if $p > 0.05$. Because most data were not normally distributed, non-parametric tests were used for most comparisons. Longitudinal changes in IRG expression during treatment were tested using Friedman tests, followed by Wilcoxon signed ranks test. The comparisons of COBRA and COBRA-light therapy and responders and non-responders were performed using Mann-Whitney U test. Correlations between IRG expression and ΔDAS were assessed using Spearman correlation and correlations between IRG expression and ^{2}log-transformed CRP and ESR ratios were assessed using Pearson correlation. $P < 0.05$ were considered statistically significant.

RESULTS

Patient Characteristics

Demographic and clinical data are shown in **Table 2**. No significant differences were observed in clinical characteristics between the COBRA and the COBRA-light group. After 26 weeks, the COBRA-light group displayed a higher DAS value and a lower percentage of patients with DAS values below 2.4. However, these differences did not reach significance, neither at later time points (data not shown, $p \geq 0.45$), which is in line with previously demonstrated non-inferiority of COBRA-light versus COBRA therapy (3–5).

Dynamics of the IFN Response During Immunosuppressive Therapy

In order to gain insight into the dynamics of the IFN response during COBRA and COBRA-light therapy, we first analyzed the expression of 6 IRGs at baseline (T0) and after 4 weeks (T4) and 13 weeks (T13) of treatment in the complete group of COBRA and COBRA-light combined.

As shown **Figure 1** and **Supplementary Table 1**, expression of all measured IRGs except *IFITM1* and *IL1RN* displayed significant changes over all time points (Friedman test, $p \leq 0.016$, vs. $p \geq 0.057$ for *IFITM1* and *IL1RN*). These changes were most pronounced at T4, with median fold changes ranging from only 1.1-fold and 1.3-fold for *IFITM1* and *IL1RN*, respectively, up to 2.5-fold for *RSAD2* (**Supplementary Figure 1A**). In the significant genes, i.e., *IFI6, IFI44L, MX1,* and *RSAD2,* 69–77% of patients displayed a more than 1.2-fold decrease, whereas only 46 and 57% of the patients showed a more than 1.2-fold decrease in *IFITM1* and *IL1RN*, respectively (**Supplementary Table 1**). As displayed in **Supplementary Figure 2**, the extent of the fold

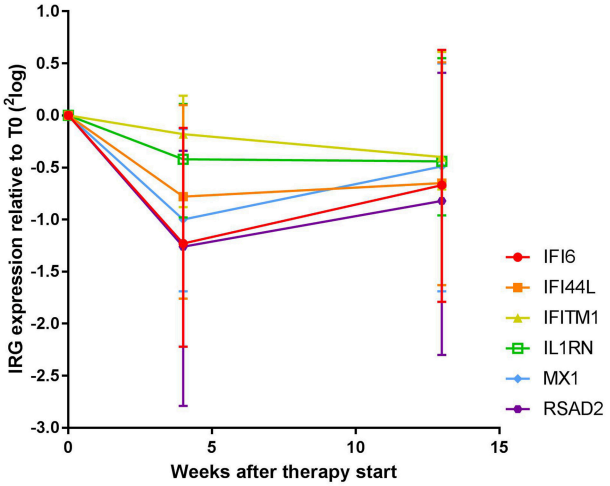

FIGURE 1 | Expression dynamics of individual IRGs during COBRA and COBRA-light therapy. Both cohorts were merged for initial analysis.

change of T4 and T0 was partly dependent on the gene expression levels at baseline, i.e., higher baseline expression generally led to higher fold decreases. However, several patients displayed relatively low baseline expression and relatively high fold changes and vice versa, indicating that the extent of the fold change could not be fully explained by the baseline expression values.

Between T4 and T13, changes in IRG expression were either non-significant or displayed a moderate increase at the group level (1.0 to 1.4–fold increase, $p = 0.012$–0.29), indicating stabilization or even reversal of the IRG decrease that occurred after 4 weeks of treatment. Of note, overall dynamics were largely variable between patients (see **Supplementary Figure 1B**). Individual dynamics over time are displayed in **Supplementary Figure 3**.

Relation Between Transcription Factor Binding Sites and Sensitivity to Immunosuppressive Downregulation

Remarkably, the two genes that appeared least affected by the COBRA and COBRA-light therapy, *IFITM1 and IL1RN*, both lacked binding sites for IRF-transcription factors and STAT1 (see **Table 1**). This implies that the therapy-related IRG reduction might be IRF-dependent and/or STAT1-dependent. In order to test this hypothesis, an additional selection of IRGs was made, based on the presence of binding sites for either IRF or STAT1 (see **Table 1**). As shown in **Figure 2** and **Supplementary Table 2**, the additional IRG that lacked a TFBS for IRF proteins or STAT1, i.e., *IFITM2* displayed only moderate changes upon treatment, similar to *IL1RN* and *IFITM1* ($p = 0.49$). Accordingly, the additional genes with a TFBS for IRF proteins and/or STAT1 showed a considerable downregulation at the group level ($p \leq 0.012$). This further suggests that the therapy-related IRG reduction is largely IRF- and STAT1-dependent.

Differences in Dynamics of IFN Response Between COBRA and COBRA-Light Therapy

Since the main difference between COBRA and COBRA-light therapy is the dose of prednisolone and the use of SSZ, and previous studies have shown a potential suppressing effect of those two agents on IRG expression (7, 13, 15), we next analyzed the two therapy groups separately. The 7 IRGs with most distinct dynamics over time (*HERC5, IFI6, IFI44L, LY6E, MX1*, *RSAD2,* and *SERPING1*) were highly correlating (Spearman $r \geq 0.53$, $p < 0.001$), hence expression levels of these genes were averaged into a 7-IRG score for visualization purposes.

As shown in **Figure 3**, both the COBRA and the COBRA-light group displayed a similar median decrease in IRG expression between T0 and T4, despite the difference in prednisolone dose and SSZ use (Comparison of fold changes, $p \geq 0.19$). However, IRG dynamics between T4 and T13 appeared strikingly different; whereas in the COBRA-treated group IRG expression displayed only minor changes (median 1.1-fold, maximum 1.6-fold increase), the majority of the patients in the COBRA-light-treated group displayed an increase in expression (median 1.8-fold, up to maximum 9.9-fold.Comparison of fold changes in 7-IRG score $p = 0.029$). Significantly more COBRA-light-treated patients displayed an increase of at least 1.2-fold (chi-square $p = 0.019$). Similar results were found for the individual IRGs (**Supplementary Figure 4**). There was no significant correlation between T13/T4 ratio and baseline IRG expression in these groups ($p \geq 0.12$, data not shown), indicating that these dynamics are dependent on the treatment rather than on the baseline expression levels.

Dynamics of IFN Response in Relation to Clinical Response to Therapy

Despite the significant changes in the IFN response observed at the group level, we also observed substantial variation in IRG expression between individuals. For example, some patients did not display downregulation in any of the IRGs between T0 and T4, or only in a part of them (data not shown). Therefore, we also investigated whether these inter-individual variations could be related to the clinical response to COBRA and COBRA-light therapy.

Non-response was defined as DAS > 2.4 at T26. As such, the merged cohort consisted of 7 non-responders and 28 responders. Due to low numbers, the two cohorts could not be analyzed separately. In line with previous reports, no correlation was observed between baseline DAS and IRG expression (15, 17) (data not shown). As shown in **Table 3** and **Supplementary Table 3**, no significant differences in the 7-IRG score or any of the treatment-sensitive IRGs were observed between responders and non-responders, at baseline nor in the expression and dynamics after 4 and 13 weeks ($p \geq 0.059$). Furthermore, no significant correlation was observed between IRG expression and dynamics and the change in DAS after 13 and 26 weeks (unadjusted p-values ≥ 0.045).

At T4, where the maximum IRG decline was observed, DAS was not determined. Instead, we investigated CRP and ESR at T4

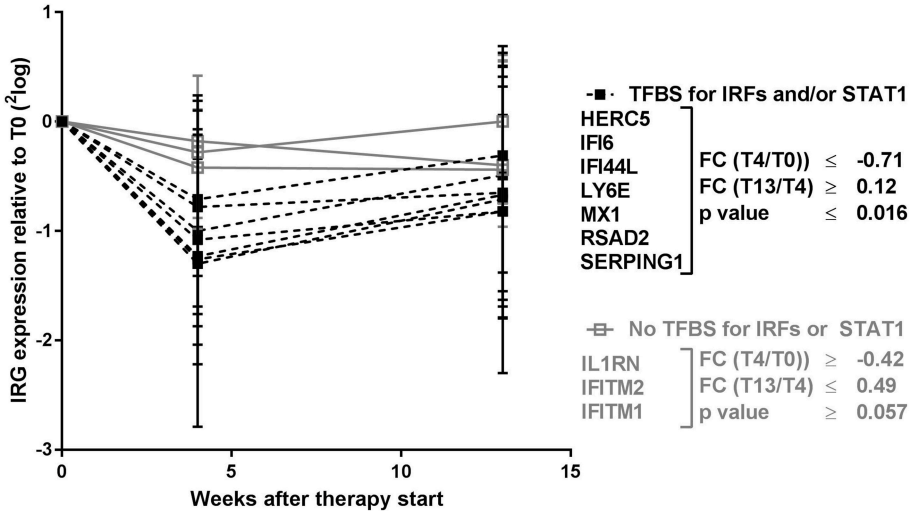

FIGURE 2 | Expression dynamics of individual IRGs during COBRA and COBRA-light therapy. IRGs were categorized based on the presence or absence of transcription factor binding sites (TFBS) for IRF proteins and/or STAT1. FC, fold change expressed in ^{2}log values. *P*-values are indicated for longitudinal analysis by Friedman test.

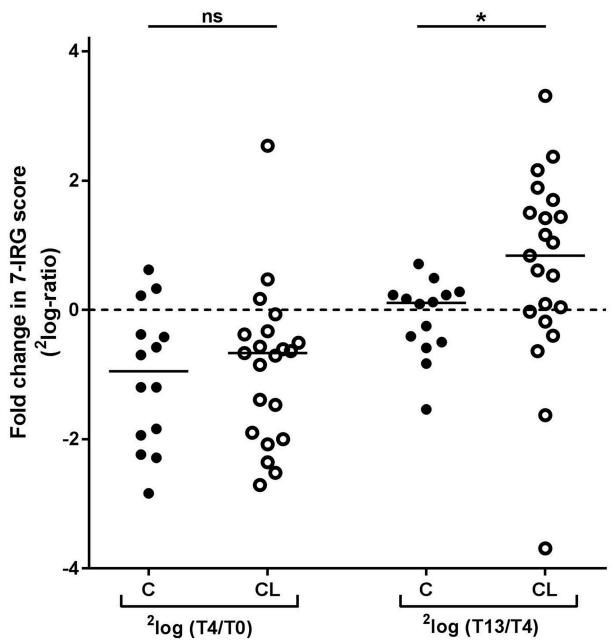

FIGURE 3 | Comparison of longitudinal changes in 7-IRG score between COBRA (C) and COBRA-light(CL)-treated RA patients. *$p < 0.05$.

TABLE 3 | Assessment of 7-IRG score values and dynamics in relation to clinical response to COBRA and COBRA-light therapy.

	7-IRG score at time point			^{2}log-ratios in 7-IRG score	
	T0	T4	T13	T4/T0	T13/T4
R vs. NR (DAS ≤2.4 or >2.4 at T26)	0.17	0.23	0.56	0.86	0.53
ΔDAS at T13 (correlation)	0.18	0.21	0.72	0.43	0.29
ΔDAS at T26 (correlation)	0.70	0.32	0.56	0.58	0.93
^{2}Log-ratio CRP (T4/T0)	0.31	0.34	0.81	0.010(+)	0.22
^{2}Log-ratio CRP (T13/T0)	0.087	0.68	0.54	0.066	0.25
^{2}Log-ratio CRP (T26/T0)	0.12	0.17	0.30	0.61	0.90
^{2}Log-ratio ESR (T4/T0)	0.23	0.49	0.68	0.013(+)	0.84
^{2}Log-ratio ESR (T13/T0)	0.083	0.85	0.55	0.038(+)	0.36
^{2}Log-ratio ESR (T26/T0)	0.063	0.36	0.63	0.16	0.75

Table indicates p values. Details of the statistical analyses are described in the methods section. The direction of the significant correlations is indicated between brackets.

and later time points as indicators of inflammation. Interestingly, a significant positive correlation was observed between the change in IRG expression and change in both CRP and ESR between T0 and T4 ($p \leq 0.051$, Pearson $r \geq 0.42$ for 7-IRG score, see **Table 3** and see **Supplementary Tables 4, 5** for the individual IRGs). However, this correlation was diminished at later time points, suggesting that there is no relation with the eventual clinical response to COBRA and COBRA-light therapy. Separate analysis of the COBRA and COBRA-light group revealed similar results (data not shown).

DISCUSSION

In previous studies using cross-sectional data from RA patients, we observed lower IRG expression in patients using GCs, SSZ and hydroxychloroquine, but not in patients using MTX (7, 15). The unique and virtually complete longitudinal collection of PAXgene blood enabled us to investigate the influence of immunosuppressive therapy on the IFN response in a

longitudinal setting. To our knowledge, the present study is the first to do so.

Using blood collected at baseline and after 4 and 13 weeks of COBRA or COBRA-light treatment, we observed a substantial downregulation of the IFN response within 4 weeks of therapy. This reduction was irrespective of the therapy group, but was not equally strong for each IRG. Between 4 and 13 weeks, however, IRG expression changes were highly variable between patients, which appeared partly dependent on the treatment.

The extent of the downregulation after 4 weeks of treatment was similar between COBRA and COBRA-light-treated patients. Most probably, this decline is due to the prednisolone treatment, as its dose is relatively high in both groups, and it acts more rapidly than MTX and SSZ. The absence of differences between COBRA and COBRA-light treatment at this time point suggests that prednisolone dose of 30 mg/day prednisolone already causes maximum downregulation. The expression dynamics seemed to be restricted to IRGs that contained one or more binding sites for IRF transcription factors and/or STAT1. Conversely, three genes that lacked such binding sites, displayed considerably less downregulation during treatment. Previous *in vitro* studies have shown that the GC signaling pathway, which is activated by prednisolone, is able to compete with the IFN signaling pathway for certain IRF proteins (12, 13) and to inhibit STAT1 activation (14), which could explain our observations.

Between T4 and T13, the IRG dynamics were more variable and differed between the two patient groups. Whereas normalization of IRG expression toward baseline levels was observed in the COBRA-light-treated group, IRG expression remained rather stable in the COBRA-treated group. This is particularly remarkable, as the prednisolone dose is equal between both groups at after 12 weeks (7.5 mg/day), and the only difference is the MTX dose (7.5 mg/week in COBRA and 25 mg/week in COBRA-light) and the addition of 2 g SSZ in the COBRA-treated groups. The total received dose of prednisolone, however, is 1.5-fold higher in the COBRA-treated group at this point. Possibly, the combination of SSZ and higher total prednisolone dose causes a more prolonged downregulation of the IFN response in the COBRA group. However, due to the combination of agents, it is not possible to strongly conclude which agent is responsible for the observed differences in dynamics.

Unfortunately, no untreated control-group with longitudinal follow-up was available, hence it cannot be fully excluded that the IRG dynamics we observed were a consequence of natural fluctuation. However, the correspondence with previously published *in vitro* data (12, 13) as well as our previous *in vivo* data (7, 15) and the observed differences between COBRA and COBRA-light strongly suggest that the observed changes in IRG expression are not spontaneous but truly mediated by the treatment.

The observation that not all IRGs appeared equally sensitive to the immunosuppressive agents of COBRA and COBRA-light therapies, and the putative influence of total prednisolone dose, could particularly be important when using the IFN response as a biomarker, which has been described for several biologics, including TNF inhibitors, rituximab, and tocilizumab (8, 10, 11, 19, 20). For example, we have demonstrated that the predictive performance of the 8-IRG geneset for non-response to rituximab is reduced when patients use prednisolone at the moment of blood collection, presumably because of a prednisolone-mediated reduction in IRG expression (7). Correspondingly, for 5 of the 8 genes in this geneset we have now shown that they indeed are sensitive to immunosuppressive treatment, including prednisolone.

Remarkably, the observation that the IRG downregulation attenuated in COBRA-light-treated patients implies that the IFN response could normalize upon reduction of the prednisolone dose. Hence, the 8-IRG geneset might still be applicable as a predictor for rituximab in patients who are tapering their prednisolone.

Moreover, it would be particularly interesting to investigate whether the IRGs that were less affected by COBRA and COBRA-light treatment could serve as alternative predictors for the response to biologics, since they do reflect IFN activity in RA (17), hence they might still play a role in the response to biologics. Interestingly, the gene *IFITM1*, which appeared less sensitive to prednisolone interference, has already been described as a predictor of rituximab nonresponse in a transcriptomics study (21). Alternatively, one study demonstrated an association between IFN-related gene variants and the response to rituximab (22). Although the predictive value was rather low, the concept of using IFN-related gene variants, which are naturally insensitive to therapy interference, would be interesting to study in further detail and with more IFN-related SNPs (23).

Besides the differential sensitivities of individual IRGs to the treatments, we also observed high heterogeneity in the IRG dynamics between patients. As described before, IRG expression in RA patients is generally highly heterogeneous, which we observed both at baseline and upon therapy. Although we observed a linear relation between baseline IRG expression and the extent of the downregulation after 4 weeks, the variation in IRG dynamics could not be fully explained by the baseline variation in IRG expression. This indicates that besides the type of treatment and the administered doses of treatment, there are also other factors that could influence the IFN response in RA. It has been well-discussed that the IRG response in RA patients is the result of several factors combined, such as extracellular stimuli (24), receptor expression (25) and genetic variation in signaling proteins (22, 23, 26). Considering the putative mechanism of IRG downregulation by prednisolone as described above, particularly the variation in signaling proteins could also contribute to a patient's sensitivity to the observed IRG downregulation. In addition, many other factors, independent of baseline IRG expression, such as therapy adherence and the patient's sensitivity to glucocorticoids (27) could hypothetically affect the extent of the IRG downregulation.

Despite this heterogeneity in the IFN response between patients, we did not observe an association between the IRG expression or dynamics and the response to COBRA and COBRA-light therapy. Considering the differences in IRG dynamics between COBRA and COBRA-light, the potential relation between IRG expression and clinical response should ideally be analyzed for both treatment groups separately.

Since methotrexate has no proven interference with IRG expression, while prednisolone and sulfasalazine have, the use of IRG-interfering agents is considerably higher with COBRA-treatment compared to COBRA-light treatment. Moreover, as all agents have different modes of action (28–30), hence clinical response for each agent is probably achieved via different mechanisms. Consequently, it is possible that the relation between IRG expression and clinical response is different between COBRA and COBRA-light. Unfortunately, the current cohorts were too small to study this in detail.

Since DAS information was not available at T4, a direct comparison of DAS dynamics and IRG dynamics could not be made. Instead, we additionally investigated CRP and ESR as indicators of changes in inflammation in relation to IRG dynamics. Interestingly, a significant correlation was observed between IRG decline and CRP and ESR decline at T4, but not at later time points. At this early time point, clinical effects are mostly attributed to the prednisolone treatment, whereas at later time points more influence is anticipated from MTX and SSZ. As a consequence, the IRG dynamics at T4 could reflect the initial clinical response to prednisolone, but it does not predict the eventual clinical response as this is the result of the combination of agents. It would be interesting to study the potential relation between IRG dynamics and clinical response in patients using prednisone as monotherapy compared to patients using MTX and/or SSZ monotherapy.

In summary, we have demonstrated that both COBRA and COBRA-light therapy are able to downregulate the IFN response in RA. The dynamics of this downregulation were partly dependent on the presence of TFBS within the IRGs and the combination and dosages of agents, but they were irrespective of the clinical response to therapy. Altogether, these results shed a new light on the behavior of the IFN response in RA.

ETHICS STATEMENT

This study was approved by the medical ethics committee of VU University Medical Center and Reade, Amsterdam, The Netherlands, and informed consent was obtained from all donors.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. TdJ and WL: study concept and design. TdJ, TS, EM, and WL: acquisition of patient material and data. TdJ, TS, EM, CvdL, RvV, and WL: analysis and interpretation of data.

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SUPPLEMENTARY MATERIAL

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Glucocorticoid Therapy of Multiple Sclerosis Patients Induces Anti-inflammatory Polarization and Increased Chemotaxis of Monocytes

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Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system (CNS), characterized by the infiltration of mononuclear cells into the CNS and a subsequent inflammation of the brain. Monocytes are implicated in disease pathogenesis not only in their function as potential antigen-presenting cells involved in the local reactivation of encephalitogenic T cells but also by independent effector functions contributing to structural damage and disease progression. However, monocytes also have beneficial effects as they can exert anti-inflammatory activity and promote tissue repair. Glucocorticoids (GCs) are widely used to treat acute relapses in MS patients. They act on a variety of cell types but their exact mechanisms of action including their modulation of monocyte function are not fully understood. Here we investigated effects of the therapeutically relevant GC methylprednisolone (MP) on monocytes from healthy individuals and MS patients *in vitro* and *in vivo*. The monocyte composition in the blood was different in MS patients compared to healthy individuals, but it was only marginally affected by MP treatment. In contrast, application of MP caused a marked shift toward an anti-inflammatory monocyte phenotype *in vitro* and *in vivo* as revealed by an altered gene expression profile. Chemotaxis of monocytes toward CCL2, CCL5, and CX3CL1 was increased in MS patients compared to healthy individuals and further enhanced by MP pulse therapy. Both of these migration-promoting effects were more pronounced in MS patients with an acute relapse than in those with a progressive disease. Interestingly, the pro-migratory GC effect was independent of chemokine receptor levels as exemplified by results obtained for CCR2. Collectively, our findings suggest that GCs polarize monocytes toward an anti-inflammatory phenotype and enhance their migration into the inflamed CNS, endowing them with the capacity to suppress the pathogenic immune response.

Keywords: multiple sclerosis, methylprednisolone therapy, monocytes, M2 polarization, chemokines

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INTRODUCTION

Multiple Sclerosis (MS) is an inflammatory autoimmune disease of the central nervous system (CNS) involving different types of immune cells including T cells, B cells, and monocytes. The most common disease course is characterized by acute relapses with complete or incomplete remission. This relapsing-remitting phenotype (RRMS) is observed in the majority of the MS patients, with young adults being most affected. RRMS can convert into a secondary-progressive form (SPMS) later in life, which is characterized by progressive worsening of the disease with or without additional relapses. The hallmark of the third form of MS, termed primary-progressive (PPMS), is a continuous worsening of the symptoms without intermittent improvements (1). Although numerous new drugs have been developed within the last decade, the most widely used treatment of acute relapses is still high-dose methylprednisolone (MP) pulse therapy to which most patients respond well, resulting in an amelioration of symptoms within a few days (2). Patients suffering from SPMS and PPMS are also treated with MP pulse therapy in the case that the disease is not stable. Mechanistically, various activities of glucocorticoids (GCs) affecting immune cells but also non-hematopoietic cell types are discussed (3, 4). Furthermore, the GC response was recently reported to be highly cell type-specific, both in magnitude and even direction of transcriptional regulation (5). In the context of MS therapy it is believed that patients profit most from direct or indirect dampening effects on T cells. It has been reported that GCs down-regulate expression levels of pro-inflammatory cytokines and adhesion molecules required to pass the blood-brain barrier (BBB). They also promote apoptosis induction in immune cells, inhibit T cell activation, and additionally exert inhibitory effects on inflammatory mediators such as nitric oxide (NO) (6). Our own preclinical studies using the animal model experimental autoimmune encephalomyelitis (EAE) further revealed that T cells are the major target cells of free administered GCs (7, 8). However, effects on myeloid cells were also shown to be crucial if GC were encapsulated in liposomes (9) or nanoparticles (10). In addition, we found that altered T cell migration along chemokine gradients was a mechanism accounting for the therapeutic activity of GCs in the treatment of neuroinflammation, whereas apoptosis induction in T cells unexpectedly turned out to be of minor importance (11).

T cells are the target of most current immunotherapies for MS patients, highlighting the importance of this cell population for the pathogenesis of MS. Nevertheless, myeloid cells including monocytes play important roles for innate immune responses and indirectly also influence adaptive immune responses by serving as antigen-presenting cells, and with both functions they also play a crucial role in MS and EAE (12). They are found in CNS lesions in EAE and MS and often outnumber infiltrating T cells. In animal models it has been shown that monocytic infiltration contributes to disease progression (13), and monocyte-derived macrophages are key players in the reactivation of infiltrating T cells (14). Consequently, elimination of macrophages (15–17) or selective depletion of $CCR2^{+}$ Ly-$6C^{hi}$ monocytes (18) reduced CNS inflammation. Alterations in the composition of monocyte subpopulations in the peripheral blood and cerebrospinal fluid (CSF) of MS patients have been reported as well, thus further highlighting their substantial role in human neuroinflammation (19).

In humans, monocytes are a heterogeneous cell population, constituting ∼10% of total leukocytes in the blood. They have a short life span and evolve in three different subsets: the most prevalent being $CD14^{++}CD16^{-}$ classical (or inflammatory) monocytes, $CD14^{++}CD16^{+}$ intermediate state monocytes, and $CD14^{+}CD16^{++}$ non-classical monocytes (20). They can give rise to macrophages that encompass a dynamic spectrum of phenotypes with classical or M1 macrophages (producing IL-12, IL-1β, NO and reactive oxygen species, and acting in a pro-inflammatory fashion) and alternatively activated or M2 macrophages (expressing CD163, CD206, Arg1, and acting in an anti-inflammatory fashion) being the extreme ends of this spectrum (21). M2 myeloid cells were found to contribute to an improvement of autoimmune diseases such as MS and EAE (22–25). Similarly, skewed proportions of the different monocyte subsets have been reported for many human inflammatory and autoimmune diseases. For instance, in rheumatoid arthritis, systemic lupus erythematodes, sepsis, uveitis and sarcoidosis, intermediate-state monocytes were expanded (26–31). In contrast, data for MS patients concerning monocyte subsets are less consistent (19, 32, 33). Classical pro-inflammatory $CD14^{++}CD16^{-}$ monocytes are recruited to the CNS in response to CCL2. Non-classical $CD14^{+}CD16^{++}$ monocytes, however, are not necessarily beneficial in the context of MS. Namely, it has been shown that the latter cells can adhere to the endothelium and help T cells to extravasate at the site of inflammation and thereby contribute to MS pathology. Accordingly, they are found in active and demyelinating lesions and the CSF (33). Beyond their disease-promoting activity, however, monocytes are also able to dampen inflammation depending on their subtype and status.

The influence of different drugs used in the long-term treatment of MS such as glatiramer acetate (23), dimethyl fumarate (34), or fingolimod (35) on myeloid cell function has been intensively investigated. In contrast, effects of GCs on myeloid cells in the context of MS are less clear. Treatment of monocytes with GCs *in vitro* induces a stable anti-inflammatory gene expression profile (36). Consequently, such monocytes interfere with T-cell-mediated inflammation *in vivo*, where they were shown to directly suppress the secretion of IL-17 and IFNγ without inducing a direct Th2 shift. Additionally, treatment with GCs enables monocytes to induce regulatory T cells (Treg) at the site of inflammation (37, 38).

Here we investigate the influence of MP—it being the most widely applied GC in MS therapy—on human monocytes from healthy individuals and MS patients *in vitro* and *ex vivo*. We found evidence that monocyte polarization becomes skewed toward the M2 phenotype by MP treatment and that the migration of monocytes along chemokine gradients is increased without any significant changes in the level of their respective receptors. These findings suggest that GCs

also exert their beneficial effects on MS bouts by tuning monocyte function and not necessarily solely by suppressing T cells.

MATERIALS AND METHODS

Patients

Thirty patients with established diagnosis of MS according to the McDonald Criteria revised in 2017 were included in the current study (14 RRMS, 8 SPMS, 8 PPMS). All patients received high-dose MP (1,000 mg) intravenously on three consecutive days according to medical indication (due either to MS relapse or progressive worsening of neurologic symptoms in patients with progressive MS). Peripheral blood was drawn in Li-Heparin monovettes (Sarstedt, Nürnbrecht, Germany) before and 24 h after the first injection of MP. Due to the small volume of blood that could be obtained from each patient and due to the sometimes limited recovery of blood after MP therapy, not all types of analyses were performed for every patient. The number of patients included in each experiment is therefore indicated in the figure legends.

Information about MS patients included in this study (disease subtype, age, gender, severity of clinical symptoms as assessed by the Expanded Disability Score Scale (EDSS), acute relapse, disease duration, treatment) are summarized in **Table 1**. SPMS patients that were treated with MP due to an acute relapse (**Table 1**) were combined with the RRMS group and collectively referred to as "MS patients with acute relapse." In contrast, SPMS patients without an acute relapse (**Table 1**) were combined with the PPMS group and referred to as "MS patients with progressive disease." In addition, 24 healthy donors (age and gender summarized in **Table 1**) were included. The investigations were conducted according to the *Declaration of Helsinki* and national and international guidelines. The study was approved by the local ethics committee of the University Medical Center Göttingen. Informed written consent was obtained from each subject prior to the collection of blood.

TABLE 1 | Summary of the characteristics of patients and healthy individuals included in the study.

	Healthy individuals	RRMS	SPMS	PPMS
Number	24	14	8	8
Age (years ± SD)	29.4 (8.8)	39.4 (8.9)	53.1 (7.5)	57.1 (10.2)
Females, number (%)	11 (45.8)	7 (50)	5 (62.5)	6 (75)
Mean EDSS score (±SD)	n.a.	2.54 (1.05)	6.12 (1.21)	5.5 (1.23)
Disease duration (Mean ± SD)	n.a.	4.79 (4.92)	22.63 (13.57)	12.5 (3.2)
Acute relapse, number (%)	n.a.	13 (92.8)	4 (50)	–
Disease modifying therapy, number (%)	n.a.			–
Fingolimod		4 (28.6)	–	–
IFNβ		4 (28.6)	–	–
Glatirameracetate		2 (14.3)	2 (25)	–
Dimethylfumarate		1 (7.1)	–	–
Teriflunomide		1 (7.1)	–	–
GC		–	2 (25)	5 (62.5)
Rituximab		–	1 (12.5)	–
None		2 (14.3)	3 (37.5)	3 (37.5)

Age, EDSS, and disease duration are presented as mean ± SD. n.a., not applicable.

Purification and Short-Term Culture of Human Monocytes

Peripheral blood lymphocytes were enriched using a lymphoprep gradient (Axis Shield, Oslo, Norway) as described (11), and monocytes were purified with magnetic beads (Stemcell Technologies, Köln, Germany). Purity was assessed on the basis of CD14/CD16 staining by flow cytometry using a FACSCanto II device (BD Biosciences, Heidelberg, Germany), and routinely >95% (**Figure 1**). Monocytes were analyzed directly or cultured for 3 h in RPMI 1640 medium supplemented with 0.5% fatty acid-free BSA under serum-starved conditions in the presence or absence of 10^{-6} M MP. One portion of the cells was used for RNA isolation and surface marker analyses and the other portion served to assess the migratory capacity.

Flow Cytometry

Flow cytometric analysis of monocytes was performed as previously described (11). To this end, cells were stained with the following monoclonal antibodies (BioLegend, Uithoorn, The Netherlands) in PBS supplemented with 0.1% BSA and 0.01% NaN_3: anti-human CD14-PE/Cy7 (clone: HCD14), anti-human CD16-APC/Cy7 (clone: 3G8), anti-human CD163-PE (clone GHI/61), anti-human CD192 (CCR2)-PerCP/Cy5.5 (clone: K036C2), and anti-human CX3CR1-FITC (clone: 2A9-1). Data were acquired on a FACS Canto II device (BD Bioscience) and analyzed using FlowJo® software (Tree Star, Ashland, OR).

Boyden Camber Assay

After 3 h *in vitro* cultivation with or without MP (see above), 5 × 10^5 monocytes per well were subjected to a transwell assay using a pore size of 5 μm (Corning Life Sciences, NY, USA) as previously described (11). Cells were allowed to migrate along a gradient of 10 ng/ml CCL2, 10 ng/ml CCL5, or 1 ng/ml CX3CL1 (ImmunoTools, Friesoythe, Germany) for 1 h. The medium in the lower chamber was harvested and the transmigrated monocytes attached to the plate were incubated with 2 mM EDTA in PBS for 20 min at 37°C. Detached cells were scratched off the well bottom and pooled with the harvested medium for analysis. Finally, cells were quantified by flow cytometric analysis using Calibrite Beads (BD Bioscience).

Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described (11). To this end, total RNA was isolated using the Quick-RNA MiniPrep Kit (Zymo, Irvine, CA) and cDNA was prepared with the iScript Kit (Bio-Rad, Munich, Germany). Quantitative RT-PCR was performed on an ABI 7500

instrument (Applied Biosystems, Darmstadt, Germany) using the SYBR mastermix from the same company. Results were normalized to the mRNA expression of *HPRT* and evaluated using the ΔΔCt method. Primer sequences are depicted in **Table 2**.

Statistical Analysis

Data sets were initially subjected to the Shapiro-Wilk normality test to analyze Gaussian distribution. Depending on the results, either a parametric or a non-parametric test was employed, and in the case of matched data, a paired test was used. Accordingly, the experimental groups were compared with a *t*-test, Mann Whitney test, Wilcox matched-pairs signed rank test, or a One-way ANOVA followed by Newman-Keuls Multiple Comparison test as outlined in the figure legends. Analyses were performed with GraphPad Prism software (San Diego, CA). Data are depicted as box-and-whiskers plots showing the minimum, maximum and median, or as the mean ± SEM in all other types of graphs. Levels of significance are as follows: n.s. $p \geq 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

RESULTS

The Abundance of Classical CD14^{++}CD16^{-} Monocytes Is Increased in MS Patients Independently of Disease Activity

Monocytes were purified from the peripheral blood of healthy individuals and MS patients and analyzed for the distribution of cellular subsets by flow cytometry (**Figure 1**). Classical CD14^{++}CD16^{-} monocytes were significantly more abundant in MS patients than in healthy control subjects whereas non-classical CD14^{+}CD16^{++} monocytes were less frequent in MS patients (**Figure 2**). In contrast, the percentage of intermediate state CD14^{++}CD16^{+} monocytes was unaltered. Noteworthy, these findings are in line with previous reports (19). Furthermore, we did not observe any differences concerning the abundance of monocyte subtypes between MS patients with progressive disease and those undergoing an acute relapse (**Figure 2**).

GCs Have Only a Minor Impact on Monocyte Subset Distribution

Monocytes were isolated from MS patients with progressive disease or an acute relapse before they received a bolus injection of MP. To study short term effects of GCs, the *ex vivo* retrieved cells were incubated for 3 h *in vitro* in the absence (control) or presence of 10^{-6} M MP. In addition, monocytes were isolated from the same MS patients again 24 h after MP pulse therapy to determine long term effects of GC treatment *in vivo*. In the case of patients with an acute relapse, monocyte subset distribution remained unaltered by MP treatment with regard to both short and long term effects (**Figure 3**). In contrast, we observed an increased frequency of classical CD14^{++}CD16^{-} monocytes in patients with progressive disease after long term MP pulse therapy, and a concomitant but non-significant reduction of non-classical CD14^{+}CD16^{++} monocytes (**Figure 3**).

TABLE 2 | Primer sequences used for quantitative RT-PCR analysis.

Gene name	Forward primer	Reverse primer
NR3C1	AAG AGC AGT GGA AGG ACA GC	CCA GGT TCA TTC CAG CCT GA
IL1B	AAC AGG CTG CTC TGG GAT TC	AGT CAT CCT CAT TGC CAC TGT
CD163	GGC TTG CAG TTT CCT CAA GA	AGC TGA CTC ATG GGA ATT TTC TG
CD206	CGA TCC GAC CCT TCC TTG ACT	AGT ATG TCT CCG CTT CAT GCC
IL10	AAG ACC CAG ACA TCA AGG CG	AAT CGA TGA CAG CGC CGT AG
ARG1	GGA GTC ATC TGG GTG GAT GC	GGC ACA TCG GGA ATC TTT CCT
HPRT	CCT GGC GTC GTG ATT AGT GA	CGA GCA AGA CGT TCA GTC CT

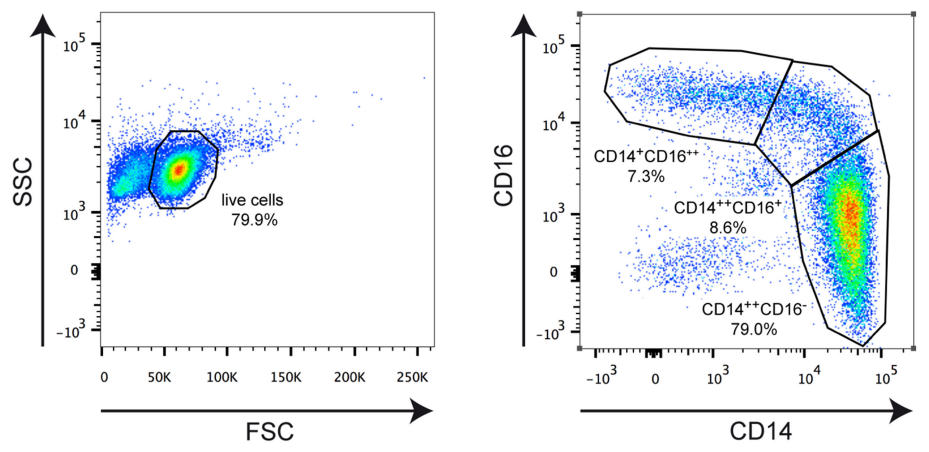

FIGURE 1 | Representative FACS analysis illustrating the applied gating strategy. Monocytes were isolated from an MS patient and stained for CD14 and CD16 surface expression using fluorochrome-conjugated monoclonal antibodies. The left plot depicts the gating for living cells based on forward scatter (FSC) and side scatter (SSC). The right plot shows gating for classical CD14^{++}CD16^{-} monocytes, intermediate state CD14^{++}CD16^{+} monocytes, and non-classical CD14^{+}CD16^{++} monocytes. The borders of the gates and the percentages of cells therein are indicated in each plot.

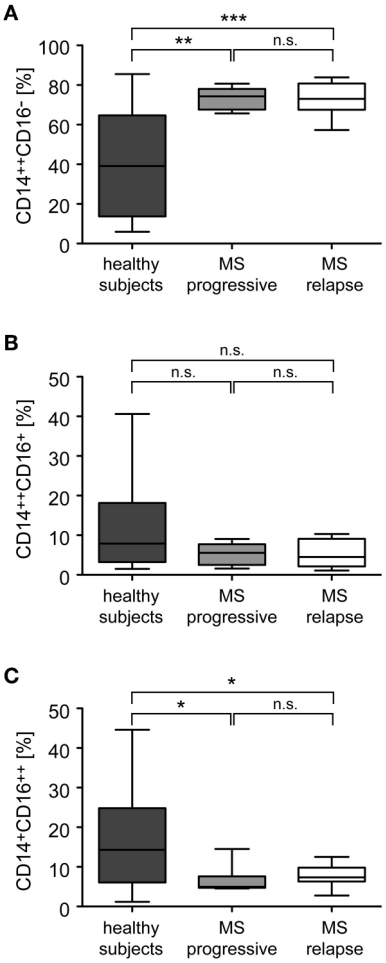

FIGURE 2 | Distribution of monocyte subsets in the peripheral blood of healthy subjects and MS patients with progressive disease or an acute relapse. Monocytes were isolated from the peripheral blood and the percentages of $CD14^{++}CD16^{-}$ inflammatory monocytes **(A)**, $CD14^{++}CD16^{+}$ intermediate state monocytes **(B)**, and $CD14^{+}CD16^{++}$ non-classical monocytes **(C)** were determined by flow cytometry. MS patients were divided into two groups according to their disease activity (progressive, relapse). Data are presented as box-and-whiskers plots showing the minimum, maximum and median; $n = 20$ (healthy subjects), $n = 8$ (MS progressive), $n = 12$ (MS relapse). Statistical analysis was performed using a One-way ANOVA and Newman-Keuls Multiple Comparison test. Levels of significance: n.s. $p \geq 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

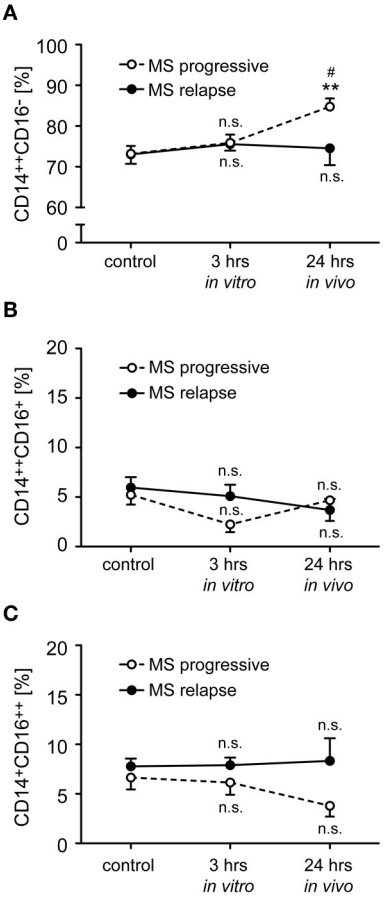

FIGURE 3 | Impact of GC treatment on monocyte subset distribution in MS patients with progressive disease or an acute relapse. Monocytes were isolated from MS patients before MP pulse therapy and incubated for 3 h without (control) or with 10^{-6} M MP *in vitro*. A second blood sample was obtained from the same MS patients 24 h after MP pulse therapy *in vivo*. The percentages of $CD14^{++}CD16^{-}$ inflammatory monocytes **(A)**, $CD14^{++}CD16^{+}$ intermediate state monocytes **(B)**, and $CD14^{+}CD16^{++}$ non-classical monocytes **(C)** were determined by flow cytometry. MS patients were divided into two groups according to their disease activity (progressive, relapse). Data are presented as the mean ± SEM; $n = 8$ (MS progressive), $n = 12$ (MS relapse). Statistical analysis was performed using a One-way ANOVA and Newman-Keuls Multiple Comparison test. Levels of significance: n.s. $p \geq 0.05$; $^{**}p < 0.01$ (control vs. 24 h); $^{\#}p < 0.05$ (3 vs. 24 h).

GCs Induce Monocyte Polarization Toward an Anti-inflammatory M2 Phenotype

In addition to the classification of monocytes on the basis of cell surface receptors, their phenotype can be characterized by determining their gene expression profile. To this end, we performed an mRNA expression analysis of genes that have been linked to either an M1 or M2 polarization. Monocytes were isolated from healthy subjects and MS patients, incubated with or without 10^{-6} M MP *in vitro* for 3 h and analyzed by quantitative RT-qPCR. In addition, long term GC effects were investigated 24 h after MP pulse therapy *in vivo* (only patients). Initially, we analyzed mRNA levels of *NR3C1*, the gene encoding the GC receptor (GR). *NR3C1* expression did not significantly differ between healthy subjects and MS patients and was reduced by MP treatment as expected (39, 40). However, the latter effect reached statistical significance only in the case of the *in vivo* therapy (**Figure 4A**). Expression analysis further revealed that mRNA levels of *IL1B*, a pro-inflammatory cytokine that is typical for an M1 polarization of monocytes, were reduced by MP treatment in healthy subjects and MS patients both *in vitro* and *in vivo* (**Figure 4B**). Concomitantly, the M2 marker genes *ARG1*, *CD163,* and *CD206* as well as the gene encoding the anti-inflammatory cytokine *IL10* were all increased in monocytes of healthy subjects and MS patients following MP treatment, although the differences were not always

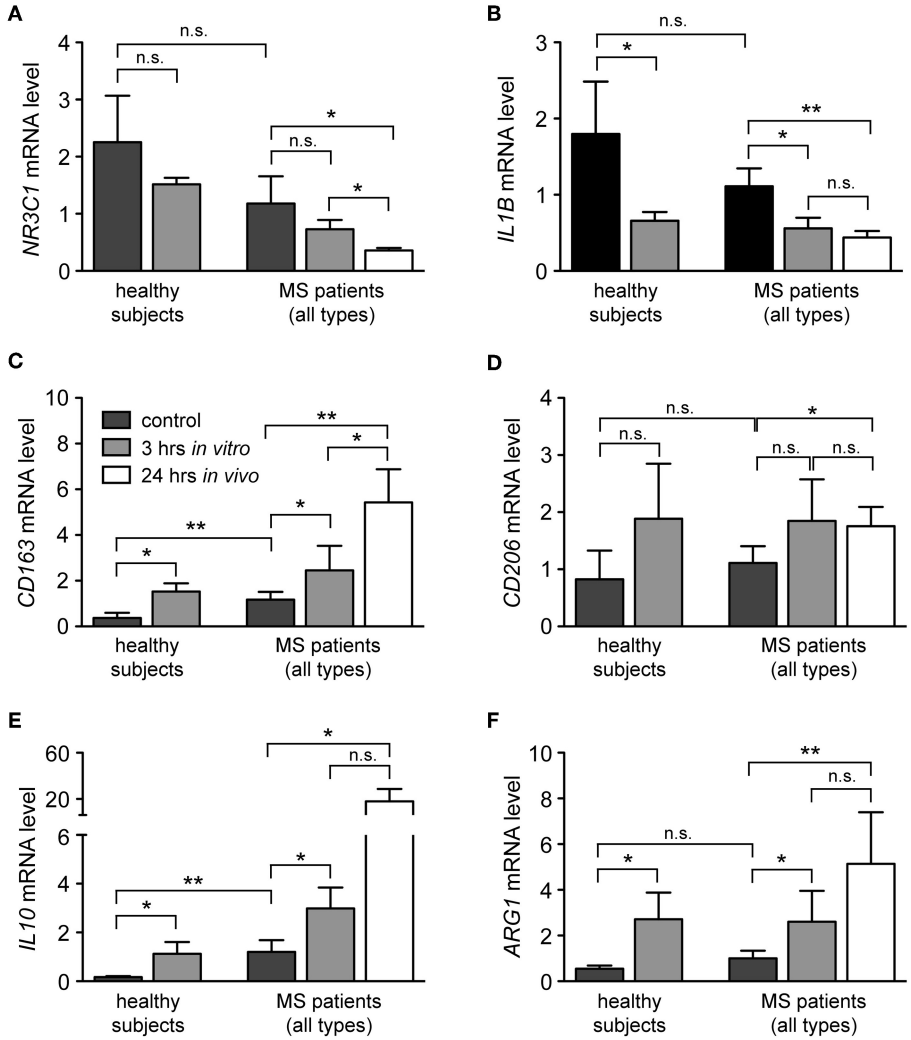

FIGURE 4 | Modulation of the phenotype of monocytes from healthy subjects and MS patients by GCs. Monocytes were isolated from the peripheral blood and cultured without (control) or with 10^{-6} M MP for 3 h *in vitro*. A second blood sample was obtained from the same MS patients 24 h after MP pulse therapy *in vivo*. Thereafter, RNA was prepared and analyzed by quantitative RT-PCR for mRNA levels of *NR3C1* **(A)**, *IL1B* **(B)**, *CD163* **(C)**, *CD206* **(D)**, *IL10* **(E)**, and *ARG1* **(F)**. Gene expression was evaluated using the ΔΔCt method and normalized to *HPRT*. Data are presented as the mean ± SEM; $n = 6$ (healthy individuals), $n = 9$ (MS patients). Statistical analysis was performed using a paired *t*-test (*IL1B*, CD206) or a Wilcox matched-pairs signed rank test (*NR3C1, CD163, IL10, ARG1*). Levels of significance: n.s. $p \geq 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$.

statistically significant (**Figures 4C–F**). It is noteworthy that in general, GC effects were more pronounced after high-dose MP pulse therapy than following *in vitro* culture (**Figure 4**). Gene expression levels for *CD163* and *IL10* were found to be elevated in MS patients compared to healthy individuals in the steady state (**Figure 4**). To confirm our results at the protein level, we analyzed surface expression of CD163 as an example by flow cytometry. There were no differences in CD163 levels after short term MP treatment *in vitro*, either for healthy subjects or MS patients (**Figure 5A** and data not shown). However, 24 h after MP pulse therapy *in vivo*, CD163 surface levels were strongly elevated in a subgroup of MS patients (**Figure 5**). Interestingly, 6 out of 7 patients in whom CD163 surface expression on monocytes was upregulated were suffering from an acute relapse. Collectively, MP induces a shift toward the anti-inflammatory M2 monocyte phenotype, which is most evident in MS patients receiving high-dose MP pulse therapy.

GCs Enhance the Migratory Capacity of Monocytes Along Chemokine Gradients

Transmigration of monocytes across the BBB and infiltration into the meninges and parenchyma is a hallmark of MS and guided by a set of pro-inflammatory chemokines (41). It is against this background that we determined the migratory capacity of monocytes from healthy subjects and MS patients after GC treatment *in vitro* and *in vivo*. The spontaneous basal migration rate of monocytes in the absence of a chemokine gradient was low and independent of disease status and MP

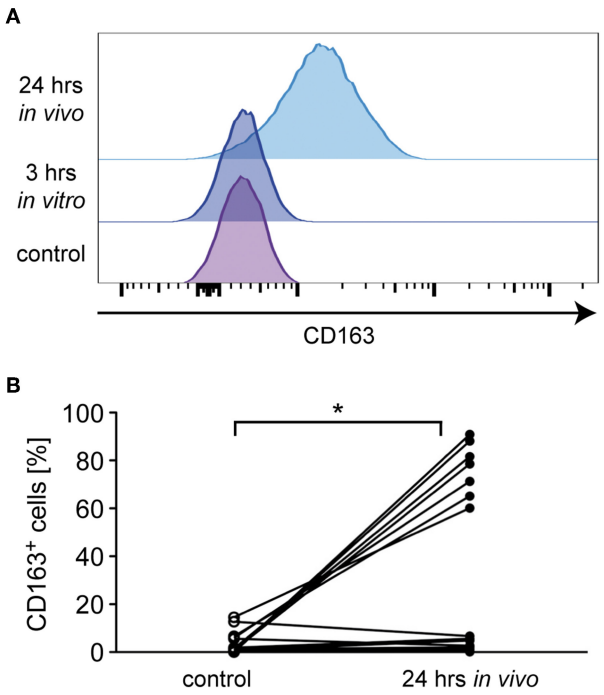

FIGURE 5 | Analysis of monocyte CD163 surface levels in MS patients. Monocytes were isolated from MS patients before MP pulse therapy and cultured without (control) or with 10^{-6} M MP for 3 h *in vitro*. A second blood sample was obtained from the same MS patients 24 h after MP pulse therapy *in vivo*. CD163 surface expression was analyzed by flow cytometry on all cells independently of the CD14/CD16 status. **(A)** Representative stacked histograms are depicted for an MS patient in which CD163 surface levels were upregulated after MP pulse therapy. **(B)** Percentages of $CD163^+$ monocytes before (control) and 24 h after MP pulse therapy *in vivo*. The corresponding values for each patient are connected by a line. $n = 15$. Statistical analysis was performed using a Mann Whitney test. Levels of significance: $^*p < 0.05$.

treatment (**Figure 6A**). Expectedly, monocytes migrated toward the chemokines CCL2, CCL5, and CX3CL1, with a higher migratory activity observed for monocytes from MS patients compared to healthy individuals (**Figures 6B–D**). Short term *in vitro* culture slightly increased the migratory capacity of monocytes retrieved from healthy subjects and MS patients, although significance was missed in most cases (**Figures 6B–D**). In contrast, *in vivo* MP pulse therapy of MS patients strongly and significantly enhanced the migratory capacity of monocytes in response to all three chemokines (**Figures 6B–D**). In addition, we further dissected the migratory capacity of monocytes toward CCL2, the chemokine that caused the largest effects, for MS patients according to their individual disease activity. It turned out that the basal migration was the same in both groups, whereas MS patients with progressive disease had a lower CCL2-directed migration than MS patients with an acute relapse (**Figure 7**). Importantly, the results for monocyte migration toward CCL2 were comparable for both groups with regard to short and long term MP effects (**Figure 7B**). Furthermore, the same tendency was observed for patients from different MS subtypes (RRMS, SPMS, PPMS), although statistical significance was not reached here due to limited numbers of patients (data not shown). In summary, our data indicate that high-dose MP pulse therapy of MS patients enhances monocyte chemotaxis.

The Frequency of $CCR2^+$ Monocytes and Their CCR2 Surface Expression Levels Are Unaffected by GC Treatment

Monocyte migration along chemokine gradients depends on the surface expression of the respective receptors as well as intracellular signaling pathways and cytoskeletal rearrangements. To distinguish between these mechanisms, we tested alterations in chemokine receptor expression levels exemplified for CCR2, the receptor of CCL2 which is the chemokine that induced the most robust migration and alteration by GC treatment (**Figures 6**, **7**). The percentage of $CCR2^+$ monocytes in MS patients was significantly higher than in healthy subjects (**Figure 8A**), which is in agreement with their higher percentage of classical inflammatory $CD14^{++}CD16^-$ monocytes (**Figure 2**). In contrast, the surface density of this receptor was not significantly changed (**Figure 8B**). Importantly, MP pulse therapy of MS patients neither altered the abundance of $CCR2^+$ monocytes nor the surface expression levels of the receptor (**Figures 8A,B**), indicating that the increased migration of monocytes toward CCL2 after MP treatment was unrelated to GC effects on the chemokine receptor itself. Notably, $CX3CR1^+$ monocytes in MS patients were less abundant than in healthy subjects and unaffected by MP pulse therapy (data not shown), which is also in line with the lower abundance of non-classical $CD14^+CD16^{++}$ monocytes in MS patients regardless of their treatment (**Figure 2**).

DISCUSSION

MS is a complex disease involving multiple interactions between different immune cell populations. Although T cells undoubtedly play a very important role in the pathogenesis of MS, monocytes are implicated in disease pathogenesis too and therefore represent potential therapeutic targets. They can contribute to inflammatory processes by influencing Th17 cell differentiation (30) and impact T-cell activation and differentiation, e.g., by down-regulation of cytokine production or induction of Treg cells at the site of inflammation (37). Although progress has been made in the understanding of these processes (42, 43), the role of different monocyte subsets in autoimmune diseases such as MS remains incompletely understood. Monocytes are rapidly mobilized in large numbers to inflamed sites and also possess T cell-independent effector functions such as phagocytic activity and the secretion of pro-inflammatory cytokines and chemokines. Interestingly, these cells can even be found in the healthy human brain. Especially intermediate state $CD14^{++}CD16^+$ monocytes are present in the CSF of healthy subjects, where they account for >50% of all monocytes (19), which highlights their importance for the immune surveillance of the CNS. In pathological conditions such as MS, monocytes are found in active and early demyelinating lesions and thus may contribute to the breakdown of the BBB. Nevertheless,

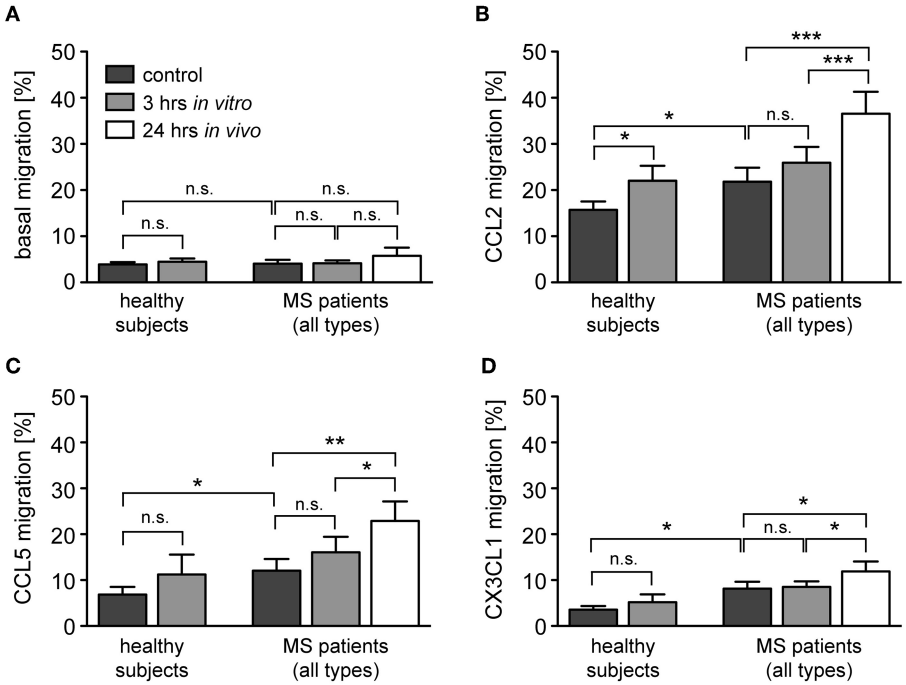

FIGURE 6 | Monocyte migration along chemokine gradients in healthy subjects and MS patients under the influence of GCs. Monocytes were isolated from healthy subjects and MS patients before and after (24 h *in vivo*) MP pulse therapy. Cells were cultured in the absence (control) or presence of 10^{-6} M MP for 3 h *in vitro* and then transferred into the upper part of a Boyden chamber. Basal monocyte migration without a chemokine gradient **(A)** and migration toward a gradient of CCL2 **(B)**, CCL5 **(C),** or CX3CL1 **(D)** into the lower part of the Boyden chamber were analyzed by flow cytometry and results are depicted as the percentage of transmigrated cells (mean ± SEM). $n = 19/19/11/9$ (healthy subjects), $n = 13/15/12/17$ (MS patients). For statistical analysis, untreated samples were compared to each other using a *t*-test, comparison of untreated vs. MP-treated samples from healthy subjects was performed using a paired *t*-test, and comparison of samples from MS patients to each other was performed using a One-way ANOVA and Newman-Keuls Multiple Comparison test. Levels of significance: n.s. $p \geq 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

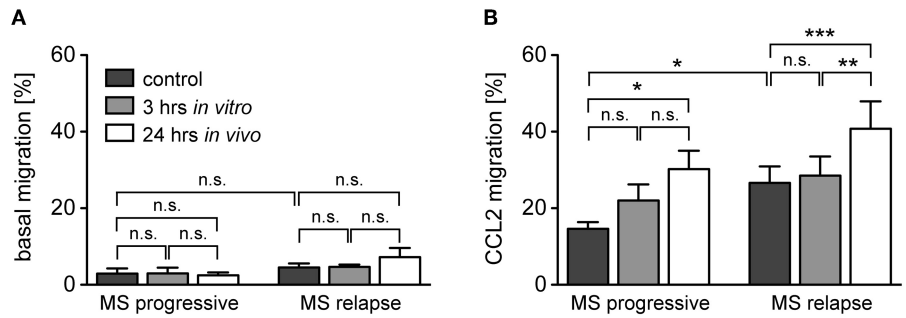

FIGURE 7 | Monocyte migration along a CCL2-gradient in MS patients with progressive disease or an acute relapse under the influence of GCs. The data are the same as in the experiment presented in **Figure 4**, but the MS patients are now divided into two groups according to their disease activity. Basal monocyte migration without a chemokine gradient **(A)** and migration toward a gradient of CCL2 **(B)** into the lower part of the Boyden chamber were analyzed by flow cytometry and are depicted as the percentage of transmigrated cells (mean ± SEM); $n = 4/6$ (progressive), $n = 9/9$ (relapse). For statistical analysis, untreated samples were compared using a *t*-test and comparison of samples from MS patients to each other was performed using a One-way ANOVA and Newman-Keuls Multiple Comparison test. Levels of significance: n.s. $p \geq 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

depletion of this cell type is not advisable as they can also have beneficial effects in the resolution phase of inflammation and repair processes. For instance, when myeloid cells transduced with the innate immune receptor TREM2 were applied in EAE mice, they created an anti-inflammatory milieu in the CNS resulting in the amelioration of clinical symptoms and reduced structural damage (44). It is further noteworthy that a removal of monocytes would be difficult to achieve because they only have a short half-life of a few days. Selectively employing the anti-inflammatory capacity of monocytes while avoiding a general immune suppression might, however, be favorable for the treatment of autoimmune diseases like MS.

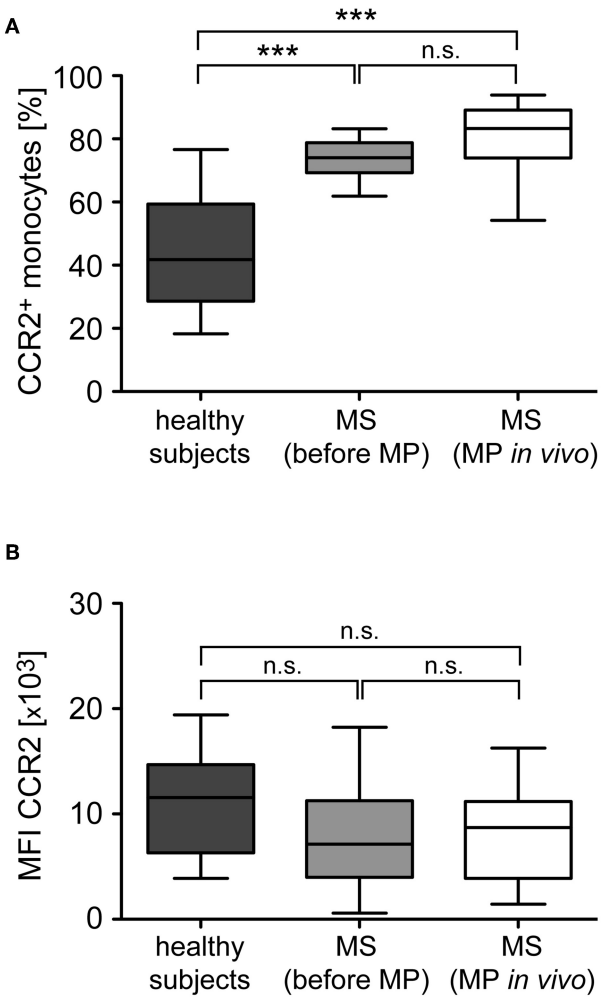

FIGURE 8 | Analysis of CCR2 surface expression levels in monocytes from healthy subjects and MS patients before and after GC treatment *in vivo*. Monocytes were isolated from healthy subjects as well as MS patients before and 24 h after MP pulse therapy *in vivo*. CCR2 surface expression was analyzed by flow cytometry and subsequently the percentage of $CCR2^+$ monocytes **(A)** and the surface level of CCR2 based on the mean fluorescence intensity (MFI) were determined **(B)**. Data are presented as box-and-whiskers plots showing the minimum, maximum and median; $n = 13/22/17$. Statistical analysis was performed using a One-way ANOVA and Newman-Keuls Multiple Comparison test. Levels of significance: n.s. $p \geq 0.05$; ***$p < 0.001$.

In this respect it is relevant that GCs induce a stable anti-inflammatory phenotype in mouse monocytes following their treatment *in vitro* (36). After transfer into recipient mice, such monocytes were found to maintain their polarization and were able to repress T-cell-mediated inflammation even when it was already established (37). Astonishingly, relatively little is known about the mechanisms by which GCs impact this immune cell population during MS pulse therapy, albeit myeloid cells are known to contribute to the pathogenesis of MS and EAE. Expression of the GC receptor in myeloid cells was found to be dispensable for the treatment of EAE with free dexamethasone (7). However, when the GCs were targeted to myeloid cells by encapsulation into liposomes (9) or nanoparticles (10), their effects on monocytes/macrophages turned out to be crucial for their therapeutic efficacy in the same EAE model. In this case, GC treatment resulted in a strong M2 polarization of myeloid cells, which was essential for an amelioration of the disease symptoms (9, 10).

In this study, we report that GCs also have pronounced effects on human monocytes, especially those from MS patients. *In vitro* culture of monocytes from MS patients with MP resulted in an increased gene expression of the M2 markers *ARG1*, *CD163,* and *CD206* and the anti-inflammatory cytokine *IL10*, and the concomitant down-regulation of the mRNA level of the pro-inflammatory cytokine *IL1B*. This effect was even more pronounced after MS patients were subjected to 24 h of MP pulse therapy. Hence, GCs induce an anti-inflammatory M2 monocyte phenotype in MS patients. Surprisingly, we found that the migration of monocytes toward several pro-inflammatory chemokines was enhanced after the exposure to MP *in vitro* and *in vivo*. Presumably, this effect is mostly independent of a modulation of the expression levels of the respective chemokine receptors. Neither the frequency of $CCR2^+$ monocytes nor the surface level of this receptor were significantly increased after MS patients underwent MP pulse therapy. Hence it is likely that GCs influence processes other than chemokine receptor levels leading to an enhanced chemotaxis of monocytes. It is noteworthy that we previously described a similar phenomenon for the impact of GCs on the migratory behavior of T cells toward CCL19 and CXCL12, which turned out to be independent of the levels of the respective chemokine receptor as well (11). Therefore, it appears likely that downstream signaling pathways are responsible for the altered migration of monocytes after GC treatment. It has been reported that phospholipase C and phosphokinase C are involved in CCR2 signaling (45), leading to an activation of focal adhesion kinase (FAK) (46). The observation that FAK is phosphorylated in response to GCs in T cells (11) provides a possible explanation for synergistic effects of GCs and CCL2 on monocyte migration. Further support for this notion comes from a report that paxillin, a downstream signaling molecule of FAK, is induced by GCs in human mesenchymal stem cells and thereby enhances their migration (47), and that GCs influence the cytoskeleton of T cells via phospholipase C (48), an effect which could also impact the migratory behavior of monocytes.

Interestingly, frequencies of inflammatory and non-classical monocytes were not substantially influenced by MP treatment although our gene expression analysis revealed a shift toward an anti-inflammatory phenotype under these conditions. Apparently, the cell populations defined by either surface expression of CD14 and CD16 or gene expression of anti-inflammatory molecules are different. It has been shown that human M2 polarized macrophages display a more motile phenotype and migrated more directed and over longer distances toward CCL2 as compared to M1 or M0 macrophages (49). Additionally, it has been hypothesized that the inflammatory chemokine CCL2 might have a beneficial role in MS because its levels are higher in the remission phase than during relapses, although no clear explanation for this phenomenon could be provided (50). This suggests that in phases of disease remission

M2 polarized macrophages and monocytes are recruited to the site of inflammation by CCL2, where they promote repair and remyelination. We postulate that the application of GCs enhances this natural repair mechanism by affecting two different aspects of this process. First, GC application promotes M2 polarization of monocytes and second, it enhances the migration of these M2 polarized monocytes toward different chemokines. It is tempting to speculate that under these circumstances anti-inflammatory monocytes already reach the CNS at time points when natural repair mechanisms have not yet been initiated, thereby accelerating and optimizing the repair process and facilitating the remission of the disease.

Although T cells are still widely considered to be the major target cells of MP pulse therapy, resulting in changes in cytokine expression, adhesion molecule expression, migration and apoptosis (51), some effects of GCs on myeloid cells have already been described in the past. For instance, the phagocytic potential of human monocytes was enhanced by the incubation with dexamethasone *in vitro* (52). Interestingly, enhanced phagocytosis of macrophages *in vitro* is associated with an M2 polarization (53, 54). Furthermore, high-dose MP pulse therapy resulted in a decreased frequency of monocytes producing IL-8, which is typical for the inflammatory $CD14^{++}CD16^{-}$ subset (55). While we did not observe a change in the frequency of inflammatory or non-classical monocytes 24 h after MP pulse therapy, it is noteworthy that the aforementioned decrease of IL-8-producing monocytes was described 5 days after treatment, suggesting that such a change might be evident only at a later time point. In addition, GC treatment of EAE in mice resulted in a reduced expression of beta-arrestin-1 and enhanced mRNA levels of *A1AR* (56), which is thought to regulate cytokine expression and release and NO production in myeloid cells (57). In fact, mRNA levels of cytokine genes were at least partially affected by GCs in our study: we observed a reduced expression of the pro-inflammatory cytokine *IL1B* and an increased expression of the anti-inflammatory cytokine *IL10*. Interestingly, previous reports indicated that IL-6 levels were not changed by GCs in human monocytes (36), which is in contrast to mouse monocytes (58). The reason for this species difference, however, is unclear. Furthermore, analysis of human monocyte-derived dendritic cells showed that GCs induced IL-10 secretion *in vitro* (59), and analysis of human monocyte-derived macrophages revealed that GCs repressed IL-6 and TNFα responses induced by LPS stimulation *in vitro* (60). Collectively, these findings are in line with our finding that GC treatment modulates cytokine expression by human monocytes.

Our data suggest that CCL2 is the chemokine that controls monocyte migration into the CNS to a higher degree compared to the other chemokines tested in this study. The migration rate of untreated monocytes from healthy subjects and MS patients toward CCL2 was higher compared to CCL5 and CX3CL1, which confirms previous data also showing higher migration rates of human monocytes toward CCL2 in comparison to CX3CL1 (19). Hence, it does not come as a surprise that CCL2-directed migration is also the predominant target of GCs in the context of chemotaxis. Still, it is somewhat contradictory at first sight that chemotaxis toward an inflammatory chemokine is increased by GCs rather than decreased. We believe that this observation needs to be interpreted in the light of the concurrent phenotypic changes that lead to an anti-inflammatory polarization of monocytes. It appears that GCs promote the infiltration of those monocytes into the CNS that are able to terminate inflammation and initiate repair processes, thus contributing to an amelioration of disease symptoms after MP pulse therapy of MS patients. Of note, the occurrence of anti-inflammatory activity of myeloid cells in inflammatory CNS diseases has been reported previously (61, 62) but the exact mechanisms remained incompletely understood.

In summary, GCs exert marked effects on monocytes from MS patients, which could in part explain the therapeutic efficacy of MP pulse therapy. Apparently, these effects are achieved by a combination of M2 polarization and enhanced chemotaxis and certainly play an important role in addition to the well-described impact of GCs on T-cell function. Therefore, GCs should not only be considered as T-cell suppressors but also as modulators of myeloid cells in MS therapy.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the local ethics committee of the University Medical Center Göttingen with written informed consent from all subjects, in accordance with the *Declaration of Helsinki*.

AUTHOR CONTRIBUTIONS

HF performed and analyzed most of the experiments, and wrote the manuscript. TF performed and analyzed experiments. HP provided blood samples of MS patients. HR designed the project, analyzed experiments, and wrote the manuscript. FL designed the project, analyzed experiments, and wrote the manuscript.

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REVIEW
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Extra-Adrenal Glucocorticoid Synthesis in the Intestinal Mucosa: Between Immune Homeostasis and Immune Escape

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Glucocorticoids (GCs) are steroid hormones predominantly produced in the adrenal glands in response to physiological cues and stress. Adrenal GCs mediate potent anti-inflammatory and immunosuppressive functions. Accumulating evidence in the past two decades has demonstrated other extra-adrenal organs and tissues capable of synthesizing GCs. This review discusses the role and regulation of GC synthesis in the intestinal epithelium in the regulation of normal immune homeostasis, inflammatory diseases of the intestinal mucosa, and the development of intestinal tumors.

Keywords: glucocorticoids, intestinal mucosa, intestinal immune homeostasis, inflammatory bowel disease, colorectal cancer, liver receptor homolog-1, tumor necrosis factor

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GENERAL ASPECTS OF GLUCOCORTICOIDS

Glucocorticoids

Glucocorticoids (GCs) are immunoregulatory hormones synthesized in the adrenal cortex and secreted into the blood in a circadian mode under physiological and stress conditions (1). GCs regulate fundamental body functions in mammals including control of cell growth, development, metabolic homeostasis, cognition, mental health, immune homeostasis, and apoptosis (2–5). In the 1940s GCs were discovered as extracts of the adrenal cortex. This was followed by the isolation of adrenocorticotropic hormone (ACTH) from pituitary gland extracts. In 1950, Kendall, Reichstein, and Hench were awarded the Nobel Prize in Physiology and Medicine for their pioneering work in describing that GCs had a powerful anti-inflammatory effect in the treatment of rheumatoid arthritis (6, 7). Since the 1950s, and owing to their strong anti-inflammatory and immunosuppressive activities, GCs have been widely used for the treatment of inflammatory disorders and autoimmune diseases, such as asthma, rheumatoid arthritis, dermatitis, inflammatory bowel disease (IBD), sepsis, lupus erythematosus, and multiple sclerosis (7–11). GCs are also used as immunosuppressive drugs following organ transplantation and in the treatment of leukemia (11, 12).

Immunological, environmental, and emotional stress induces the release of GCs to mediate immunoregulatory activities, mostly immunosuppressive, on distant tissues and cells, in particular in immune cells (4). For example, GCs have an immunosuppressive activity on T cell-mediated immune responses (13) and this is why they are frequently used for the treatments of T cell-mediated immunopathologies.

The synthesis of adrenal GCs is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (**Figure 1**), and controlled by the main circadian oscillator located in the suprachiasmatic nucleus (SCN) of the hypothalamus (1). Basal and stress-inputs to the hypothalamus promote the release of corticotropin-releasing hormone (CRH) from neurosecretory cells of the paraventricular nucleus (PVN), which stimulates the synthesis and secretion of ACTH (corticotropin) from the anterior pituitary gland. ACTH in turn promotes the production and secretion of GCs (cortisol in humans and corticosterone in rodents) from the adrenal cortex (14) (**Figure 1**). Afterwards, GCs target the hypothalamus and the anterior pituitary to inhibit the release of CRH and ACTH in a negative feedback loop (**Figure 1**). GCs act on almost all types of cells in the body to maintain homeostasis both, in response to normal diurnal changes in metabolism and in response to stress (2, 3). Noteworthy, inflammatory cytokines including interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor alpha (TNF) were also reported to stimulate the release of ACTH and CRH further indicating the bidirectional communication between immune and neuroendocrine systems (15).

Adrenal GC Synthesis

Adrenal GCs are synthesized and released by the *zona fasciculata* of the adrenal cortex in a circadian manner, as well as in response to environmental and immunological stress (16). GCs are synthesized from the precursor cholesterol and the synthesis is regulated by the transcriptional control of the steroidogenic enzymes that involve cytochrome P450 (CYP) oxidative enzymes and hydroxysteroid dehydrogenase (HSD) enzymes (1) (**Figure 1**). The first step in steroidogenesis takes place within mitochondria, where cholesterol is transported from the outer to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR) (17). The first and rate-limiting step in steroid synthesis is the conversion of cholesterol to pregnenolone by the action of side-chain cleavage enzyme, P450scc, encoded by the *CYP11A1* gene (**Figure 1**). Thus, it is the expression of P450scc that renders a cell steroidogenic, i.e., able to synthesize steroids *de novo*. Supporting this notion, mice with a deletion of the *Cyp11a1* gene suffer from steroid deficiency (17–19). In humans, once pregnenolone is produced from cholesterol, it undergoes 17α-hydroxylation by P450c17 (CYP17) to yield 17α-hydroxypregnenolone. Next, pregnenolone is converted to progesterone by 3β-HSD (20). Afterwards, 21-hydroxylase (CYP21) converts progesterone into 11-deoxycortisol (humans) or 11-deoxycorticosterone (rodents), then 11β-hydroxylase encoded by the *CYP11B1* gene catalyzes the last hydroxylation step in the GC synthesis. The last step comprises the conversion of 11-deoxycortisol to cortisol in humans, and 11-deoxycorticosterone to corticosterone in rodents, since the rodent adrenals lack CYP17 enzyme (20, 21) (**Figure 1**).

Several factors have been shown to contribute to and modify the cellular and organismal responses to GCs. Notably, most of the secreted cortisol in the blood (∼90%) is bound to proteins (corticosteroid-binding globulins and albumin). This binding regulates the general availability of GCs to tissues and/or direct the delivery of hormones to specific sites (22–24).

It is known that the presence of an 11β-hydroxyl group is essential for the anti-inflammatory and immunosuppressive effects of GCs and for the sodium-retaining effects of the mineralocorticoids (MCs). Therefore, it has been shown that the isoenzymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) critically regulate the conversion between the active and the inactive form of a steroid in target cells. 11β-HSD2 catalyzes the conversion of cortisol, the biologically active form, to the inactive cortisone, whereas 11β-HSD1 converts cortisone to cortisol. Thus, 11β-HSD1, which is expressed in a wide range of tissues and predominantly in the liver, facilitates GC hormone actions whereas the major role of 11β-HSD2 is to prevent cortisol from gaining access to high-affinity MC receptors. Therefore, 11β-HSD2 is predominantly expressed in the MC responsive cells of the kidney and other MC target tissues such as the colon (11).

Adrenal GC synthesis is regulated by the orphan nuclear receptor (NR) steroidogenic factor 1 (SF-1), encoded by the *NR5A1* (nuclear receptor subfamily 5, group A, member 1) gene. SF-1 plays a key role in the development and function of steroidogenic tissues, and has emerged as a key regulator of endocrine function within the hypothalamic-pituitary-gonadal axis and adrenal cortex, and as an essential factor in sex differentiation. SF-1 was first identified as an essential regulator of endocrine development and function, including steroid hormone biosynthesis, via induction of the expression of steroidogenic enzymes, including CYP11A1, CYP17, CYP21, CYP11B1, and 3β-HSD. Similarly, SF-1 has been reported to regulate the expression of StAR as well as the ACTH receptor (25, 26).

Glucocorticoid Receptor Activation

GCs act via genomic (transcriptional) and non-genomic (transcription-independent) mechanisms (27). Most cellular actions of GCs are primarily mediated via binding to their cognate intracellular receptor, the classic glucocorticoid receptor (GR) protein, GRα. GR is a ligand-regulated transcription factor

Abbreviations: ACTH, Adrenocorticotropic hormone; AF-1, activation function 1; AP-1, activator protein 1; APC, Adenomatous polyposis coli; cAMP, cyclic adenosine monophosphate; CD, Crohn's disease; CREB, cAMP response element binding protein; CRH, corticotropin-releasing hormone; CTL, cytotoxic T lymphocyte; CTLA-4, CTL-antigen 4; CYP, cytochrome P450; DBD, DNA-binding domain; DC, dendritic cell; DLPC, dilauroyl phosphatidylcholine; DSS, dextran sulfate sodium; GC, Glucocorticoids; GILZ, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; GRE, GC response element; HPA, Hypothalamus-pituitary-adrenal (gland); HSD, hydroxysteroid dehydrogenase; IBD, Inflammatory Bowel Disease; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; IFN-γ, interferon gamma; ISC, intestinal stem cell; LCMV, lymphocytic choriomeningitis virus; LBD, ligand-binding domain; LPL, lamina propria lymphocyte; LRH-1, liver receptor homolog-1; MAPK, mitogen activated protein kinase; MC, mineralocorticoid; NF-κB, nuclear factor "kappa-light-chain-enhancer" of activated B cells; NR, nuclear receptor; NR5A1, nuclear receptor subfamily 5 group A member 1; PD-1, programmed death-1; PMA, phorbol myristate acetate; PPARγ, peroxisome proliferator-activated-receptor gamma; PVN, paraventricular neuron; SCN, suprachiasmatic nucleus, SF-1, steroidogenic factor 1; SHP, small heterodimer partner; StAR, steroidogenic acute regulatory protein; STAT, signal transducer and activator of transcription; TCR, T cell receptor; TEC, thymic epithelial cell; TF, transcription factor; TGF, transforming growth factor; TJ, Tight junction; TNBS, 2,4,6-trinitrobenenesulphonic acid; TNF, tumor necrosis factor; TNFR, TNF-receptor; UC, ulcerative colitis.

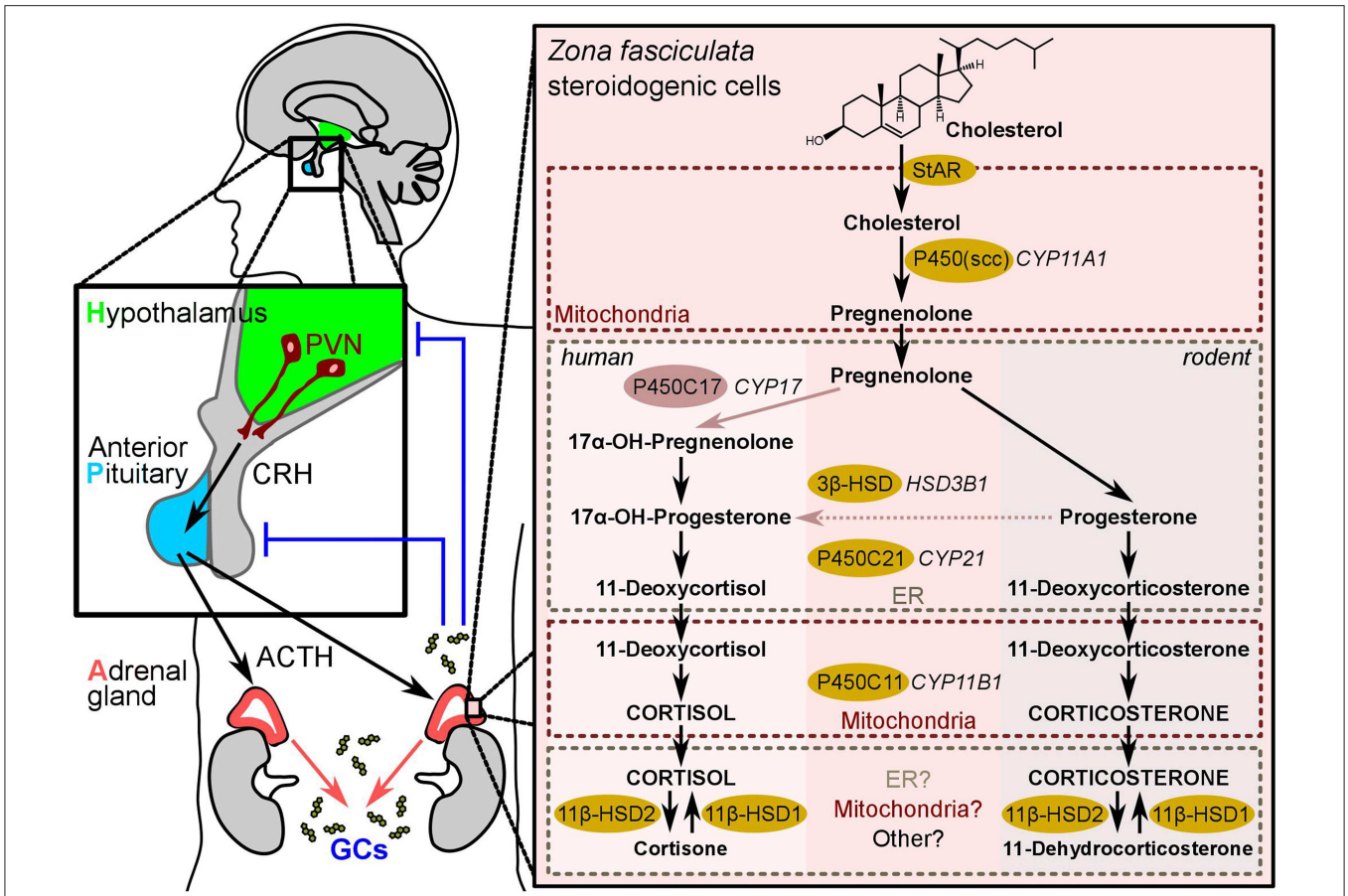

FIGURE 1 | The HPA-axis and adrenal glucocorticoid synthesis. The Hypothalamus-pituitary-adrenal-axis (also known as "stress axis") represents the sequence of endocrine events between the hypothalamus (green), the anterior pituitary gland (blue), and the cortex of the adrenal gland (red). Corticotropin-releasing hormone (CRH) secreted from the paraventricular neurons (PVNs) of the hypothalamus stimulates adrenocorticotropic hormone (ACTH, corticotropin) release from the anterior pituitary, which consequently stimulates the production of glucocorticoids in the steroidogenic cells of the *zona fasciculata* in the adrenal cortex. Blue lines indicate negative feedback. The right-hand panel shows the biochemical reactions leading to glucocorticoid-synthesis in humans and in rodents. The synthesizing enzymes are shown in yellow (and light-red for the human CYP17). The (so far known) subcellular localization of the steroidogenic enzymes in the mitochondria or the ER is highlighted by dotted-line boxes.

(TF) that belongs to the NR subclass 3C and is therefore known as NR3C1 (nuclear receptor subfamily 3, group C, member 1). In line with the pleiotropic actions of GCs, GR is expressed in nearly every cell of the body and is essential for life after birth. Alternative mRNA splicing results in a second GR isoform, GRβ. GRβ does not bind to GC agonists, resides constitutively in the nucleus, and is inactive by itself. However, when co-expressed with GRα, GRβ functions as a dominant negative inhibitor of GRα (2, 28–31).

The GRα shares common structural and functional domains with other NRs. These domains include an N-terminal ligand-independent transactivation domain, also called activation function 1 (AF-1), which is responsible for the transcription activation, a highly conserved DNA-binding domain (DBD) that is important for GR homodimerization and DNA-binding specificity, a C-terminal ligand-binding domain (LBD) that contains the ligand-binding site and a second ligand-dependent transactivation domain (AF-2), and a flexible hinge region separating the DBD and the LBD (32–34). In addition to the known dimerization function of the DBD, *in vivo* evidence has shown that LBD mutation severely compromised GR dimerization, whereas no correlation between oligomerization state, DNA binding, and transcriptional activity could be established (35). These data clearly indicate that multiple domains are involved in GR dimerization.

In the absence of ligand, the GRα is sequestered predominantly in the cytoplasm as an inactive multi-protein complex formed by chaperonic molecules, including heat shock proteins Hsp90, Hsp70, Hsp23, and immunophillins p59 and calreticulin (28, 29, 36). These proteins maintain the receptor in a conformation that is transcriptionally inactive, but favors high affinity ligand binding (2). Binding of endogenous or synthetic GCs to the LBD of GRα induces receptor conformational change leading to the dissociation of the multi-protein complex and allows the translocation of the GC/GR complex to the nucleus where it regulates gene transcription (21). Upon translocation to the nucleus, the GRα binds DNA sequences, known as GC response elements (GREs), to positively or negatively regulate

gene transcription by direct DNA-binding or by interaction with other proteins (3, 37).

In addition to the transcription activation, the GR represses a wide variety of genes. This repression function is mediated by negative GREs (nGRE) in the promoter regions of target genes. nGREs contribute to the negative feedback of HPA axis, bone, and skin function, inflammation, angiogenesis, and lactation. Moreover, GR inhibits glycoprotein hormone promoter, which is positively regulated by the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and contains binding sites for CREB and GR. Upon DNA binding, GR inhibits transcription activation directly by preventing CREB binding (28, 38, 39).

Accumulating evidence suggests that GCs can act via non-genomic mechanisms to elicit more rapid cellular responses (within seconds to minutes) that do not require nuclear GR-mediated changes in gene expression. The non-genomic effects of GCs are considered to be mediated through binding to membrane-bound GR, binding to cytosolic GR, or by interactions with cellular membranes (30, 40, 41). Bartholome et al. showed that membrane GRs are expressed in human monocytes and B cells (42). Additionally, they monitored a strong positive correlation between the frequency of membrane GR-positive monocytes and various parameters of disease activity in patients with rheumatoid arthritis. This observation prompted the authors to suggest that immunostimulation induces the expression of membrane GR in immune cells such as monocytes that in turn triggers rapid signal cascades leading to a significantly higher percentage of cells to undergo GC-induced apoptosis to limit excessive immune reaction (42). GCs can also bind to their cytosolic GR to induce rapid non-genomic effects resulting in interactions with signaling pathways. For example, GCs were shown to activate endothelial nitric oxide synthase in a non-genomic manner and mediated by stimulated phosphatidylinositol 3-kinase and protein kinase Akt phosphorylation (41, 43). High concentrations of GCs have been shown to induce quantitative increase in the intercalation of GC molecules in the membrane, influencing the membrane fluidity, membrane associated proteins and cation uptake, as measured by the reduction of cation transport ATPase activity (44, 45).

Glucocorticoid Functions

Anti-inflammatory Functions of GCs

Upon tissue injury, irritants or pathogen invasion, immune cells of the innate, and adaptive immune systems are activated and recruited to the site of inflammation (12, 46). Immune cells activation and recruitment is mediated by cytokines and chemokines, which are regulated by inflammatory TFs, including the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), signal transducer and activator of transcription proteins (STATs) and activator protein 1 (AP-1) (46). These TFs are crucial regulators of a variety of cellular functions, including cell survival, proliferation, differentiation, and apoptosis (47–50). In the presence of pro-inflammatory stimuli, these TFs trigger activation of pro-inflammatory cytokines, such as TNF, IL-1β, and IL-6 among others, to induce inflammation and promote cell survival (51). GR induces anti-inflammatory activities by direct interaction with other TFs, including NF-κB (51), STAT3 (52), STAT5, and AP-1, leading to their inhibition, thus repressing the expression of pro-inflammatory genes and thereby promoting the resolution of inflammation (29) (**Figure 2**). Since this interaction does not require DNA binding, the term tethering GRE is often used to describe these elements. Interestingly, tethering GREs do not contain DNA binding sites for GRs, but instead contain binding sites for other DNA-bound regulators, including NF-κB and AP-1, that recruit GRs (28, 53).

GCs also induce proteins with anti-inflammatory activities, including glucocorticoid-induced leucine zipper (GILZ), resulting in the inhibition of the mitogen-activated protein kinase (MAPK) pathway (27). MAPK activation is associated with cell proliferation, differentiation, migration, senescence and apoptosis [reviewed in (54)]. Another mechanism, by which GILZ dictates its anti-inflammatory function, is via inhibition of NF-κB and AP-1 activities (27, 55).

Immunosuppressive and Metabolic Functions of GCs

GCs have powerful immunosuppressive activities mediated by acting on almost all types of cells, in particular on immune cells (33). GCs induce apoptosis in a variety of immune cells, including developing thymocytes as well as circulating and tissue-resident T cells, mediated by the pro-apoptotic proteins Puma and Bim (56–58). GCs also promote dendritic cell (DC) apoptosis (29). Additionally, GCs favor the expansion of immunosuppressive regulatory T cells (Tregs) by upregulating the expression of FoxP3, the master regulator of Tregs (59, 60). Moreover, GCs promote the shift from T helper 1 (Th1) to Th2 immune responses by differentially regulating apoptosis of Th1 and Th2 cells (13, 61–63).

GCs also control the function of innate immune cells, including monocytes and macrophages, in order to regulate tissue homeostasis. GCs have been shown to induce the differentiation and promote the survival of anti-inflammatory (M2) macrophages, evident by the induced expression of the immunomodulatory cytokine IL-10. This effect is mediated by prolonged activation of the MAPK pathway resulting in inhibition of caspase activities, and expression of anti-apoptotic genes. On the other hand, GCs efficiently suppress classical pro-inflammatory macrophage (M1) activation, as evidenced by the inhibition of the pro-inflammatory cytokines TNF, interferon gamma (IFNγ) and IL-1β (64–67) (**Figure 2**). These cytokines are highly upregulated in many inflammatory disorders, and their crucial role in the pathogenesis of IBD is well-established (68, 69). GCs potently inhibit the differentiation of DCs and their capacity to stimulate T cells (70, 71).

The resulting immune reaction in pathophysiological conditions depends on the balance between effector cells promoting inflammation and its modulation by regulatory mechanisms (72). In this context, the discussed anti-inflammatory and immunosuppressive properties of GCs are necessary to restore homeostasis following successful elimination of the injurious agent, ultimately leading to the resolution of inflammation and tissue repair after tissue damage caused by excessive inflammation (12).

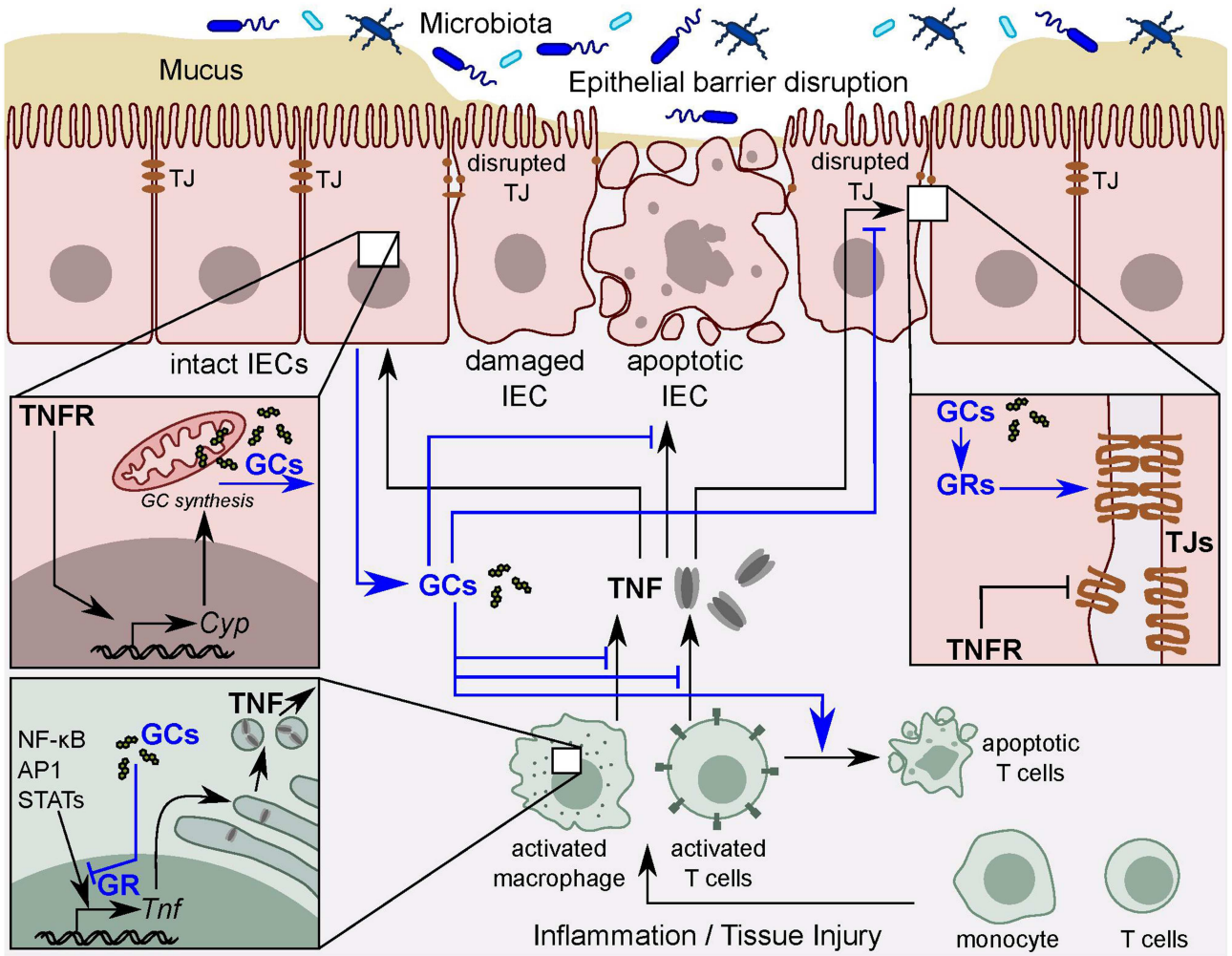

FIGURE 2 | TNF and intestinal GC synthesis. Intestinal epithelial barrier disruption leads to permeability defects and the subsequent interaction of intestinal immune cells with the luminal contents. Activated immune cells release pro-inflammatory cytokines, such as TNF. In turn, TNF results in tight junction (TJ) disruption and intestinal epithelial cell (IEC) apoptosis and thereby exacerbates local inflammation. TNF also directly stimulates IECs to synthesize and release immunoregulatory glucocorticoids (GCs) to counter-balance excessive tissue damage. GCs act via the glucocorticoid receptor (GR) to inhibit TNF-mediated tissue damage in a negative feedback loop. The GR also inhibits pro-inflammatory transcription factors, including NF-κB, AP-1, and STATs leading to the resolution of the inflammation.

Another main biological function of adrenal GCs includes the control of energy metabolism and glucose homeostasis. GCs promote gluconeogenesis in the liver and decrease glucose uptake by antagonizing the response to insulin. Whereas, physiological levels of GCs are required for proper metabolic control, excessive GC action has been linked to a variety of metabolic diseases, such as type II diabetes and obesity (73, 74).

EXTRA-ADRENAL GC SYNTHESIS

Overview of Extra-Adrenal GC Synthesis

The substantial capacity of the adrenal glands to produce enormous amounts of GCs and to release them into the systemic circulation in response to stress hampered the discovery of other GC-producing organs. In fact, strong systemic immune cell activation upon removal of the adrenal glands in mice results in rapid death due to shock (75). Therefore, for long time GC synthesis and secretion was thought to be exclusively confined to the adrenal glands. However, increasing evidence has shown that other extra-adrenal organs are also capable of producing GCs [reviewed in (76)]. Evidence for local GC synthesis comprises the detection of steroidogenic enzymes and high levels of local GCs in different tissues, even upon adrenalectomy. Moreover, the physiological relevance of local GC synthesis has been shown by the major impact of the inhibition of local GCs synthesis even in adrenal-intact scenarios (76–79). Thus, whereas systemic adrenal-derived GCs coordinate multiple organ functions and whole body metabolism, locally synthesized GCs play a highly specific role in regulating local homeostasis, cell development and immune cell activation (31, 80).

In the past two decades the thymus (81–83), the skin (84, 85), the brain (78), the vasculature (86), the lung

(79), and the intestine (77, 87–90) have been shown to produce substantial amounts of GCs, and thereby regulate local immunological responses.

Pioneering work by the group of Ashwell in the thymus provided the first proof for extra-adrenal GC production and opened an exciting field of research for the identification of other GC-producing organs (83). They showed that bioactive GCs are *de novo* synthesized by thymic epithelial cells (TECs), and that they play an important role in antigen-specific thymocyte development by opposing cell death induction from too strong TCR signaling during negative selection, thereby allowing positively selected T cells to survive. This is supported by the finding that inhibition of thymic corticosterone production increased TCR activation-induced cell death and enhanced negative selection of thymocytes (13, 58, 83). Of note, thymocytes (91) and mature T cells (92, 93) were also reported to synthesize GCs, yet it is presently unclear whether this reflects *de novo* synthesis or conversion of serum-derived inactive derivatives.

Interestingly, the skin locally produces CRH, ACTH and expresses the steroidogenic enzymes. Therefore, the skin is considered to have its own local HPA axis. *De novo* synthesis of GCs in the skin is thought to play an important role in local homeostasis as indicated by the deficiency of the steroidogenic enzymes in skin biopsies from patients with inflammatory skin diseases (94). Other organs that express the GC-synthesizing machinery and therefore are capable to *de novo* synthesize bioactive GCs from cholesterol include the brain, the vasculature and the intestine (95). Interestingly, although the lung expresses all the steroidogenic enzymes required for *de novo* synthesis, analysis of lung GC synthesis revealed that the predominant pathway by which corticosterone is produced is by reactivation from inactive serum-derived dehydrocorticosterone via 11β-HSD1 enzyme (79).

Differential Modes of Synthesis of Extra-Adrenal GCs

Most of extra-adrenal GC-synthesizing organs express both the enzymes required for *de novo* GC synthesis as well as the reactivating enzymes from inactive metabolites. However, interestingly different extra-adrenal organs synthesize bioactive GCs via different mechanisms, possibly reflecting local environmental needs. For example, TECs have been shown to have the mRNA, protein, and activities of enzymes required for *de novo* GC synthesis, including StAR, CYP11A1, 3β-HSD, CYP17, and CYP11B1. Furthermore, fetal thymic organ culture demonstrated the conversion of a cholesterol analog to pregnenolone and 11-deoxycorticosterone. Similar to adrenal GCs, TEC-derived GC synthesis was stimulated by ACTH (76, 83). In contrast, ACTH inhibited GC synthesis in thymocytes by downregulation of *Cyp11b1* mRNA expression. This opposite effect of ACTH in thymocytes is not yet fully understood but could possibly represent a function to limit damage to the gland by down-regulating GC synthesis during a strong activation of the HPA axis (91).

Like the thymus, the skin mainly synthesizes GCs *de novo* under the control of the local HPA axis, and the synthesis is regulated by several factors including ACTH, CRH and IL-1β (85). Noteworthy, the skin neuroendocrine system is able to crosstalk with the systemic HPA axis and thus with the adrenal GC synthesis. Interestingly, although the skin also expresses the reactivating enzyme 11β-HSD1, GC reactivation by keratinocytes seems to play a minor role in immune cell activation and contact hypersensitivity compared to the essential role of the *de novo* synthesized GCs. The reason for this could be due to its large dependence on the availability of the GC metabolite from the circulation (94).

As mentioned before, the lung largely depends on the reactivation pathway for generating bioactive GCs. Our group reported that upon immune cell activation by lipopolysaccharide (LPS) or anti-CD3 antibody increased production of corticosterone in *ex vivo* lung cultures was observed (79). Interestingly, only *Cyp11a1* has been shown to be upregulated whereas other steroidogenic enzymes expression remained unchanged. Strikingly, whereas *Hsd11b1* gene was strongly upregulated, *Cyp11b1* was barely detectable indicating that reactivation of serum-derived inactive metabolite (dehydrocorticosterone) is a more prominent pathway of local GC synthesis in the lung. In line with this, adrenalectomized mice failed to produce local GCs in the lung upon immune cell activation. This finding further supports the dependence of lung GC synthesis on adrenal GCs (79).

Our group also described and characterized the *de novo* synthesis of intestinal GCs for the first time. In the intestinal mucosa, GC-synthesizing enzymes were detected at low levels, however, they were strongly upregulated in response to immunological stress resulting in the detection of corticosterone in the supernatant of *ex vivo* cultured intestinal tissue (77). Recently, we also demonstrated a relevance for the GC reactivation impairment in the pathogenesis of IBD (96). In this review, we will discuss the synthesis of GCs in the intestine in more detail.

Taken together, it seems that various extra-adrenal organs synthesize GCs differently in order to cope with local immunological stress and to regulate local immune homeostasis.

INTESTINAL EPITHELIAL STRUCTURE AND HOMEOSTASIS

The intestinal epithelium represents the largest mucosal surface in the human body covering an area of almost 200 m^2 (21). This surface represents the physical barrier that separates the epithelium not only from potential pathogens and food antigens but also from harmless commensal bacteria termed microbiota (97, 98). The gut is anatomically divided into the small intestine and the colon. The small intestine can be subdivided into the duodenum, the jejunum, and the ileum. The architecture of the intestine is organized into crypts of Lieberkühn and epithelial protrusions, called villi, in the small intestine, whereas the colon consists mainly of crypts and has no villi, but a flat surface instead (99, 100). The main function of the epithelium is water and nutrient absorption and the maintenance of effective barrier function in order to maintain tissue homeostasis (101, 102). The

intestinal epithelium promotes these functions by a single layer of intestinal epithelial cells (IECs) organized along the crypt-villus axis (99, 103). IECs are constantly regenerated from intestinal stem cells (ISCs) at the bottom of the crypt columnar cells. The intestinal epithelium has a higher self-renewal rate than any other mammalian tissue, with a fast turnover of <5 days (104–106). ISCs give rise to transient amplifying daughter cells that migrate upward while differentiating into one of the specialized epithelial lineages (100). This fast proliferation of IECs is eventually balanced by cell death resulting from loss of attachment at the tip of the villus followed by subsequent shedding of apoptotic cells into the lumen, a process known as anoikis (106). The differentiated epithelial cell types include absorptive enterocytes, secretory cells (Paneth cells, goblet cells, enteroendocrine cells, and tuft cells), and the M cells of Peyer's patches (105). Paneth cells escape the upward migration and migrate downward instead to constitute the niche for ISCs (107). These cells secrete anti-microbial peptides to prevent bacterial infection (103), whereas tuft cells act as sensors for luminal contents (108). Additionally, enteroendocrine cells secrete various hormones to coordinate digestion and metabolism (108).

Contributing to the effective physical and biochemical barrier function is the mucus secreted by goblet cells, anti-microbial proteins that eliminate bacteria penetrating the mucous and IgA secreted by lamina propria plasma cells, in addition to the tight junctions (TJ) proteins. These TJ are junctional complexes that connect epithelial cells to each other and thereby forming tight intracellular seals (109, 110). The intestinal mucosa also produces high levels of the immunosuppressive cytokines transforming growth factor beta (TGFβ) and IL-10 to maintain local homeostasis. In fact, TGFβ- and IL-10-deficient mice develop spontaneous inflammation (111, 112).

IECs separate the intestinal lumen containing 10^{14} gut microbiota cells from the underlying lamina propria and the rest of the body (113, 114). In addition to the microbiota, the gut epithelium hosts the largest number of immune cells in the body (115). These immune cells include the so-called intraepithelial lymphocytes (IELs) (116), resident macrophages, DCs, plasma cells, lamina propria lymphocytes (LPLs), and neutrophils (115, 117, 118). This direct contact of immune cells with the microbiota, that has great potential to provoke immune cell stimulation, requires fine-tuning to find the appropriate balance between protective immune responses and tolerance toward the microbiota. Disruption of the intestinal epithelial barrier leads to permeability defects, and subsequent interaction between luminal microorganisms and cells of the immune system (**Figure 2**). The barrier breakdown exacerbates inflammation leading to severe tissue damage, as in the case of IBD (98).

IBD comprises a group of intestinal inflammatory disorders, namely ulcerative colitis (UC) and Crohn's disease (CD). Although the etiology is currently not fully understood, it has been associated with a complex interaction between the host genetics, environmental or microbial factors and the immune system (119–121). These interactions result in chronic relapsing inflammation of the intestine as a consequence of inappropriate immune cell activation (117). UC causes inflammation of the mucosa of the colon and rectum, whereas CD causes inflammation of the full thickness of the bowel wall and may involve any part of the digestive tract from the mouth to the anus (122).

Chronic inflammation has emerged as one of the hallmarks of cancer. Many cancers arise following prolonged inflammation or display inflammatory characteristics throughout progression (123, 124). For example, the relative risk of colorectal cancer in patients with IBD has been estimated to increase by up to 20-fold (125, 126). Notably, the risk correlates directly with the duration and extent of inflammation (127, 128).

Increasing lines of evidence have shown that the synthesis of GCs by IECs plays an important role in the regulation of intestinal immune homeostasis under pathophysiological conditions (21, 77, 129, 130). Supporting this notion, defective local intestinal GC synthesis or metabolism has been shown to be involved in the pathogenesis of intestinal inflammation (90, 96, 131, 132).

EXTRA-ADRENAL GLUCOCORTICOIDS IN THE INTESTINE

First evidence for the steroidogenic potential of the gut was suggested in 1995 following the detection of *Cyp11a1* and *Hsd3b1* mRNA in the gut of mouse embryos by *in situ* hybridization (133). Further evidence originated from our own work while studying IEL apoptosis. It was observed that IELs rapidly undergo apoptosis when cultured *ex vivo*, an effect that was accelerated following GC treatment in mice. Interestingly, while adrenalectomy significantly reduced IEL *ex vivo* apoptosis, a stronger effect was observed upon *in vivo* administration of the GR inhibitor RU-486. This observation prompted us to speculate that another source of GCs, likely in the intestinal mucosa, primed the IELs already *in vivo* to undergo *ex vivo* cell death (56).

Subsequent studies characterized the *de novo* GC synthesis in the murine intestinal mucosa in response to immunological stress following anti-CD3 injection or viral-activated T cells (77). It was shown that the intestinal mucosa constitutively expressed many of the steroidogenic enzymes required for the *de novo* synthesis of corticosterone from cholesterol and for the reactivation of corticosterone from dehydrocorticosterone. Moreover, expression of the steroidogenic enzymes including *Cyp11a1*, *Cyp11b1,* and *Hsd11b1* was strongly induced upon immunological stress. The source of the aforementioned three enzymes and therefore intestinal GCs was shown to be the crypt region of the IECs (77). This was demonstrated by a further study that linked the expression of *Cyp11a1* and *Cyp11b1* to the cell cycle, thus restricting the production of GCs to the proliferating cells of the intestinal crypts (134).

The basal expression of steroidogenic enzymes might suggest that GC production, though at very low levels, is possibly fulfilling an important function in the regulation of local immune homeostasis and epithelial barrier integrity (75). In line with this, *in vitro* data revealed the importance of GCs in the maturation and differentiation of the IECs (135). Additionally, GCs have been shown to play a role in the expression of TJ proteins and the maintenance of the intestinal epithelial barrier integrity, in

particular antagonizing the TJ-destructing effect of TNF during inflammation (109) (**Figure 2**).

Cima et al. used adrenalectomized mice to exclude the contribution of systemic GCs, and measured by radioimmunoassay the corticosterone release into the supernatant of *ex vivo* cultured intestinal tissue from anti-CD3-injected mice (77). The *in situ* corticosterone synthesis was confirmed since metyrapone, a potent inhibitor of 11β-hydroxylase and 11β-HSD1 (136, 137), blocked corticosterone release (77). Similarly, stimulation of the innate immune system with LPS induces GC synthesis in a macrophage-dependent manner, since it also occurred in $RAG^{-/-}$ mice lacking T and B lymphocytes (89). Furthermore, administration of TNF, infection of mice with viruses, or chemically induced intestinal inflammation promote the expression of *Cyp11a1* and *Cyp11b1,* and strongly induces the synthesis of intestinal GCs (95). Although most of the studies of GC synthesis were conducted in mice, subsequent research showed that the human intestinal tissue also expresses the steroidogenic enzymes and is capable of synthesizing GCs (96, 138–140).

Intestinal GC Triggers and the Role of TNF

TNF is a pro-inflammatory cytokine with a wide range of pleiotropic functions. TNF interacts with two different receptors, designated TNF receptor (TNFR) 1 and TNFR2, which are differentially expressed on cells and tissues, and initiate both distinct and overlapping signal transduction pathways. These diverse signaling cascades lead to a range of cellular responses, which include cell death, inflammation, survival, differentiation, proliferation, and migration (141, 142). In the intestinal epithelium, TNF demonstrates variable and very complex functions in physiological as well as pathological conditions (143). TNF has been shown to drastically promote epithelial cell death (144) and increase the epithelial barrier permeability via a direct effect on the expression and organization of TJ proteins, thereby leading to intestinal inflammation (**Figure 2**). In fact, TNF is considered as one of the most important effector molecules in the pathogenesis of IBD (145). Moreover, TNF signaling has been shown to drive colonic tumor formation after sustained chronic colitis. Consequently, TNFR deficiency or the treatment of wild type mice with the specific pharmacological inhibitor of TNF, etanercept, markedly reduces colitis-associated colon cancer (146).

Although the main cellular source for TNF is immune cells, fibroblasts and epithelial cells have also been shown to produce TNF (147). Macrophage and T cell activation results in massive release of TNF, which contributes to the damage of the epithelial layer (148). Therefore, TNF-neutralizing antibodies have been efficiently used for the treatment of IBD (142, 149). This is mainly due to inhibition of IEC cell death, but also due to the downregulation of pro-inflammatory processes that might contribute to local tissue damage (101) (**Figure 2**).

Despite the well-characterized pro-inflammatory properties of TNF, accumulating evidence for anti-inflammatory roles of TNF is increasingly appreciated. For example, Naito et al. demonstrated that the absence or neutralization of TNF in a mouse model of dextran sulfate sodium (DSS)-induced colitis exacerbated intestinal inflammation (150). Further studies revealed that TNF induces intestinal GC synthesis by direct activation of IECs, thus contributing to intestinal immune homeostasis. In this regard, TNF plays an anti-inflammatory role (90) that could be in part through sensitizing activated T cells to undergo apoptosis, thus resulting in accelerated resolution of the inflammation (151). Interestingly, TNF seems to be the master regulator of intestinal GC synthesis irrespective of the trigger (**Figure 2**). Noti et al. investigated the intestinal GC synthesis following macrophage and T cell activation in TNFR-deficient and wild type mice. They showed that, while immune cell activation resulted in robust induction of intestinal GCs in wild type mice, it was significantly decreased in TNFR-deficient mice (89). Similarly, intestinal GC synthesis was lacking in mice with TNF deficiency or in TNFR-deficient mice treated with the inflammatory agent DSS or the hapten 2,4,6-trinitrobenzenesulphonic acid (TNBS). In marked contrast, oxazolone, a hapten that promotes a Th2 cytokine-mediated intestinal inflammation that does not involve TNF, fails to promote intestinal GC synthesis (90). These observations clearly indicate that inflammation *per se* is not sufficient to promote intestinal steroidogenesis, but rather the type of inflammation appears to be critical. It also points out the dependence of intestinal GC synthesis on TNF (90, 95).

Taking into consideration the mutual antagonistic action of TNF and GCs, this GC-regulatory function of TNF might appear confusing at a first glance. Nevertheless, local intestinal GC synthesis may counterbalance the deleterious effects of TNF in two ways: (1) an increase in barrier resistance by promoting the expression of TJ proteins and (2) by dampening overwhelming immune responses and the associated immune cell activation that are triggered by epithelial barrier disruption. Hence, although TNF is involved in the disruption of the epithelial barrier integrity, it is also involved in restoring intestinal epithelial barrier function by the induction of GC synthesis as a negative feedback loop (**Figure 1**). Moreover, since TNF is not only produced by immune cells but also by IECs, it is feasible to believe that this regulatory system may even work in an epithelial layer-autonomous manner (75, 89).

Taken together, TNF seems to function as a sensor of intestinal immune responses and a master regulator of intestinal GC synthesis in response to activation of the innate and adaptive immune system. Furthermore, TNF mediates a novel anti-inflammatory function via the induction of intestinal GC synthesis (89) (**Figure 2**).

Intestinal GCs Functions

Under steady-state conditions, GCs have been implicated in the maturation and the maintenance of the intestinal epithelial barrier integrity. For instance, results from *in vitro* experiments revealed that synthetic GCs had a protective effect against the TNF-dependent increase of intestinal permeability. Microarray data analysis demonstrated that GCs differentially regulate the expression of enterocyte markers that are involved in the polarization and TJ formation (152).

Given the potent immunoregulatory activities of GCs, extra-adrenal GC synthesis in the intestine is assumed to play an

important role in the regulation of local immune homeostasis. Indeed, in the intestinal mucosa GCs are synthesized in response to immunological stress. Local GCs then inhibit the activation of immune cells in a negative feedback leading to the resolution of inflammation and associated tissue damage (77, 89, 90). Following anti-CD3 antibody injection, *in situ* produced GCs exhibited a regulatory activity on intestinal T cells that are in close contact with the GC-producing IECs, i.e., IELs and Peyer's patches lymphocytes (PPLs) (77). Likewise, infection of mice with the lymphocytic choriomeningitis virus (LCMV) results in the activation and expansion of virus-specific intestinal T cells and the subsequent release of GCs. GCs in turn suppress anti-viral immune responses. In fact, inhibition of intestinal GC synthesis accelerated the expansion of antigen-specific cytotoxic T cells, further confirming the immunoregulatory role of locally produced GCs (77, 130).

In another study, experimental colitis induction via DSS or TNBS resulted in epithelial erosion, loss of goblet cells, and strong immune cell infiltration into the intestinal mucosa. Simultaneously, it promoted the upregulation of pro-inflammatory mediators such as TNF, steroidogenic enzymes and the synthesis of intestinal GCs. Notably and in line with the discussed role of TNF in the induction of intestinal GC synthesis, the injection of TNF triggered intestinal GC synthesis and resulted in the amelioration of oxazolone-induced colitis in mice. Interestingly, inhibition of intestinal GC synthesis by metyrapone abrogated the observed anti-inflammatory effect of TNF (89).

More recently, in a mouse model of DSS-induced colitis, mice with IEC-specific deletion of the microsomal P450 reductase enzyme (null mice) exhibited a significant decrease of colonic GC synthesis compared to wild type mice. This was associated with an exacerbated colonic inflammation, as evidenced by the presence of higher levels of pro-inflammatory cytokines, increased weight loss, colon shortening and colonic tissue damage in the null mice. Remarkably, restoration of colonic GC synthesis resulted in amelioration of the colitis (153). This clearly indicates that intestinal GCs are synthesized as a mechanism to counterbalance local inflammation. Supporting this notion, the expression of *CYP11A1* and *CYP11B1* were robustly reduced in the inflamed colon biopsies of patients with IBD compared to healthy controls (138).

Furthermore, intestinal GCs critically regulate the expression of colonic peroxisome proliferator-activated-receptor-gamma (PPARγ). PPARγ is a critical regulator of the inflammatory responses by transrepressing TFs, such as NF-κB and AP-1. Consequently, disruption of PPARγ expression in mouse colonic epithelial cells increases susceptibility to DSS-induced colitis. In line with the anti-inflammatory role of PPARγ, reduced expression was observed in IBD patients. That also correlated with a significant reduction in colonic GC synthesis and the expression of steroidogenic enzymes (140).

We recently demonstrated a significant downregulation of *HSD11B1* gene expression, with a simultaneous upregulation of *HSD11B2*, in colons from pediatric IBD patients compared to healthy controls (96). This opposite transcriptional regulation of 11β-HSD isoenzymes could indicate a possible role of defective local GC reactivation in the pathogenesis of IBD by limiting the local levels of the active immunomodulatory GCs, thus hindering the resolution of inflammation. However, in a murine model of acute colitis we observed the opposite, where we found a significant upregulation of *Hsd11b1* and a downregulation of *Hsd11b2* upon colitis induction (96). Interestingly, these correlations were also reported when comparing inflamed tissue to non-inflamed colonic tissue in IBD patients, suggesting that dysregulation of the 11β-HSD enzyme system could play a role in the pathogenesis of IBD (132, 154). Taken together, in view of the discussed immunoregulatory roles of intestinal GCs, it is conceivable to believe that defective intestinal GC synthesis represents a potential key mechanism in the pathogenesis of IBD.

Intestinal GC Synthesis Regulation

Transcriptional Regulation

Whereas, the regulation of adrenal GC synthesis has been extensively studied and most of the pathways are well-defined, the molecular pathways for the regulation of extra-adrenal GC synthesis await further investigation (21). Mueller et al. investigated the molecular basis of steroidogenesis in the intestine and found substantial differences in the mode of regulation of intestinal GC synthesis as compared to the adrenals. This distinct regulation of intestinal GC synthesis could possibly reflect an adaptation to the local environment (88). For example, in marked contrast to the well-known regulatory role of SF-1 in adrenal GC synthesis [reviewed in (26)], SF-1 expression was found to be absent in the intestine. Interestingly, SF-1 activity was replaced by its close homolog, the NR liver receptor homolog-1 (LRH-1, NR5A2) (87, 88).

LRH-1 is expressed in tissues derived from endoderm, including intestine, liver, exocrine pancreas, and the ovary (155). Moreover, LRH-1 is expressed in macrophages (156) and T cells (157). LRH-1 plays vital roles in early embryonic development as evidenced by the embryonically lethal phenotype of the LRH-1-null mice (158). Other functions of LRH-1 comprise cholesterol and bile acid homeostasis, glucose metabolism and steroidogenesis in adulthood (159, 160). In the intestinal epithelium, LRH-1 contributes to crypt cell proliferation and epithelial cell renewal through the induction of cell cycle genes, namely cyclin D1 and cyclin E1 (161). Therefore, LRH-1 has been suggested as an oncogene and implicated in the development of colon cancer (162).

LRH-1 is constitutively active, though its function is regulated by several mechanisms. These include ligand binding, interactions with co-activators and co-repressors, as well as posttranslational modifications, such as phosphorylation and SUMOylation (160, 163, 164). Although LRH-1 is considered as an orphan NR since no endogenous ligands are identified yet, phospholipids such as dilauroyl phosphatidylcholine (DLPC) have been shown to activate LRH-1. Thus, it is very likely that endogenous ligands exist (165, 166). Among the most studied co-repressors of LRH-1 is the NR small heterodimer partner (SHP) (167), which is also a transcriptional target of LRH-1 (168). Structural studies have shown that SHP preferentially inhibits LRH-1 over other NRs, including the LRH-1 close homolog SF-1 (169, 170).

Differences in Regulation of Intestinal vs. Adrenal GC Synthesis

The differential regulation of intestinal vs. adrenal GC synthesis, i.e., LRH-1 vs. SF-1, is likely reflecting different needs for the systemic vs. intestinal GC synthesis (21). In this regard, another major difference is the differential response of adrenal and intestinal epithelial cells to cAMP and phorbol myristate acetate (PMA). In the adrenals, it is well-established that the activation of ACTH receptors leads to the activation of adenylate cyclase and the formation of cAMP. In turn, cAMP activates protein kinase A leading to the induction of steroidogenic enzyme expression. Surprisingly, cAMP mediated the opposite effect in intestinal epithelial cells by causing a profound inhibition of both basal and LRH-1-driven steroidogenesis. Remarkably, a reciprocal effect was shown upon treatment with PMA that activates protein kinase C. PMA has been shown to substantially promote both basal and LRH-1-induced steroidogenic enzymes expression and GC synthesis in intestinal epithelial cells (88). As PMA is a potent activator of the MAPK pathway, it is likely that PMA affects LRH-1 activity by inducing its phosphorylation (21, 171).

LRH-1 Function in Intestinal Homeostasis

In the murine intestinal epithelial cell line mICcl2, that displays a crypt cell-like phenotype, overexpression of LRH-1 induced the expression of *Cyp11a1* and *Cyp11b1* in a dose-dependent manner. This was accompanied by robust induction of GC synthesis (87). Since LRH-1 is critical for embryonic development, Mueller et al. used LRH-1 haplodeficient mice to investigate the role of LRH-1 in the regulation of intestinal GCs *in vivo*. They showed that although anti-CD3 injection strongly induced the expression of *Cyp11a1* and *Cyp11b1,* and the synthesis of intestinal GCs in wild type mice, it was blunted in LRH-1 haplodeficient mice. These findings confirm the critical role of LRH-1 in the regulation of intestinal GC synthesis (87).

In humans, LRH-1 transcriptionally regulates the expression of the steroidogenic enzymes CYP11A1, CYP17, HSD3B2, and CYP11B1 as well as StAR (172). The importance of LRH-1 in the regulation of intestinal GC synthesis and intestinal immune homeostasis has been demonstrated by the fact that LRH-1 haplodeficient mice and mice with intestine-specific deletion of LRH-1 exhibited strongly reduced GC synthesis, and consequently suffered from exacerbated colitis (87, 96, 138) (**Figure 3**). Furthermore, colon biopsies from patients with IBD show reduced expression of LRH-1 and steroidogenic enzymes. That was inversely correlated with the expression of pro-inflammatory cytokines (138). Additionally, it has been shown that cortisol production and the expression of LRH-1 and 3β-HSD1 were significantly decreased in colonic epithelial cells from patients with UC (140). Recently, we demonstrated a strong correlation between the expression of LRH-1 and steroidogenic enzymes in pediatric IBD patients (96). Importantly, we monitored a significantly reduced expression of *HSD11B1* in colons from IBD patients compared to healthy controls suggesting that defective reactivation of GCs could represent an underlying mechanism in intestinal inflammation. Additionally, in a murine model of colitis we confirmed that colitis-induced expression of the steroidogenic enzymes *Cyp11a1*, *Cyp11b1,* and *Cyp21* is LRH-1-dependent since their induction was significantly reduced in LRH-1 intestine-specific knockout mice (96). These data suggest that the presence of LRH-1 protects the intestinal epithelium against inflammation and underscores a possible role for defective local GC synthesis in the etiology of IBD.

Interestingly, SHP inhibits LRH-1-induced *Cyp11a1* and *Cyp11b1* expression and GC synthesis in mICcl2 cells (88). This indicates a potential role of SHP in the regulation of intestinal immune homeostasis by regulating LRH-1-induced GC synthesis. Recently, Huang et al. investigated the role of the NRs SHP and LRH-1 in the regulation of intestinal GC synthesis and its relevance in intestinal immune homeostasis in the context of viral infection (130). They showed that systemic deficiency of SHP results in increased intestinal GC synthesis during viral infection that suppressed the expansion and activation of virus-specific T cells. In contrast, intestine-specific deletion of LRH-1 strongly reduced intestinal GC synthesis and accelerated the expansion of cytotoxic T cells upon viral infection (130). Noteworthy, Bayrer et al. recently showed that intestinal organoids lacking LRH-1 exhibit reduced expression of the LRH-1 target genes *Shp*, *Cyp11a1,* and *Cyp11b1*, as well as increased crypt cell death and epithelial permeability (173). They also showed that overexpression of LRH-1 mitigated inflammation-induced damage of murine and human intestinal organoids, including those from IBD patients, and decreased the disease severity in a T cell transfer model of colitis (173).

Of note, the expression of steroidogenic enzymes is linked to the cell cycle, thus implicating a restriction of the intestinal GC synthesis to the proliferating cells at the bottom of the crypts (134, 152). Similar to steroidogenic enzymes, LRH-1 expression is confined to the proliferating cells of the crypts, suggesting a cell cycle-dependent regulation of intestinal GC synthesis (87, 134, 161).

LRH-1 seems to contribute to intestinal epithelium homeostasis via two mechanisms: (1) by stimulating the synthesis of anti-inflammatory GCs and thereby resolution of inflammation and associated tissue damage, (2) by enhancing crypt cell proliferation and hence the regeneration of the damaged epithelium (**Figure 4**).

Interestingly, LPS-induced GC synthesis seems not to be regulated by LRH-1, since it was not affected by LRH-1 deficiency. Surprisingly, LRH-1 haplodeficient mice expressed even higher levels of *Cyp11b1* and showed a tendency toward increased GC synthesis in response to LPS exposure compared to wild type mice (89). This clearly indicates that other signals and TFs are regulating GC synthesis in response to innate immune system stimulation. Furthermore, TNF has been shown to suppress LRH-1 and thereby reduce local GC synthesis in sustained chronic colitis (174).

Of interest is the finding that under basal conditions the microbiota also contribute to the regulation of intestinal GC synthesis. Furthermore, intestinal GC synthesis has been shown to regulate systemic metabolism, indicating a so far unrecognized role for intestinal GC synthesis in not only regulating local but also systemic homeostasis (114).

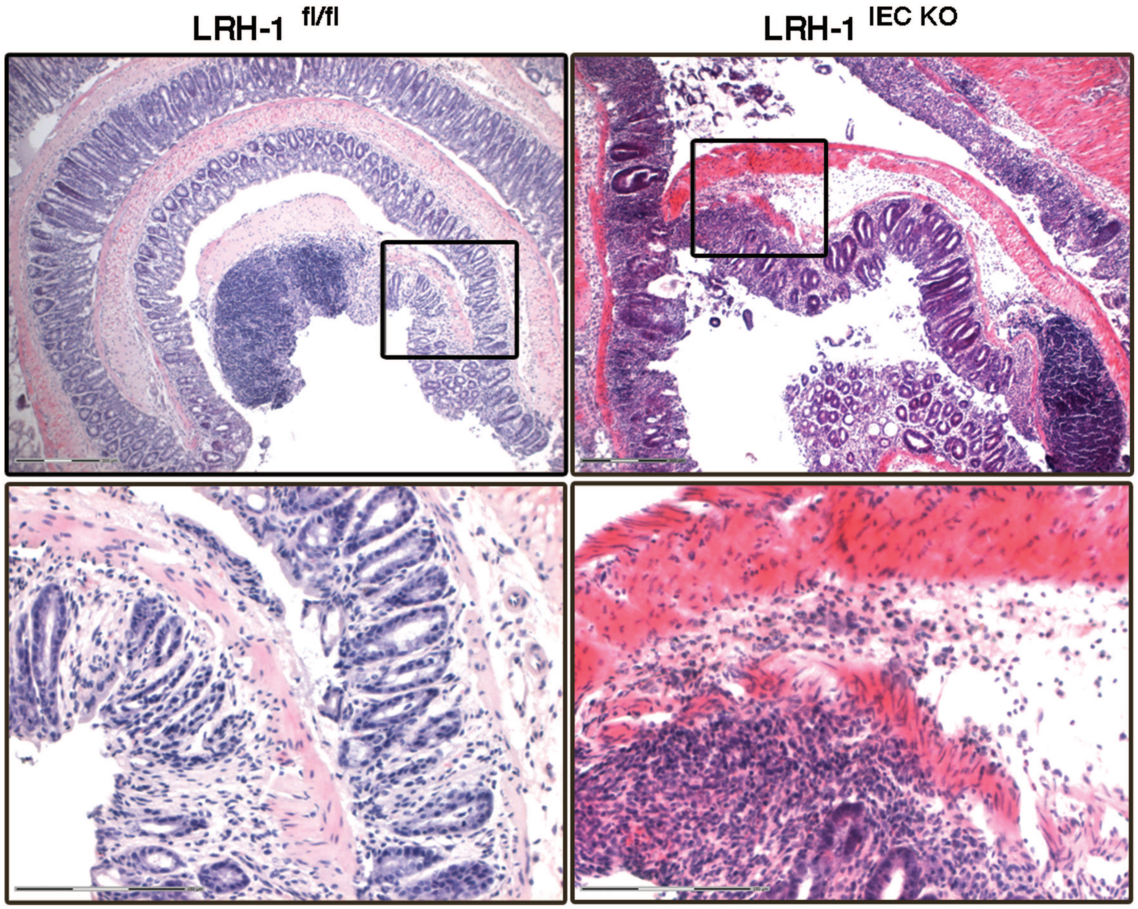

FIGURE 3 | LRH-1 is critical for intestinal immune homeostasis. Colitis was induced in female 8–9 weeks-old wild type (LRH-1$^{fl/fl}$) mice and intestine epithelial cell-specific knockout mice (LRH-1$^{IEC\ KO}$) by administration of 2.2% (w/v) DSS in the drinking water for 5 days followed by normal drinking water for 2 days. Representative H&E staining of Swiss-rolled colon sections of mice treated with DSS at day 7 showing the exacerbated colitis in LRH-1$^{IEC\ KO}$ mice compared to LRH-1$^{fl/fl}$ mice. Scale bars: 300 μm overview, 150 μm inlay.

In summary, despite the well-established roles of TNF and LRH-1 in the regulation of intestinal GC synthesis, their interaction in this process is still unclear. It could be possible that multiple pathways and interaction partners are involved in LRH-1-regulated intestinal GC synthesis. Moreover, we cannot exclude that TNF and LRH-1 are acting via independent mechanisms to stimulate intestinal GC synthesis. Nonetheless, our understanding of these interactions is far from being established and other regulatory mechanisms for intestinal GC synthesis are yet to be defined. It would also be relevant to investigate the possible crosstalk between local intestinal GCs and systemic GCs, and how this is regulated.

LRH-1 IN INTESTINAL TUMORS

In the intestinal epithelium, LRH-1 regulates not only steroidogenesis (87, 89), but also crypt cell proliferation (161). Thus, LRH-1 has been shown to contribute to intestinal tumor formation (162) (**Figure 4**). LRH-1 induces cell proliferation through the concomitant induction of the cell cycle-regulating gene products cyclin D1 and E1, and c-Myc, which is further potentiated by its interaction with β-catenin. Whereas, β-catenin co-activates LRH-1 after direct binding of LRH-1 to the cyclin E1 promoter, LRH-1 acts as a co-activator for β-catenin/TCF4 (T cell factor 4) on the cyclin D1 promoter (161, 162). Due to its role in proliferation and the maintenance of pluripotency, LRH-1 has emerged as an oncogene implicated in the development of a variety of cancers, including pancreatic (175), prostate (176), breast (177, 178), gastric (179), and colorectal cancer (CRC) (162, 180). LRH-1 exhibited an increased expression pattern in high-grade prostate cancer, and has been reported to promote prostate cancer growth by inducing intra-tumoral steroidogenesis (176). LRH-1 also contributed to metastasis development in pancreatic cancer (175).

LRH-1 has been shown to drive colon cancer cell growth by repressing the expression of the cell cycle inhibitor p21 in a p53-dependent manner (180). Consistent with the role of LRH-1 in CRC development, it has been shown that LRH-1 heterozygous mice developed significantly less tumors compared to wild type in two independent models of CRC,

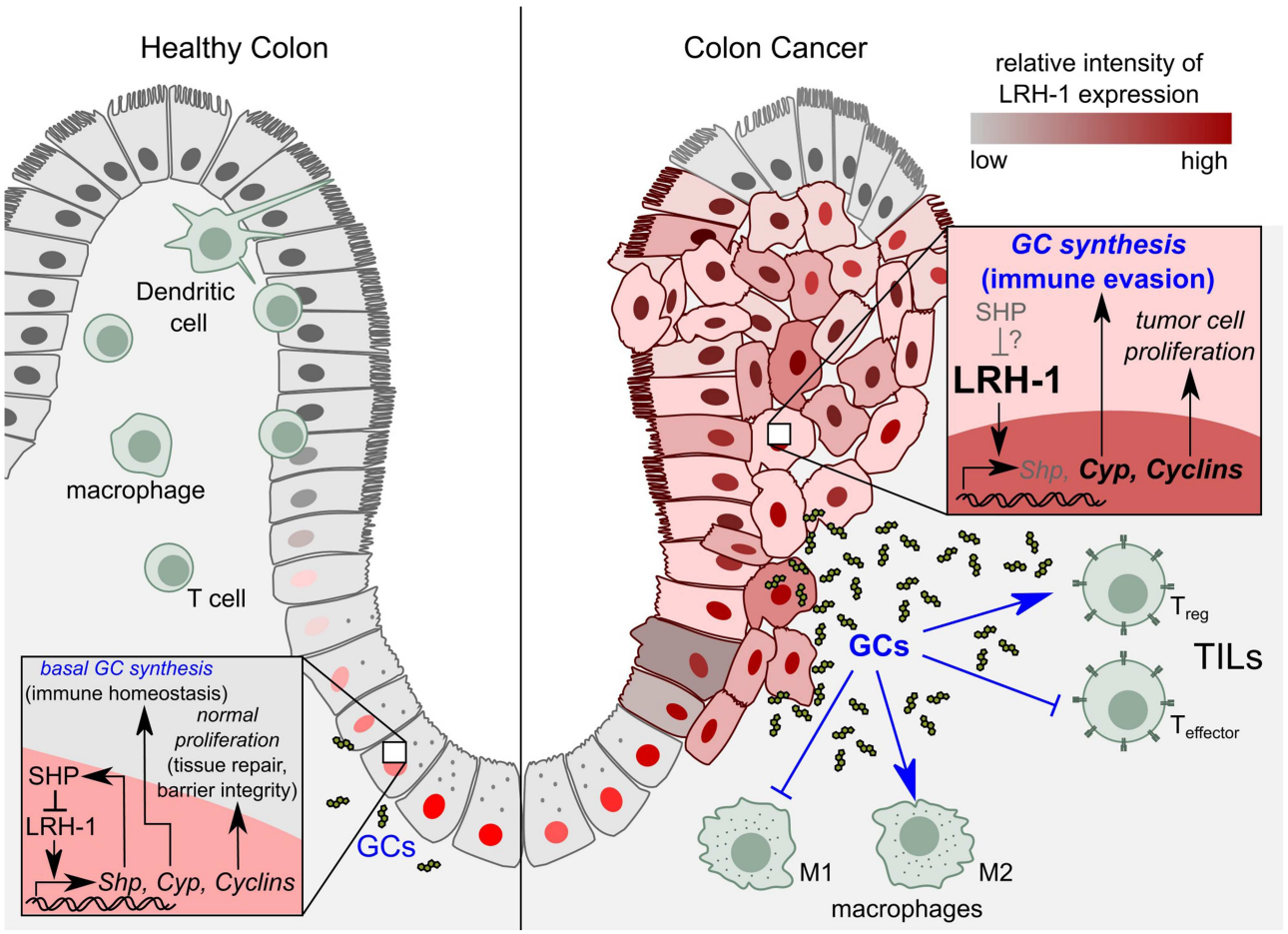

FIGURE 4 | Role of LRH-1 in healthy colon vs. colon cancer. Left panel: In healthy colon, LRH-1 is expressed in the nucleus of cells at the bottom of the intestinal crypts, where it regulates intestinal immune homeostasis by the regulation of cell proliferation through cyclins on the one hand, and the synthesis of immunoregulatory glucocorticoids (GCs) on the other hand. Right panel: In colon cancer, LRH-1 exhibits a nuclear as well as cytoplasmic expression pattern. LRH-1 induces colon tumor cell proliferation by upregulating expression of cyclins. LRH-1 is proposed to play a role in tumor immune evasion by the synthesis of immunosuppressive GCs that leads to the inhibition of anti-tumor immune responses. While in healthy tissue SHP imposes a negative feedback loop to LRH-1 signaling, the role of SHP in the molecular events during colon cancer development remains to be elucidated. SHP, Small heterodimer partner; TILs, tumor infiltrating lymphocytes; T_{reg}, regulatory T cells; $T_{effector}$, effector T cells; M1, pro-inflammatory macrophages (anti-tumor); M2, anti-inflammatory (tumor promoting) macrophages.

the azoxymethane-induced and $APC^{min/+}$ mice model (162). Unlike the nuclear expression of LRH-1 at the bottom of the normal colonic crypts, immunostaining of neoplastic colon from patients with high-degree dysplasia showed significantly higher cytoplasmic levels. Additionally, in neoplastic lesions, staining of LRH-1 was no longer limited to the cells lining the crypts but also present in the surface epithelial cells (**Figure 4**). These alterations in LRH-1 expression and subcellular localization further indicate the important role of LRH-1 in CRC development (162). Moreover, if and how the LRH-1-induced SHP, which in healthy colon tissue counterbalances LRH-1 function, contributes to the molecular events during colon cancer development, remains unknown (**Figure 4**).

In contrast to the known role of LRH-1 in intestinal tumorigenesis, LRH-1 expression has been shown to be significantly downregulated in murine adenoma tissue compared to adjacent normal mucosa. The expression of LRH-1 gene was reduced in tumors that express elevated levels of the pro-inflammatory cytokine TNF. Reciprocally, decreased LRH-1 expression in heterozygous mice attenuates TNF expression (162). However, the relevance of this inverse correlation is so far unknown and again points out the complex interaction between TNF-induced signaling pathways and LRH-1.

Recently, a large CRC patient cohort revealed that immunohistochemical detection of LRH-1 expression was drastically enhanced in colon cancer tissue compared to adjacent non-cancerous tissue from the same patient, and this correlated with a more advanced disease stage. In fact, patients with positive LRH-1 expression displayed significantly lower overall survival rate. Consequently, the authors proposed LRH-1 as a possible prognostic marker and a novel therapeutic target in CRC (181). These observations were confirmed in another recent study that revealed marked overexpression of LRH-1 in CRC tissue compared to paired non-cancerous tissue (182). Taken together,

LRH-1 represents a novel and promising therapeutic target for the treatment of cancer.

INTESTINAL GC SYNTHESIS AS A TUMOR IMMUNE ESCAPE MECHANISM

The notion that the immune system can recognize and destroy transformed cells is known as cancer immune surveillance. However, since the role of the immune system in controlling cancer growth and recurrence remains highly controversial, this term has been replaced by "cancer immunoediting" to describe the dual roles of the immune system in promoting host defense and facilitating tumor growth and immune escape (183, 184). Several mechanisms by which cancer cells evade the immune system have been described. These include: (1) immune suppression at the tumor microenvironment mediated by Tregs or other types of suppressive cells (the major mechanism of tumor immune escape), (2) induction of apoptosis in tumor-specific cytotoxic T lymphocytes (CTLs) by the expression of pro-apoptotic ligands e.g., Fas ligand and TRAIL, (3) defective antigen presentation, (4) release of immunosuppressive cytokines such as IL-10 and TGFβ, and (5) inducing tolerance and immune deviation by mechanisms including, among others, shifting the balance of Th1 immune responses to Th2, and expression of immune inhibitory molecules such as PD-1 (programmed death-1) and CTLA-4 (CTL antigen-4) (95, 185).

Colorectal tumors are highly immunogenic. Therefore, anti-tumor immune responses may significantly limit tumor growth. In fact, a strong correlation between anti-tumor immune responses and CRC patient survival has been demonstrated (186–188). On the other hand, immune escape mechanisms have been recognized as one of the hallmarks of cancer (123, 189). Pagés et al. studied the correlation between pathological signs of early metastatic invasion and the local immune response within the tumor in a cohort of 959 resected colorectal tumors using flow cytometry, gene expression profiling and *in situ* immunohistochemistry (186). In this study, the authors reported up to 15 years clinical follow-up of the patients for the presence or absence of early signs of metastasis. Remarkably, they showed that tumors without such signs had increased infiltrates of CD8+ T cell numbers and increased gene expression for CD8, T-box transcription factor 21, interferon regulatory factor 1, IFN-γ, granulysin, and granzyme B, that correlated with increased survival. Likewise, the presence of high levels of infiltrating memory T cells, as measured by immunohistochemistry, correlated with increased survival (186). The same group confirmed these results in two other independent cohorts of CRC patients (187). Furthermore, in 566 CRC patients a significant positive correlation between markers of innate immune system and early activated T cells has been linked to protection from relapse. Additionally increased densities of CTLs and effector memory T cells within the primary tumor significantly protected CRC patients from tumor recurrence (188). In another study, CRC patients with high expression of Th17 markers had a poor prognosis, whereas patients with high expression of the Th1 markers had prolonged disease-free survival (190). These data provide compelling evidence for the role of the immune system in limiting CRC development and clearly suggest that immune evasion could represent an important mechanism by which colorectal tumor cells prevent their destruction by the immune system.

Supporting this hypothesis, Sidler et al. described the first evidence for a novel LRH-1-dependent GC synthesis in CRC cell lines as well as primary tumors, that exerted inhibitory effects on activated T cells (139). They showed that colon cancer cell lines express the enzymes required for *de novo* synthesis of bioactive GCs, including CYP11A1, CYP11B1, and CYP17. Consequently, cortisol production as measured by thin layer chromatography, radioimmunoassay, and bioassay was detected in culture supernatants (139).

The expression of steroidogenic enzymes in CRC cells is dependent on endogenous LRH-1, as evidenced by the diminished expression of these enzymes upon LRH-1 downregulation. Similar to intestinal GC synthesis, tumor-cell derived GC synthesis was also regulated by LRH-1 since overexpression of LRH-1 boosted cortisol production in a dose-dependent manner, whereas it was significantly inhibited following LRH-1 knockdown. Primary tumors from CRC patients also expressed high levels of LRH-1, CYP11A1, CYP11B1, and StAR, and readily synthesized cortisol following *ex vivo* culture. Interestingly, unlike the basal inducible GC production in the normal intestine, LRH-1-mediated GC synthesis in colonic tumors is constitutive since it was not further enhanced by PMA (139). This observation suggests that LRH-1 is constitutively active, or the presence of LRH-1 activators in the tumor microenvironment. Of interest, enhanced EGF signaling as demonstrated by EGFR overexpression has been shown in 60–80% of CRC patients, that was associated with poor prognosis (191). Since EGF has been shown to exert a mitogenic signal by the MAPK pathway (192), it is tempting to speculate that EGF-induced signaling pathways activate LRH-1 in CRC tumors via a MAPK-induced phosphorylation. However, this hypothesis needs to be further investigated.

Noteworthy, tumor-derived GCs suppressed T cell activation, as shown by the substantial inhibition of CD69 expression (an early activation marker of T cells) in activated $CD4^+$ and $CD8^+$ murine splenic T cells. This inhibitory effect was GC-specific since it was reversed by blocking the GR (139). Hence, besides its role in inducing tumor cell proliferation, LRH-1 could contribute to CRC tumor development via the synthesis of immunosuppressive GCs (**Figure 4**). Taken together, LRH-1-mediated synthesis of immunoregulatory GCs in CRC could represent a novel immune escape mechanism by inhibiting T cell-mediated anti-tumor immune responses and thereby favoring the tumor growth.

THERAPEUTIC POTENTIAL AND FUTURE PERSPECTIVE OF INTESTINAL GC SYNTHESIS

Targeting GCs in Intestinal Inflammation

Thus far, the importance of locally synthesized GCs has been reflected by the impairment of cortisol production as well as decreased LRH-1 expression in colonic epithelial cells from UC

patients (138, 140). Despite the advances in introducing novel therapies for the treatment of IBD, GCs remain the first-line treatment for inducing rapid remission in moderate to severe IBD with high efficacy. Nevertheless, emergence of resistance and the side-effects of systemic GCs represent a major therapeutic challenge (131, 193). Along these lines, restoring local GC synthesis in the intestine could represent an attractive approach to ameliorate the symptoms of IBD and to avoid the systemic GC side-effects. This could be achieved by enhancing LRH-1 activity in the intestine since LRH-1 controls both local GC synthesis and epithelial regeneration (87, 89, 90, 138). In fact, a recent study underlined the therapeutic potential of targeting LRH-1 by showing that restoration of LRH-1 reestablished epithelial integrity in mouse and human organoids treated with TNF or 5-fluorouracil, a chemotherapeutic agent with intestinal toxicity. Moreover, overexpression of LRH-1 protected mice from T cell-induced colitis (173). As mentioned earlier, structure-based studies identified DLPC as a potential ligand that was able to enhance LRH-1 transcriptional activity (166). Interestingly, DLPC has been shown to exert anti-diabetic effects by activating LRH-1 in the liver when used in a therapeutic setting (194, 195). Thus, it is tempting to speculate that administration of LRH-1 ligands could also ameliorate intestinal inflammation. However, this attractive idea remains to be tested.

Targeting GCs in Colorectal Cancer

In CRC, LRH-1 regulates proliferation as well as GC synthesis that could possibly represents an immune escape mechanism (139) (**Figure 4**). In line with this, LRH-1 has also been described to promote prostate cancer growth by inducing intra-tumoral steroidogenesis (176).

Consistent with the critical role of LRH-1 in tumor development, LRH-1 is overexpressed in many tumors, as discussed above. For instance, a remarkable upregulation of LRH-1 was reported in CRC tissue compared to paired non-cancerous tissue from two independent CRC patient cohorts (181, 182). Hence, suppression of LRH-1 activity in tumors is postulated to exert anti-proliferative effect that could potentially lead to tumor regression. Supporting this notion, LRH-1 knockdown resulted in impaired *in vitro* proliferation of pancreatic and CRC cell lines (175, 196). Recently, Qu et al. showed that targeting LRH-1 via microRNA inhibited *in vitro* proliferation and invasion of CRC cell lines (182). These data provide compelling evidence for the therapeutic potential of targeting LRH-1 in cancer. Advances in structure-based studies identified small molecule inhibitors of LRH-1 including 3d2 (197) and SR1848 (198). The inhibitory effect of 3d2 and SR1848 on LRH-1 was confirmed *in vitro* and *in vivo* and reported to induce anti-proliferative effects on a variety of cancer cell lines (157, 197, 198).

In conclusion, inhibition of LRH-1 activity in colon tumors with high LRH-1 expression represents an interesting therapeutic approach to be followed upon, aiming at inhibition of both LRH-1-induced proliferation as well as GC synthesis. This is of particular interest since in CRC a strong correlation between the degree of immune cell infiltrates and patient survival has been demonstrated (186, 187). Of note, *ex vivo* culture of primary colonic tumors from patients showed increased GC synthesis compared to adjacent non-tumor tissue (139). These observations further underscore that immune evasion, e.g., via the synthesis of immunoregulatory GCs, might be an important mechanism by which intestinal tumors shape the tumor microenvironment resulting on one hand in tumor support by stromal cells, on the other hand in the escape of CRC from the destruction by the immune system.

AUTHOR CONTRIBUTIONS

AA and TB designed and discussed the manuscript. AA wrote the manuscript and drafted the figures. CS finalized the figures and revised the manuscript. TB finalized the manuscript.

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Role of Dual-Specificity Phosphatase 1 in Glucocorticoid-Driven Anti-inflammatory Responses

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Glucocorticoids (GCs) potently inhibit pro-inflammatory responses and are widely used for the treatment of inflammatory diseases, such as allergies, autoimmune disorders, and asthma. Dual-specificity phosphatase 1 (DUSP1), also known as mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1), exerts its effects by dephosphorylation of MAPKs, i.e., extracellular-signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK). Endogenous DUSP1 expression is tightly regulated at multiple levels, involving both transcriptional and post-transcriptional mechanisms. DUSP1 has emerged as a central mediator in the resolution of inflammation, and upregulation of DUSP1 by GCs has been suggested to be a key mechanism of GC actions. In this review, we discuss the impact of DUSP1 on the efficacy of GC-mediated suppression of inflammation and address the underlying mechanisms.

Keywords: sepsis, infection, arthritis, bone disease, asthma, COPD, atherosclerosis

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INTRODUCTION

Glucocorticoids (GCs) are steroid hormones with immunosuppressive activity that are used to treat a wide variety of inflammatory conditions, including rheumatoid arthritis, pulmonary diseases, and acute inflammation caused by microbial infection.

Anti-inflammatory properties of GCs are partially dependent on their ability to suppress mitogen-activated protein kinases (MAPKs) (1, 2). MAPKs are a family of protein kinases that respond to a wide variety of extracellular stimuli. They are activated by phosphorylation of tyrosine and threonine residues within their active domains and are inactivated by dephosphorylation of either residue (2–4). MAPK cascades are evolutionary conserved and control a large number of cellular processes, including proliferation, differentiation, apoptosis, motility, and stress responses. The three major signaling cascades either involve extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), or p38 MAPK (2–4). Dysregulation of MAPK activity has been suggested to contribute to the onset of many pathologies, including neurodegenerative diseases, diabetes, cancer, and inflammation (4–7).

MAPKs can be dephosphorylated by tyrosine-specific phosphatases, serine-threonine phosphatases, or dual-specificity (Thr/Tyr) phosphatases (DUSPs) (8, 9). GC treatment primarily attenuates MAPK signaling via DUSP1, also known as mitogen-activated protein kinase phosphatase-1 (MKP-1) (10–12). In this review, we discuss the influence of DUSP1 on GC-mediated effects (**Figure 1**).

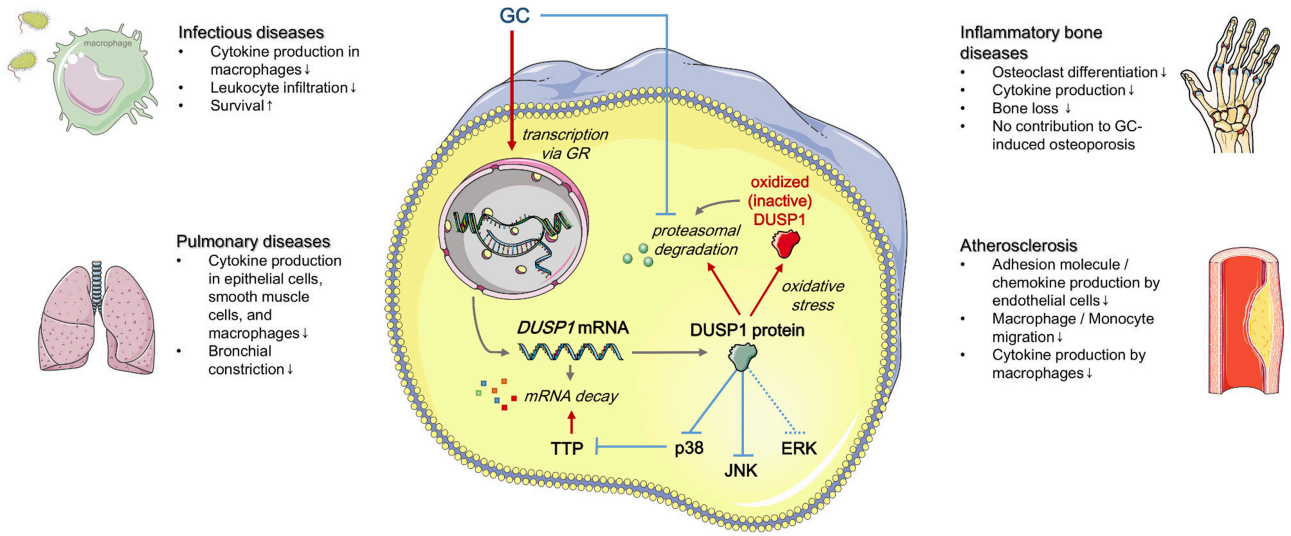

FIGURE 1 | Regulation and effects of GC-induced DUSP1 expression (simplified). Red: positive regulation, blue: negative regulation. See text for details. Templates from Servier Medical Art (http://www.servier.com) were used to generate the figure.

DUAL-SPECIFICITY PHOSPHATASE 1 (DUSP1)

Although DUSP1 was initially identified as an ERK-specific phosphatase, p38 MAPK and JNK are its preferred substrates in several cell types, including myeloid cells (13–16). Thus, DUSP1 activity limits p38 and JNK-dependent pro-inflammatory gene transcription (17–20).

However, DUSP1 is also involved in the regulation of anti-inflammatory genes. Over-production of IL-10 in *Dusp1*$^{-/-}$ mice was observed in peritonitis models after lipopolysaccharide (LPS) challenge or infection with *Escherichia coli* or *Staphylococcus aureus*, and LPS-treated *Dusp1*$^{-/-}$ macrophages. This can be explained by the interaction of DUSP1 with the RNA-binding protein tristetraprolin (TTP, gene name *Zfp36*): increased p38-mediated phosphorylation of TTP results in its inactivation, followed by accumulation of the inactive, but stable, form of TTP and enhanced stability of TTP target mRNAs. These target mRNAs comprise pro-inflammatory chemokines and cytokines, e.g., *Tnf*, *Cxcl1*, and *Cxcl2*, but also the anti-inflammatory *Il10*. Approximately 50% of the genes dysregulated in *Dusp1*$^{-/-}$ macrophages are affected by TTP inactivation (21).

The promoter region of the *Dusp1* gene contains binding sites for several transcription factors, including activator protein 1 (AP-1), nuclear factor-κB (NF-κB), cAMP response element-binding protein (CREB), and the glucocorticoid receptor (GR) (22–25). Hence, DUSP1 can be induced under various conditions, ranging from inflammatory activation to altered cellular metabolism and GC excess during stress responses. GCs may further enhance DUSP1 expression by inhibiting its proteasomal degradation (12).

DUSP1 has been shown in a few studies to be regulated via the stability of its mRNA. Several mRNA binding proteins can influence *Dusp1* mRNA stability. TTP-mediated *Dusp1* mRNA decay has been suggested to be a feedback mechanism in inflammatory responses by which TTP limits its own activity: reduced DUSP1 expression enhances p38 MAPK phosphorylation, thereby promoting TTP inactivation (26). Besides, several miRNAs, such as miR-101, have been shown to modulate DUSP1 expression (27). Posttranslational DUSP1 modifications include phosphorylation, acetylation, and oxidation. ERK-mediated phosphorylation of DUSP1 can either lead to increased or decreased DUSP1 protein stability, depending on the phosphorylation site (28, 29). Acetylation of Lys57 results in increased phosphatase activity and more effective suppression of the MAPK signaling cascades (30, 31). In contrast, oxidation of Cys258 within the active site inactivates DUSP1 and leads to its rapid degradation by the proteasome. In this manner, DUSP1 oxidation prolongs MAPK activation, ultimately resulting in enhanced inflammatory responses (32–34). S-glutathionylation of Cys258 has similar effects, indicating that DUSP1 activity is redox-sensitive (35).

Abbreviations: AP-1, activator protein-1; CASP, colon ascendens stent peritonitis; CCL, CC-chemokine ligand; CLP, caecal ligation and puncture; COPD, chronic obstructive pulmonary disease; CREB, cAMP response element-binding protein; CXCL, C–X–C motif ligand; DUSP1, dual-specificity phosphatase; ERK, extracellular-signal-regulated kinase; EC, endothelial cell; GC, glucocorticoid; GR, GC receptor; GRE, GR responsive element; ICAM1, intercellular adhesion molecule 1; ICS, inhaled corticosteroid; IL, interleukin; INF, interferon; IRFs, interferon regulatory factors; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; MKP-1, mitogen-activated protein kinase phosphatase-1; RANKL, receptor activator of NF-κB ligand; TNF, tumor necrosis factor; TLR, toll-like receptor; TTP, tristetraprolin; VCAM-1, vascular cell adhesion molecule 1.

ROLE OF DUSP1 IN INFLAMMATORY DISEASES AND ITS INFLUENCE ON GC TREATMENT EFFICACY

Infectious Diseases and Sepsis

In the context of infectious diseases and sepsis, research on the role of DUSP1 focused mainly on macrophage responses. Macrophages are a subtype of innate immune cells with high plasticity that play a crucial role in acute inflammation. They recognize pathogen- or danger-associated molecular patterns via pattern recognition receptors, such as toll-like receptors (TLRs). Stimulation of macrophages initially leads to excessive inflammation, followed by a phenotypic switch toward an anti-inflammatory and wound-healing phenotype that promotes the resolution of inflammation (36, 37).

Early studies on the role of DUSP1 in the response of macrophages to bacterial LPS suggested that DUSP1 is required to balance inflammatory responses in sepsis and infectious diseases. The ectopic expression of DUSP1 in LPS-stimulated macrophages accelerated JNK and p38 inactivation and substantially inhibited the production of TNF-α and IL-6 (38). Moreover, increased cytokine production and elevated expression of the differentiation markers CD86 and CD40 were observed in macrophages from *Dusp1*$^{-/-}$ mice when activated by TLR ligands. *Dusp1*$^{-/-}$ macrophages also showed enhanced constitutive and TLR-induced activation of p38 MAPK (39). Moreover, LPS-induced IFN-β production was increased in *Dusp1*$^{-/-}$ macrophages, both due to elevated JNK-mediated activation of cJun and *Ifnb* mRNA stabilization by TTP inactivation (20). DUSP1 induction has also been shown to be involved in endogenous feedback loops initiated by either adenosine or prostaglandin E2 signaling that skew macrophages toward an anti-inflammatory phenotype (40, 41).

Several studies confirmed the relevance of these *in vitro* findings for the *in vivo* situation. In LPS-treated mice, DUSP1 is upregulated in various tissues and cell types and limits p38 MAPK activation. In accordance, depletion of DUSP1 led to the excessive release of inflammatory cytokines, such as TNF-α, IL-6, CCL3, and CCL4, and increased LPS-induced mortality (14, 15, 39, 42). Likewise, *Dusp1*$^{-/-}$ mice showed amplified inflammatory responses and lethality after infection with either *S. aureus* (43) or *E. coli* (44).

The phenotype of *Dusp1*$^{-/-}$ mice in two sophisticated models of sepsis, i.e., caecal ligation and puncture (CLP) and colon ascendens stent peritonitis (CASP), strongly resembled those observed after LPS shock, with highly increased levels of IL-6, CCL3, and CCL4 and excess lethality (45).

Glucocorticoids induce DUSP1 in mouse macrophages, and DUSP1 is required for the inhibition of JNK and p38 MAPK by dexamethasone in these cells (10, 38). Consequently, the GC-mediated shift toward an anti-inflammatory macrophage phenotype was attenuated in cells from *Dusp1*$^{-/-}$ mice (10, 46). In a cutaneous air pouch model, the zymosan-induced production of pro-inflammatory mediators and the infiltration of leukocytes into a pre-formed dorsal cavity were inhibited by oral dexamethasone administration in wild-type, but not in *Dusp1*$^{-/-}$, mice, suggesting that DUSP1 is indeed required to unfold the full anti-inflammatory potential of GCs (10). In another study, the reduction of TNF-α-induced mortality caused by pretreatment with dexamethasone was dependent on the presence of DUSP1: whereas wildtype mice were entirely protected by dexamethasone administration, *Dusp1*$^{-/-}$ animals did not benefit from the GC treatment (1).

In conclusion, both *in vitro* and *in vivo* evidence suggests that DUSP1 critically contributes to the resolution of acute inflammatory responses and mediates protective GC effects in this context.

Inflammatory Bone Disorders

The bone mass is subject to constant remodeling orchestrated by osteoblasts and osteoclasts. In inflammatory bone disorders, e.g., autoimmune-driven rheumatoid arthritis or pathogen-induced periodontitis, the balance of osteoblast and osteoclast activity is compromised, resulting in bone loss (47).

DUSP1 was strongly downregulated in synovial biopsies from patients with rheumatoid arthritis and osteoarthritis (GEO datasets GDS5401 and GDS5403; **Figure 2A**), suggesting that DUSP1 deficiency may contribute to disease progression.

DUSP1 indeed effectively reduced osteolysis in studies utilizing mouse models of LPS-induced inflammatory bone loss and collagen-induced arthritis (CIA) (48, 49). *Dusp1*$^{-/-}$ mice showed excessive bone loss, more inflammatory infiltrates, and an increase in osteoclastogenesis at the site of LPS-injection in a model of experimental periodontitis (49). In line with these findings, adenovirus-mediated overexpression of DUSP1 was shown to protect against bone loss in a similar experimental model of periodontal disease (50). Furthermore, *Dusp1*$^{-/-}$ mice exhibited higher penetrance, earlier onset, and increased severity of experimental arthritis, accompanied by higher numbers of osteoclasts in inflamed joints and more extensive loss of bone mass. Complementary *in vitro* experiments showed that DUSP1 acts as a negative regulator of osteoclast formation and activation via suppression of p38 MAPK (48, 51). A recently published study showed that the presence of calcium crystals, which are critical factors in the pathogenesis of osteoarthritis, stimulate receptor activator of NF-κB ligand (RANKL) secretion by osteoblasts via DUSP1 downregulation, thereby promoting osteoclastogenesis (52). RANKL induction was also observed in synovial biopsies from arthritis patients in the GEO datasets mentioned above (**Figure 2B**). Moreover, overexpression of DUSP1 in fibroblast-like synoviocytes from osteoarthritis patients inhibited the expression of osteoarthritis-associated mediators (53).

However, DUSP1 depletion did not affect age-related spontaneously occurring osteoarthritis, since knockout mice showed a similar disease progression compared to controls at 21 months of age (54). Thus, the modulatory function of DUSP1 in the context of bone homeostasis seems to be most evident in the presence of a potent inflammatory trigger.

Due to their high anti-inflammatory capacity and their ability to decrease radiologic disease progression, GCs are frequently used for the treatment of rheumatoid arthritis. Paradoxically, one common side effect of GC use, primarily when used at high dosages or over prolonged periods, is a loss of bone mass,

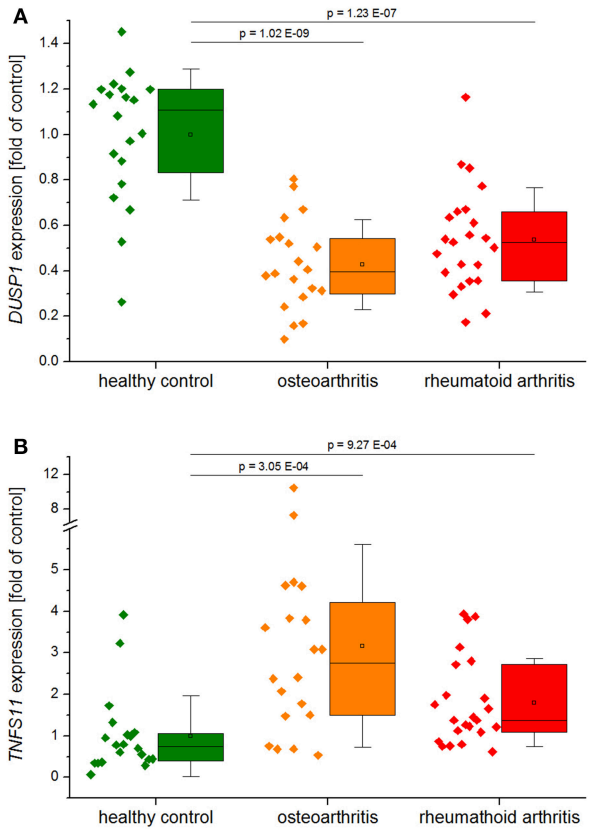

FIGURE 2 | *DUSP1* **(A)** and *TNFSF11* (RANKL, **B**) expression in synovial tissues from healthy controls, patients with rheumatoid arthritis, or osteoarthritis. Data obtained from GEO Datasets GDS5401 (Berlin dataset) and GDS5403 (Jena dataset) were normalized against their respective healthy control values before compilation. Data are shown as individual values per sample, and boxplots show the 25–75th percentiles (box), mean (square), median (line), and standard deviation (whiskers). *P*-values were generated by one-way ANOVA and Bonferroni's *post-hoc* test (**A**, normal distribution) or Mann–Whitney *U*-test (**B**, not normally distributed).

also known as GC-induced osteoporosis. This adverse effect is associated with increased osteoclastogenesis and depletion of osteoblasts (55, 56).

DUSP1 has been suggested to contribute to GC-induced bone loss since GC-inducible attenuation of osteoblast proliferation involves inhibition of the MAPK/ERK signaling pathway and can be reversed by the protein tyrosine phosphatase (PTP) inhibitor vanadate *in vitro* and *in vivo* (57–59). The assumption that the PTP in question might be DUSP1 was, however, not supported by studies with *Dusp1*$^{-/-}$ mice, which demonstrated that GC-induced bone loss was not prevented upon DUSP1 depletion: after treatment with the GC methylprednisolone for 28 days, both wildtype and *Dusp1*$^{-/-}$ mice showed a similar reduction of osteoid surfaces, volumes, and osteoblast numbers (60).

In summary, loss of DUSP1 favors bone loss, especially under highly inflammatory conditions. Further studies are required to clarify whether DUSP1 contributes to the beneficial or adverse effects of GCs in the therapy of bone-related diseases.

Pulmonary Diseases

GCs are first line anti-inflammatory medicines in chronic respiratory diseases, including asthma and chronic obstructive pulmonary disease (COPD), and are commonly used therapeutically as inhaled corticosteroids (ICS). ICS effectively control inflammation in asthma but are less effective in COPD. This is thought to be due to corticosteroid insensitivity, where the molecular pathways responsible for the effect of GCs have been modified by oxidative stress or infections (61). Moreover, a subset of asthmatics (∼10%) are refractory to ICS and classified as having severe asthma. DUSP1 has been shown to contribute to the effects of GCs in several *in vitro*, *ex vivo*, and *in vivo* studies with relevance to respiratory disease (61–63) and in some key studies, an impact on DUSP1 function has been shown to be responsible for corticosteroid insensitivity/resistance. For instance, an *ex vivo* study examined the repressive effect of GCs on stimulated production of inflammatory cytokines by alveolar macrophages from patients with severe asthma to those with non-severe asthma. GCs were less effective in macrophages from severe asthma patients, and this GC insensitivity was linked with increased p38 MAPK activation and impaired inducibility of DUSP1 (64).

The first to demonstrate that GCs upregulated DUSP1 in primary airway smooth muscle cells were Issa et al. (65). This was confirmed in a publication by the Ammit group that showed that GC-induced DUSP1 controlled cytokine mRNA stability in a p38 MAPK-mediated manner (66). Notably, knockdown of DUSP1 with siRNA showed that GC-induced DUSP1 was a significant contributor to anti-inflammatory effects at the post-transcriptional level.

Several *ex vivo* and *in vivo* studies utilizing *Dusp1*$^{-/-}$ mice highlighted the contribution of DUSP1 to GC effects in respiratory disease. For example, GC-mediated repression of the contractile response in bronchial rings from mice was abrogated by *Dusp1* depletion (67). Interestingly, the anti-inflammatory impact of DUSP1 was lost in ozone-exposed mice in a model that may recapitulate corticosteroid resistance in severe asthma (68). A plausible explanation is that GC-induced DUSP1 in the wild-type mice was oxidized by ozone and rendered non-functional. Oxidization of DUSP1 may prove to be a roadblock to further development of DUSP1 as a therapeutic target in respiratory disease as oxidative stress is a well-appreciated feature of COPD and other conditions where smoking is a risk factor (69, 70).

Finally, there are publications that note that GC-mediated effects in respiratory disease are DUSP1-independent. These include a study that detected gene expression of known GC targets in biopsies from allergen-challenged asthmatic subjects (71). Evidence from studies utilizing *Dusp1*$^{-/-}$ mice in models with relevance to asthma is somewhat equivocal and does not fully support the assertion that DUSP1 is a significant contributor to the effect of GCs *in vivo* (72).

Atherosclerosis

GCs are not a therapeutic option for the treatment of atherosclerosis, since side effects of long-term GC treatment include hyperglycemia, hypertension, dyslipidemia, and obesity

and may, therefore, promote adverse cardiovascular events (73, 74). However, as inflammation plays a significant role in the pathogenesis in atherosclerosis, GCs may exert some anti-atherosclerotic effects. Early studies demonstrated that dexamethasone reduced the severity of atherosclerosis in experimental rabbit models (75–77). Moreover, vein graft thickening was prevented by short-term dexamethasone treatment in hypercholesterolemic mice (78). The development of a drug-eluting bioadhesive gel that allowed to dissociate the systemic adverse and local anti-inflammatory effects of GC treatment. In atherosclerotic mice, inflamed plaques treated with GC-eluting adhesive gels showed reduced macrophage numbers and developed protective fibrous caps covering the plaque core. This was paralleled by lowered plasma cytokine levels and biomarkers of inflammation in the plaque (79).

The onset of atherosclerosis is triggered by proinflammatory mediators, which induce adhesion molecules in endothelial cells (ECs) by activating MAPKs, particularly p38 MAPK. Dexamethasone-induced DUSP1 upregulation caused inactivation of p38 MAPK in TNF-α-treated ECs and mediated inhibition of E-selectin expression, as shown in murine *Dusp1*$^{-/-}$ ECs and human ECs upon *DUSP1* silencing (80).

The assumption that DUSP1 is atheroprotective via inhibition of EC activation was further supported by studies investigating the influence of shear stress. ECs respond to shear stress via mechanoreceptors that translate mechanical distortions into various molecular signals, including GR translocation (81, 82). Regions of the arterial tree exposed to high shear stress are protected from endothelial activation, inflammation, and atherosclerosis, whereas regions exposed to low or oscillatory shear stress, are susceptible (83, 84). The expression of DUSP1 in cultured ECs was elevated by shear stress, whereas vascular cell adhesion protein (VCAM)-1 levels were reduced; silencing of DUSP1 restored VCAM-1 expression. *In vivo*, DUSP1 was preferentially expressed by ECs in a high-shear, protected region of the mouse aorta and was necessary for the suppression of EC activation (84).

Apart from its effect on the endothelium, DUSP1 also determines the monocyte/macrophage phenotype in atherosclerosis (35, 85, 86).

Metabolic stress was shown to induce the S-glutathionylation, inactivation, and subsequent degradation of DUSP1 in monocytes. As a result, increased p38 MAPK and ERK activity primed monocytes for chemokine-induced recruitment, thereby promoting monocyte adhesion and migration. *In vivo*, transplantation of DUSP1-deficient bone marrow into atherosclerosis-prone mice exacerbated atherosclerotic lesion formation by sensitizing monocytes to chemoattractants and polarizing macrophages toward an inflammatory phenotype (35, 86). Thus, monocyte and macrophage dysregulation by metabolic stress may drive the progression of atherosclerosis due to DUSP1 inactivation.

Interestingly, the administration of inhaled GCs has been suggested to be atheroprotective in asthma patients, although plasma levels of the drug were presumed to be very low and were not sufficient to provoke cardiovascular GC side effects (87). Whether this observation might be due to elevated DUSP1 expression or activity in ECs or the monocyte/macrophage compartment presently remains elusive.

DUSP1: A THERAPEUTIC TARGET?

As underscored by this review, there are several clinical areas where targeting DUSP1 (i.e., increasing its amount and/or activity) would be clinically beneficial. These may also comprise psoriasis or colitis, as a number of studies suggested an involvement of DUSP1 downregulation in the pathogenesis of these diseases (88–91).

Novel ligands to upregulate DUSP1 levels might represent an attractive anti-inflammatory strategy—particularly in atherosclerosis, where GCs cannot be used due to their cardiovascular side effects. Corticosteroid-sparing strategies to reduce the GC dose while achieving effective disease control have always been of clinical importance, and this is also a potential area of focus for DUSP1 upregulators. The failure of p38 MAPK clinical trials, including those recently published in COPD (92) could also bolster the search for DUSP1 modulators. The failure of targeting p38 MAPK is because while pro-inflammatory cytokines are repressed, so are the p38 MAPK-driven anti-inflammatory proteins, including DUSP1 (63, 93, 94).

However, there are challenges to overcome in the drive to develop DUSP as a therapeutic target. First and foremost, it is essential to consider that the overall impact of the MAPK-deactivator DUSP1 within the clinical context will depend on the role played by the MAPK involved. If the rationale is that MAPK needs to be inhibited, then there is a need to upregulate DUSP1 (e.g., in respiratory inflammation). Conversely, in some clinical situations, DUSP1 inhibitors may prove beneficial. For example, in some cancers, DUSP1 is overexpressed and is considered responsible for the failure of JNK-driven apoptotic pathways induced by chemotherapeutics; i.e., adjunct therapeutics with a DUSP1 inhibitor would have merit (95). The challenge in drug discovery would, therefore, be developing targeted therapies that could be delivered to the site of disease without collateral damage. Secondly, DUSP1 is sensitive to oxidative stress, and the phosphatase activity can be reduced. Notably, oxidative stress can be cause or consequence of the disease, and GCs themselves can contribute to the production of oxidative stress (96). Thus, although we may find techniques to increase DUSP1 abundance, it may be non-functional due to oxidation. Reactivation of oxidized DUSP1 function is worthy of further investigation. Thirdly, and perhaps most importantly, we need to get the timing right and ensure that that the temporal kinetics of the impact on DUSP1 on inflammatory pathways are considered. Taken together, the future utility of DUSP1 as a therapeutic strategy depends on it being active (not oxidized) and present at the right place at the right time. Treatment with exogenous DUSP1 upregulators would be akin to the usage of p38 MAPK inhibitors and as they have failed in clinical trials, restoring physiological DUSP1 activity in a manner that fully exploits dynamic regulation exerted

by the p38 MAPK/DUSP1/TTP network might even be the better option.

AUTHOR CONTRIBUTIONS

JH and AA reviewed the literature and drafted the manuscript.

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A General Introduction to Glucocorticoid Biology

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Glucocorticoids (GCs) are steroid hormones widely used for the treatment of inflammation, autoimmune diseases, and cancer. To exert their broad physiological and therapeutic effects, GCs bind to the GC receptor (GR) which belongs to the nuclear receptor superfamily of transcription factors. Despite their success, GCs are hindered by the occurrence of side effects and glucocorticoid resistance (GCR). Increased knowledge on GC and GR biology together with a better understanding of the molecular mechanisms underlying the GC side effects and GCR are necessary for improved GC therapy development. We here provide a general overview on the current insights in GC biology with a focus on GC synthesis, regulation and physiology, role in inflammation inhibition, and on GR function and plasticity. Furthermore, novel and selective therapeutic strategies are proposed based on recently recognized distinct molecular mechanisms of the GR. We will explain the SEDIGRAM concept, which was launched based on our research results.

Keywords: glucocorticoids, glucocorticoid receptor, inflammation, molecular biology, SEDIGRAM

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DISCOVERY OF GLUCOCORTICOIDS AND THE GLUCOCORTICOID RECEPTOR

The first steps leading to the discovery of glucocorticoids (GCs) took place in the 19th century when the physician Thomas Addison described that patients suffering from (chronic) fatigue, muscular degeneration, weight loss, and a strange darkening of the skin could obtain beneficial effects from adrenal extracts (1). This disease is now known as Addison's disease, which is a form of adrenal insufficiency. In 1946, Edward Calvin Kendall isolated four steroidal compounds from adrenal extracts, which he named compounds A, B, E, and F (2). Compound E, would become known as cortisol and was synthesized later that year by Sarett (3). The therapeutic potential was discovered by rheumatologist Philip Hench in a patient suffering from rheumatoid arthritis (4). Hench and Kendall were awarded the Nobel prize for Medicine and Physiology in 1950 together with Tadeus Reichstein who succeeded in isolating several steroid hormones from the adrenals, eventually leading to the discovery of cortisol. Since the discovery of their anti-inflammatory potential GCs were hailed as wonder drugs to treat various inflammatory diseases and became part of the group of most used and cost-effective anti-inflammatory drugs.

GCs bind the GC receptor (GR), a member of the nuclear receptor (NR) family of intracellular receptors, which also contains the estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), and mineralocorticoid receptor (MR) as well as several orphan receptors (with no known ligand) (5, 6). In 1966, the GR was identified as the principal receptor responsible for the physiological and pharmacological effects of GCs (7). It would take almost two more decades for the human GR-coding gene, *NR3C1* to be cloned (8, 9). The GR is very closely related to the MR

and these receptors exhibit some cross-reactivity, more specifically the MR is activated both by its own ligands, mineralocorticoids (MCs) and by GCs, but GR is activated only by GCs (10). NRs are involved in many aspects of mammalian biology, including various metabolic functions, cardiac function, reproduction and (embryonic) development, and the immune system (11).

GLUCOCORTICOID SYNTHESIS, REGULATION AND BIOLOGICAL AVAILABILITY

GCs are steroid hormones that are essential for the daily functioning of mammals. They are involved in several physiological processes, namely in metabolism (12), water and electrolyte balance (13), the immune response (14, 15), growth (16), cardiovascular function (17, 18), mood and cognitive functions (19–21), reproduction (22), and development (23). GCs are mainly synthesized in the cortex of the adrenal gland together with aldosterone (a MC) and dehydro-epi-androsterone (DHEA). The latter is the precursor of testosterone and estrogen. Aldosterone, GCs, and DHEA are synthesized by different steroidogenic enzymes in the mitochondria of, respectively, the zona glomerulosa, the zona fasciculate, and the zona reticularis of the adrenal cortex. They are however all synthesized from the same precursor, namely cholesterol (24). Extra-adrenal GC production in the thymus, vasculature, brain, and epithelial barriers has also been observed (25–30). These locally produced GCs are thought to predominantly exert local effects and contribute only minimally to the systemically circulating pool of GCs allowing a high spatial specificity of steroid actions, which are also independent of the circadian and stress induced regulation of endogenous GCs.

Adrenal GC production is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (**Figure 1**). Under basal, unstressed conditions GCs are released from the adrenal glands in the bloodstream in a circadian and ultradian rhythm characterized by peak levels during the active phase which is in the morning in humans and in the beginning of nighttime in nocturnal animals such as mice. The activity of the HPA axis is further increased upon physiological (e.g., activated immune response) and emotional stress. When the HPA-axis is stimulated, corticotropin-releasing hormone (CRH), and arginine vasopressin (AVP) are released from the hypothalamic paraventricular nucleus (PVN). Subsequently, CRH and AVP bind their receptor CRH-R1 and V1B in the anterior pituitary inducing the release of adrenocorticotrophic hormone (ACTH) in the circulation. ACTH will in turn stimulate the adrenal gland to synthetize and secrete GC hormones (cortisol) in the circulation (31).

The HPA axis is subject to a negative feedback inhibition by GCs, both in a genomic and a non-genomic way. The genomic feedback regulation is mediated through binding of GCs to the GR both at the level of the PVN and the pituitary gland, thereby repressing the *CRH, CRH-R1,* and the *POMC* gene (**Figure 1**). *POMC* codes for the proopiomelanocortin prohormone which is the precursor of ACTH. *CRH, CRH-R1,* and *POMC* gene expression are repressed by the binding of GR to negative glucocorticoid responsive elements (nGREs) (32–34). Next to this, GR is also able to physically interact with the Nur77 protein which also binds in the POMC promoter, thereby preventing it from performing its transcription function (35, 36). Non-genomically, GCs regulate the HPA axis for example via the release of endocannabinoid from CRH neurons thereby suppressing the release of glutamate from presynaptic excitatory synapses (37), or via γ-aminobutyric acid (GABA) release at the inhibitory synapses of CRH neurons (38).

Once secreted in the bloodstream GCs are bound to and transported by plasma proteins which keep the GCs inactive. Corticosteroid-binding globulin (CBG) is the main GC-binding protein in the plasma, with about 80–90% of the GCs bound to it (39). Several proteases target CBG, such as neutrophil elastase at sites of infection (40), causing the release of bound GCs. Approximately 10% of the GCs are bound to albumin that binds GCs with less affinity than CBG (39).

Due to their lipophilic nature, free GCs diffuse through the cell membrane to exert their function. However, the actual bioavailability of GCs in the cytoplasm is regulated by the balance between active and inactive forms of GCs. Two enzymes are responsible for the conversion between inactive cortisone (or 11-dehydrocorticosterone in mice) on the one hand and the active cortisol (or corticosterone, in mice) on the other hand. While 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) catalyzes the conversion of cortisone to cortisol, 11β-HSD2 carries out the opposite reaction (**Figure 2**). 11β-HSD2 is highly expressed in tissues with high MR expression, such as the kidneys, to prevent GC-induced MR activation which is known to cause salt and water dyshomeostasis (41, 42). Biologically active GCs will bind their receptor in the cytoplasm which exerts their physiological effects. This mechanism also confers a tight spatial regulation of GC actions, as the levels of these enzymes may be tissue or even cell specifically regulated and will directly determine the balance between the inactive and active form of GCs and thus the strength of the effect.

Under physiological conditions the role of endogenous GCs is not simply anti-inflammatory or immunosuppressive and shows more immunomodulation. It has been shown that GCs can also work pro-inflammatory (14). This occurs mainly in conditions of acute stress and is related to the concentration of GCs present (14, 43). Such pro-inflammatory actions were shown to include: elevation of pro-inflammatory cytokine levels (IL-1β) (44) or an exacerbation of the peripheral immune response in delayed type hypersensitivity (45).

Next to the endogenous GCs, various synthetic GCs (e.g., Prednisolone, Methylprednisolone, Fluticasone, Budesonide, and Dexamethasone) have been developed by the pharmaceutical industry that serve as treatments for various diseases. All these synthetic GCs were developed based on the structure of endogenous GCs (cortisol/hydrocortisone) (46). Experiments with structural modifications, mainly replacing side chains, resulted in synthetic GCs with optimized characteristics for medical use (pharmacokinetics, bioavailability, cross-reactivity with the MR). The most obvious differences between synthetic

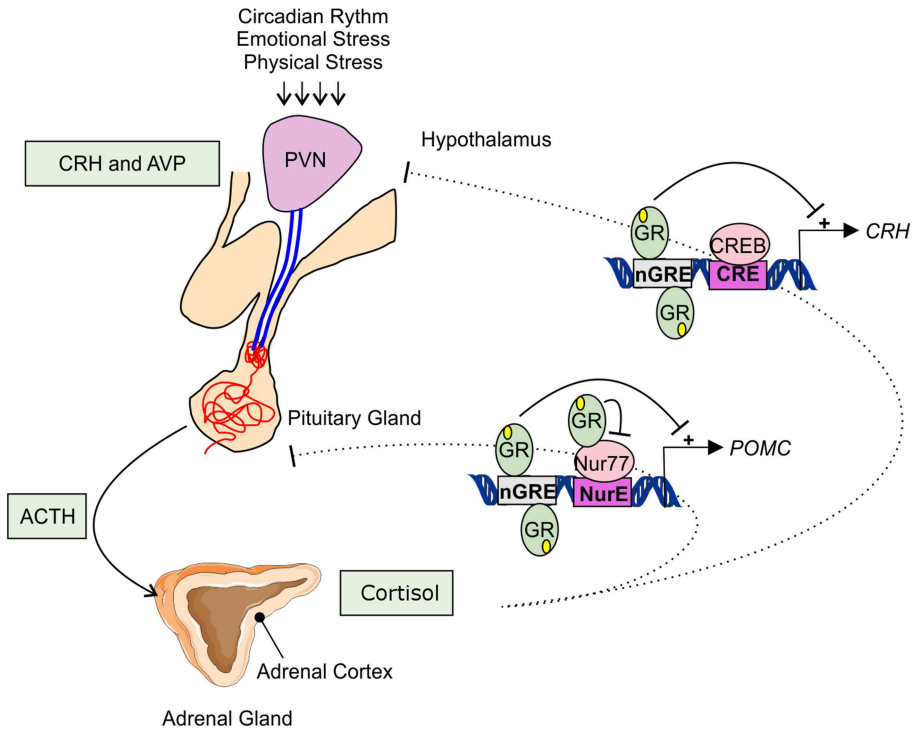

FIGURE 1 | Hypothalamic-pituitary-adrenal axis. The hypothalamic-pituitary-adrenal (HPA) axis activity is controlled by the circadian rhythm and can be induced by physiological and emotional stress. When activated, corticotrophin-releasing hormone (CRH), and arginine vasopressin (AVP) are released from the hypothalamic paraventricular nucleus (PVN). This induces the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland into the systemic circulation. ACTH will activate cortisol synthesis in the cortex of the adrenal gland. Cortisol negatively regulates the HPA-axis activity, e.g., by repressing the transcription of *CRH* and *POMC* by binding to negative glucocorticoid responsive elements (nGRE) or by binding to the transcription factor Nur77 involved in the *POMC* expression.

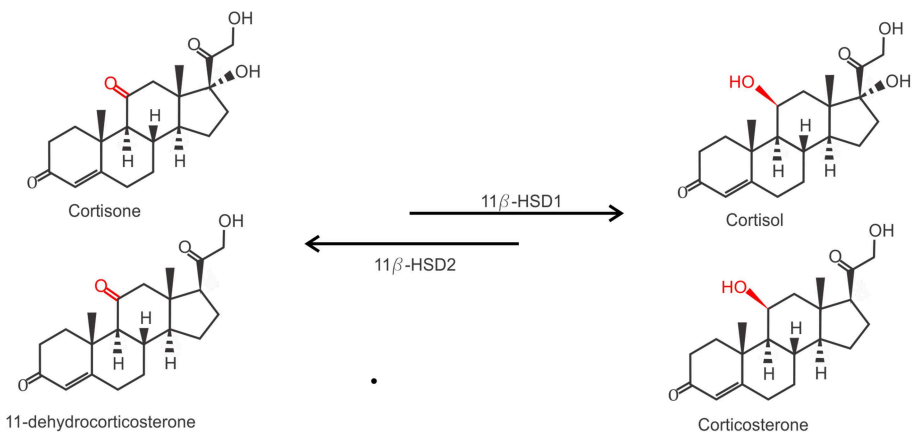

FIGURE 2 | Conversion of inactive GCs to active GCs. Inactive Cortisone (human) and 11-dehydrocorticosterone (mouse) are activated to active cortisol and corticosterone by 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1), and inactivated again by 11β-HSD2.

and endogenous GCs are (i) potency, as the synthetic variants are usually much better activators of the receptor than cortisol (4x−80x more) (47). (ii) Specificity, since endogenous GCs activate both GR and MR, but many synthetic GCs (e.g., dexamethasone, methylprednisolone) act (almost) exclusively on the GR. And (iii) synthetic GCs may (prednisolone) or may not (dexamethasone) be subject to processing by 11β-HSD1/2 which has a major impact on their bioavailability, as some synthetic GCs may (not) need to be activated by these enzymes or cannot be changed into an inactive form by them. Also, most synthetic GCs also do not bind the carrier proteins such as CBG (48–50). These facts are important to keep in

mind when giving GC treatment or performing research using synthetic GCs.

THE GLUCOCORTICOID RECEPTOR

The GR mediates the actions of GCs in cells. It belongs to the nuclear receptor superfamily of transcription factors (TFs) and is a 97 kDa protein that is constitutively and ubiquitously expressed throughout the body (51). Nevertheless, GCs exert cellular and tissue-specific effects due to the existence of different GR isoforms on the one hand and cell- and context-specific allosteric signals influencing GR function on the other hand (52–54). The GR functions by regulating the expression of GC responsive genes in a positive or negative manner. It is estimated that there are between 1,000 and 2,000 genes that are subject to GR mediated regulation, with some studies stating that up to 20% of all genes are responsive to the GR in some way (55).

GR Gene and Protein

The human gene encoding the GR is the "nuclear receptor subfamily 3 group c member 1" (*NR3C1*) gene localized on chromosome 5 (5q31.3). The mouse *Nr3c1* gene is localized on chromosome 18. The hGR gene consists of 9 exons of which exon 1 forms the 5′ untranslated region (UTR) and exons 2–9 encode the GR protein (52).

The 5′ UTR of the hGR is GC-rich, but does not contain TATA or CAT boxes (56). Thus, far 13 hGR exon 1 variants differing in upstream promoter regions have been identified (A1–3, B, C1–3, D–F, H–J) (**Figure 3**). Differential use of these promoters, located about 5 kb upstream of the transcription start site, causes varying expression levels of GR protein isoforms between cells and tissues (57–60). These promoters contain multiple binding sites for several TFs such as AP-1 (61) and Interferon Regulatory Factor (IRF) (62), but also for GR itself, thereby enabling the regulation of its own expression (63). Furthermore, these exon-1 variants are subject to epigenetic regulation. Several epigenetic modifications, such as DNA methylation and histone acetylation/methylation are known to occur in this region (or in other regions). The presence or absence of such modifications has been related to GR gene expression levels, GC resistance in certain cancers, promotion of cancer development, and mental health (64–69).

The hGR protein (**Figure 3**) is a modular protein that, like other NR family proteins, is built up out of an amino-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain (LBD) (52). The NTD is encoded by exon 2 and is the least conserved region of the NR family. It is inherently unstructured, vulnerable to proteases and only becomes structured when the protein binds DNA and forms dimers (70). In the NTD the ligand independent activation function 1 (AF1) is located. This AF1 binds cofactors, chromatin modulators, and the transcription machinery (71–73). The GR DBD is encoded by exons 3 and 4 and is important for DNA binding and GR dimerization. It is characterized by two highly conserved subdomains each containing a Cys4-type zinc finger. In the first subdomain the GR's proximal box (P box) is contained which is important for site specific GR DNA binding. The second subdomain contains the distal box (D box) which is important for GR dimerization (74). Exons 5–9 of the *NR3C1* gene encode the GR's hinge region and LBD. The former provides both flexibility between the DBD and LBD as well as a regulatory interface. The hinge region can be acetylated (lysine residues) and is a target of CLOCK/BMAL acetylation and the presence of acetyl moieties in this area reduces GR activity. Research has also shown that the interaction between the GR and CLOCK/BMAL can be uncoupled, such as by chronic stress or night shift work, which may cause hypercortisolism related pathologies (75, 76). The latter contains a ligand binding pocket, which is formed by 12 α-helices and 4 β-sheets, and the ligand-dependent AF-2 domain. The LBD has also been found important in GR dimerization (77). Further, nuclear localization (NLS), nuclear export (NES), and nuclear retention signals (NRS) have been identified in the GR protein and these are important for the subcellular distribution of the GR. Two NLS have been identified, one in the DBD and one in the LBD (78). A NES is located between the 2 zinc fingers (79) and a NRS delaying GR nuclear export overlaps with NLS1 (80).

Not a single, but multiple GR protein isoforms are identified. This is the result from alternative splicing and the use of 8 different translation initiation start sites (81). Alternative splicing at exon 9 results in two different GR splice variants, namely the classical 777 AA-long GRα or the 742 AA-containing GRβ (8). Both isoforms are identical up to AA 727, but contain non-homologous AA thereafter. Hence, GRβ has a shortened LBD lacking helix 12 and therefore it cannot bind GCs (82). Despite this, GRβ is constitutively found in the nucleus where performs several functions. It was believed and later also shown to be an antagonist to the GRα isoform. Several mechanisms have been proposed for the dominant negative action of GRβ, such as competing with GRα for GR-binding sites and co-regulators and the formation of inactive GRα/β heterodimers (82–84). The role of the GRβ is more extensive than being a simple antagonist. Other studies have shown that the GRβ regulates gene transcription of non-GRα target genes in an GRα and GC independent manner (85). Furthermore, while GRβ cannot bind endogenous GCs, it was show to bind the GR antagonist RU-468, and is modulated by it (86). Perhaps some synthetic GR agonists could also bind to this isoform. The GRβ isoform plays a role in GC resistance (insensitivity to GC treatment) in patients for several diseases. This resistance can be caused by its GRα antagonism as well as by the transcriptome changes its presence causes. A recent study showed that overexpression of GRβ in colonocytes causes dysregulation of many genes also found back in IBD patients (87). Next to GRα and GRβ, GRγ, GR-A, and GR-B splice variants have also been identified (illustrated in **Figure 3**). All splice-isoforms show diminished activity compared to GRα (88–90). Besides splicing, GR mRNA is further regulated post-transcriptionally via adenine uridylate-rich elements (ARE) in the 3′ UTR of the GR mRNA which mediate GR destabilization (91). Next to this, GR mRNA stability is also regulated by microRNAs (for example: miR-124) which bind to their binding motifs, mostly in the 3′ UTR (92, 93).

Eight GRα translation initiation variants have been identified (GRα-A, -B, -C1, -C2, -C3, -D1, D2, and D3) which is the result from the existence of 8 highly conserved AUG start codons in exon 2 (**Figure 3**) (94). The AUG start codons

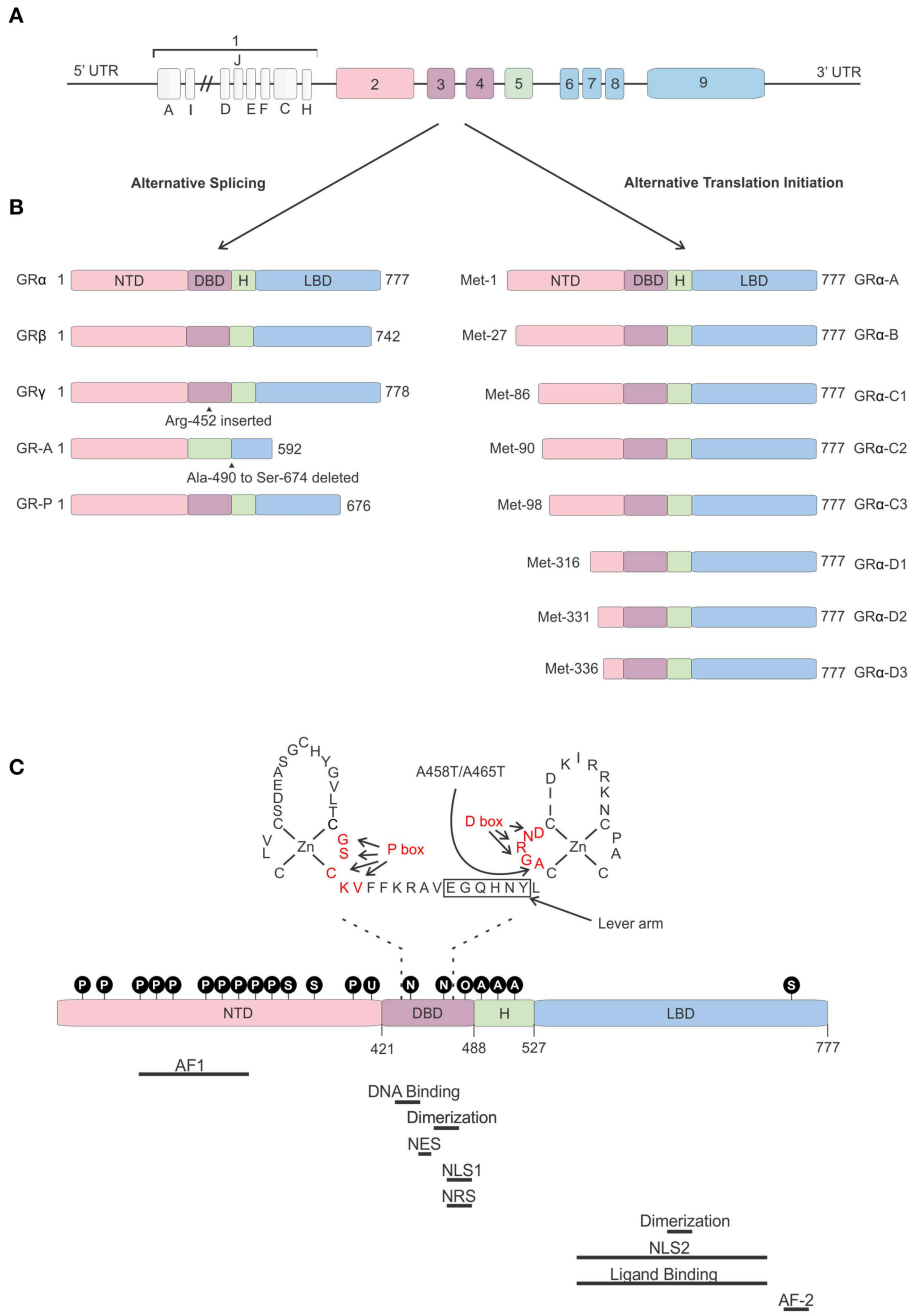

FIGURE 3 | Glucocorticoid receptor gene and protein. **(A)** Genomic structure of the glucocorticoid receptor (GR) gene. **(B)** Alternative splice and translation-initiation variants of the GR protein. **(C)** Structure of the GR protein consisting of an N-terminal domain (NTD), DNA-binding domain (DBD), a hinge region (H), and a ligand-binding domain (LBD), with a focus on the two zinc-fingers of the DBD and the GR^{Dim} mutation (A458T in human, A465T in mouse). Identified post-translational modifications of the GR are indicated in the black circles. Regions important in GR function are indicated below the protein. AF, Activation function; NES, Nuclear Export Signal; NLS, Nuclear Localization Signal; NRS, Nuclear Retention Signal; P, phosphorylation; S, sumoylation; U, ubiquitination; N, nitrosylation; O, oxidation; A, acetylation.

are differently selected due to ribosomal leaky scanning and ribosomal shunting mechanisms (94). Because the same AUG start sites are also present in the GR splice-variants, all the translation-initiation isoforms are expected to occur in each of the splice-variants (95). The GR translation variants all have a similar GC and glucocorticoid responsive element (GRE)-binding affinity, but they differ in the length of their N-termini and their transcriptional activity. They show different subcellular

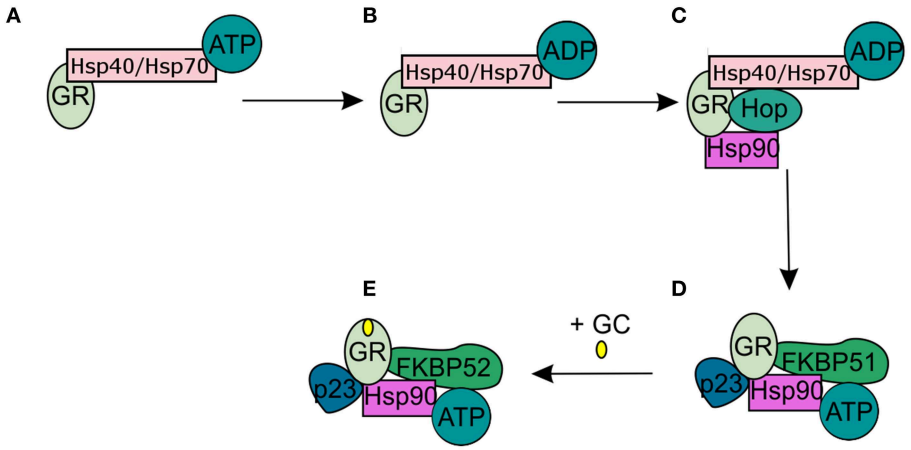

FIGURE 4 | Glucocorticoid receptor chaperone complex and maturation. **(A)** After glucocorticoid receptor (GR) translation an Hsp70-Hsp40-GR complex is formed in the cytoplasm. **(B)** A subsequent ADP-dependent Hsp70 change induces the binding of Hop. **(C)** Hop induces the binding of Hsp90. **(D)** After Hsp90 binding to Hop, Hsp70, and Hsp40 are released from the chaperone complex and replaced by p23 and FKPB51. The GR has now matured into a high affinity complex. **(E)** After binding of glucocorticoids FKBP51 is replaced by FKBP52, which is necessary for the transport of the GR to the nucleus.

localization, regulate distinct sets of genes and their relative levels vary between and within cells (94). The mechanism of regulation of alternative translation start sites and alternative splicing in response to physiological, pathological, and cell-specific signals is still poorly understood. *In vitro* work proved that these isoforms do have the capability to regulate distinct transcriptional programs (96). A later study showed that the different isoforms can regulate apoptosis with the GRα-C3 being pro-apoptotic and the GRα-D3 anti-apoptotic (97).

GR Activation and Nuclear Translocation

In the absence of intracellular bioactive GCs, the GR finds itself as a monomer in the cytoplasm where it resides in a multiprotein complex. This chaperone complex is important for GR maturation, ligand binding, nuclear transport, and activation. The composition of the chaperone complex changes during the different GR maturation/activation states (**Figure 4**) (98). After GR translation the GR is bound by Hsp70, an interaction that is accelerated by the Hsp40 co-chaperone. Once the folding process is complete GR is transferred from Hsp40/Hsp70 to Hsp90, a transfer that is mediated by Hop (99–101). Recruitment of p23 (102) and FKBP51 to the multiprotein complex leads to maturation of GR-chaperone complex into a conformation that has very high affinity for GR ligands. After GC-binding the GR-chaperone complex again reorganizes (FKBP51 is replaced by FKBP52) and a GR conformational change is induced, leading to the exposure of the GR's 2 nuclear localization signals (103). These are subsequently bound by nucleoporin and importins that carry the GR through the nuclear pore complex into the nucleus (104, 105). Initially it was believed that the GR disassociates from the cytoplasmic chaperone complex upon ligand binding. However, recent research has shown that the chaperone complex is required for efficient nuclear translocation of the receptor (106).

Once inside the nucleus, the activated GR can go on to exert its function or it can be transported back to the cytoplasm, inhibiting the GR's transcriptional activity. Nuclear export of GR is regulated by exportins and calreticulin (CRT) which binds to the GR NES, thereby disrupting the GR-DNA binding (107, 108).

The balance between nuclear import and export determines the proportion of GR protein in the nucleus and has a direct influence on the strength of GR's transcriptional activities. In the nucleus, the GR acts as a TF that can activate (trans-activation) or inhibit (trans-repression) genes as well as modulate the function of other TFs (tethering). Most of the GR functions are restricted to the nucleus, but some non-nuclear actions of GR are also known.

GR Function

In the nucleus, the GR is able to transcriptionally activate (transactivate (TA)) or transcriptionally repress (transrepress (TR)) gene-expression, both as a monomer and as a dimer, and usually via direct contact with DNA. Recently it was discovered that the GR can also bind to the DNA as a tetramer (**Figure 5**) (109, 110). The importance of this GR tetramer in transcriptional regulation is not well-understood and needs further investigation.

The GR associates with specific genomic loci and orchestrates the assembly of TF regulatory complexes containing the GR, other TFs and co-regulators that modulate the activity of the RNA polymerase II (RNApolII). Different modes of genomic GR transcriptional regulation are described (**Figure 5**).

The simplest form of GR-DNA interaction is the binding of GR to genomic glucocorticoid binding sites (GBS) containing a GRE. Classically, the GR exerts its transactivation function by binding to GREs, which are 15 bp long sequence motifs of 2 imperfect inverted palindromic repeats of 6 bp separated by a 3 bp spacer. The generally accepted GRE consensus sequence is AGAACAnnnTGTTCT. However, this may be better represented

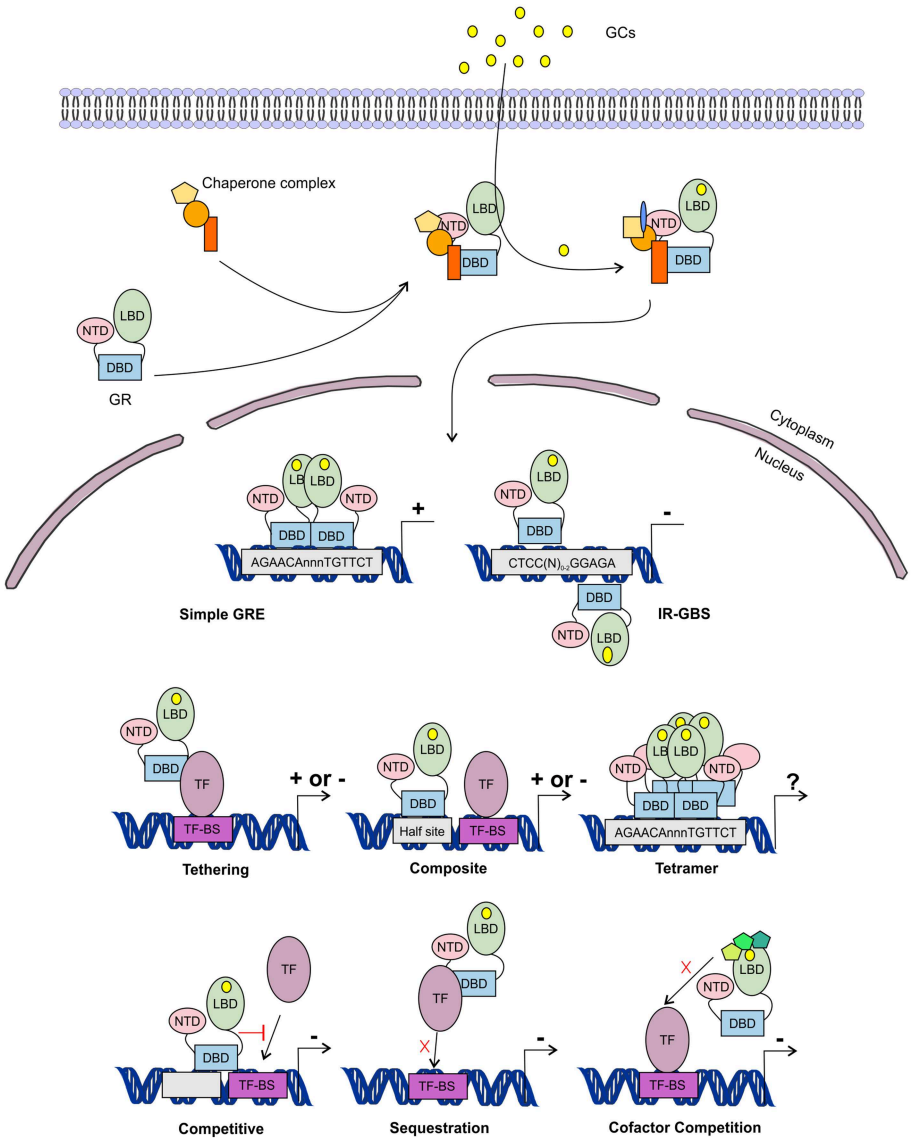

FIGURE 5 | Glucocorticoid receptor activation and function. Lipophilic glucocorticoids (GCs) diffuse through the cell membrane and bind the glucocorticoid receptor (GR) in the cytoplasm. This induces a change in the chaperone complex bound to GR, after which it translocates to the nucleus to transactivate (+) or transrepress (-) gene transcription as a monomer or a dimer. The GR can transactivate genes by binding to glucocorticoid responsive elements (GRE) as a dimer, but also as a monomer by binding to other transcription factors (TF) through tethering or by binding to composite-elements. The GR can further transrepress gene-expression by binding to inverted repeat GR-binding sequences (IR-GBS), by tethering, by composite-elements, by competing for DNA binding-sites (BS), by sequestrating TFs and by competing for cofactors with other TFs. GR might also function as a tetramer, but its function is not known.

as a sequence logo (**Figure 6**), which illustrates that some positions are much more variable than others. The GR binds to the GRE as a homodimer and each GR DBD makes contact with about 3 nucleotides in each of the half site hexamers. The two GR molecules bind the GRE in a head-to-tail fashion and 5 AA within the D box of the second GR zinc finger provide critical protein-protein contacts between the two GR partners important for stabilization of the GR DBD on the DNA. In this D box a hydrogen bond is formed between Ala458 of one dimer partner and Ile483 of the other partner (74, 111). A second interface important for dimerization (Ile628) has been identified in the LBD (77, 112). Recent research proposes that the LBD may have other dimerization interfaces related to another dimer structure (113).

GREs contain relatively few highly conserved residues and because GREs are rather short, they are abundantly present in the genome. ChIP-seq experiments with antibodies against GR showed however that only a small fraction of GRE sites are in fact occupied by the GR (114). Why this is the case is still a topic of research, but it has been shown that the chromatin structure plays

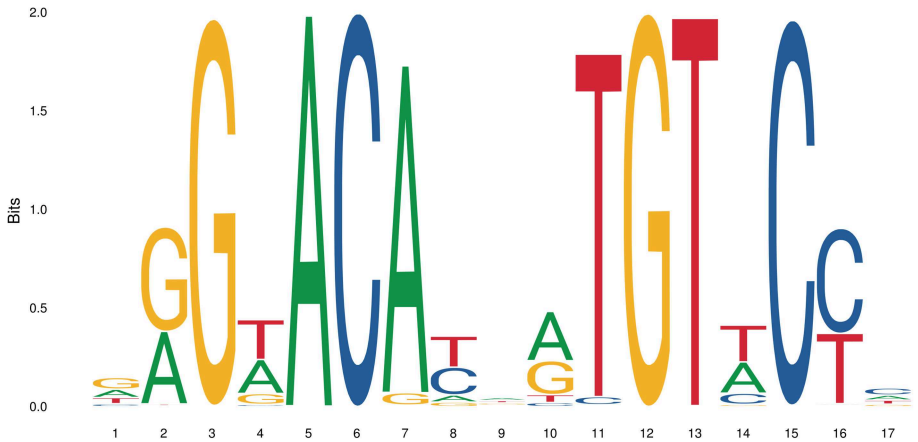

FIGURE 6 | Sequence logo of the human glucocorticoid responsive element to which the GR binds to. See text for more details.

a big role in determining which sites are accessible to GR under certain conditions (115, 116). It has also been shown that many GR binding sites can be found very far from a (known) gene or transcriptionally active sites, indicating that GR often occupies enhancer regions and/or chromatin looping is involved in GR transcriptional regulation (114).

Evidence has been found for a 2nd mode of GR-DNA interaction where GR, as a monomer, binds to half sites with an AGAACA (or the reverse complement TGTTCT) consensus sequence (117). If a binding site for another TF is nearby the GRE-half site, both elements may act as a composite site where there is an interaction (positive or negative) between the GR (monomer) and the other TF (118) (**Figure 5**). An analysis in mouse liver showed that under endogenous corticosterone levels (i.e., low concentrations) GR binding to half sites as a monomer is more prevalent than binding of full GRE sites by homodimers. In response to exogenous GCs (i.e., high concentration) the GR dimers assemble on full length GRE near known induced genes and this happens in concert with monomer removal of sites near repressed genes (119).

A third class of GR-DNA interactions involves inverted-repeat GBS (**Figure 5**). Binding to such an element leads to inhibition of gene expression. These IR-nGREs have a consensus $CTCC(N)_{0-2}GGAGA$ sequence and structural analysis showed that at these sites 2 GR monomers bind on the opposite sides of the DNA, in a head-to-tail orientation and with negative co-operativity with each other (120, 121).

Lastly, there are the indirect binding, or tethering, sites where GR is recruited to a TF complex through protein-protein interactions with heterologous DNA-bound TFs (**Figure 5**). These GBSs lack a GRE, IR-nGRE, or a GRE half site. Several TFs are known to recruit ligand bound GR via tethering including members from the AP1, STAT, and NF-κB families of TFs. These interactions directly alter the capacity of the directly DNA-bound TF to bind DNA, recruit cofactors, and activate/repress gene transcription (122, 123).

The GR can also TR gene-expression by competing with other TFs for binding to overlapping DNA-binding sequences. Indeed, recently GR half-sites were even found embedded in AP-1 response elements (124). Finally, the GR can TR gene-expression by competing with other TFs for the binding of cofactors (125–127) or by sequestrating TFs, thereby obstructing them to bind to the DNA (128) (**Figure 5**).

GR Plasticity

The GR operates in a cell- and context-specific manner. This is not only due to a different expression of GR protein isoforms but is also the cause of different signals that modulate the GR's activity at specific GBSs. Four signals are described to influence the GR's function.

A first signal that modulates GR activity is the DNA, which acts as an allosteric regulator of the GR. GRE sequences differing by only one single base pair were namely shown to affect GR conformation and regulatory activity (129). Moreover, allosteric changes provoked by one half site can be transduced via the GR lever arm (located between the P and D box, see **Figure 3**) and the receptor's D box to the dimer partner, affecting the GR's transcriptional activity (130, 131).

A second signal influencing the GR transcriptional output obviously comes from the ligand that binds to the LBD. After ligand-binding helix 12 is exposed and cofactors are recruited to the AF2 in the LBD. Depending on the ligand, the LBD will adopt another conformation and attract other cofactors thereby influencing the GR's transcriptional outcome (132, 133). The latter forms the basis of the research for "Selective GR Agonists and Modulators" (SEGRAM).

Third, the GR is heavily modified by potential post-translational modifications (PTMs). Several phosphorylation (134–140), ubiquitination (141), sumoylation (142), acetylation (76), and nitrosylation sites (143) as depicted in **Figure 3** have been identified influencing GR-localization, stability, DNA binding, ligand response, and regulatory activity.

Last, the GR's transcriptional output is influenced by its interaction partners. These include other TFs that bind direct or indirect to GR and cofactors which are recruited to GR and are involved in functions such as chromatin

regulation and regulation of the transcriptional machinery function (53, 144). The composition of the cofactor complex recruited to the GR depends on the cell specific expression of cofactors, the cell context and the integration of the previous described signals (DNA, ligand, and PTMs) that influence the GR's conformation (145). This cofactor complex eventually determines the transcriptional output of the GR.

Non-genomic GC and GR Actions

The GR is not only able to function by genomic actions, but also through non-genomic actions. Non-genomic GC/GR actions are fast and do not require transcription or protein synthesis. Limited knowledge is however available on non-genomic GC/GR actions. These include GC-mediated effects on membrane lipids, changing their physicochemical properties (146). Further, GCs have also been seen to act on a membrane-bound GR which is related to the classical GR and probably the result from differential splicing, alternative transcription initiation and PTMs (146, 147). Another membrane receptor, unrelated to the classical GR, probably also binds GCs. This protein is probably a G-coupled receptor that signals through cAMP and that binds endogenous GCs with high affinity. However, it does not bind most GC analogs such as dexamethasone (148). Other non-genomic actions, e.g., modulation of the MAPK signaling cascade, might result from components that are released from the GR chaperone complex upon the binding of GCs to the GR or from membrane bound GR (149, 150).

A final type of non-genomic action of the GR is its effect on mitochondrial function. It was show that the GR can translocate to and reside in mitochondria (151, 152). This mitochondrial GR is capable of regulating gene transcription from the mitochondrial chromosome by binding to GRE like elements alone or in complex with other factors. This was demonstrated *in vitro*, using a hepatoma cell line and in brain cell of mice and rats (153–155). A recent study showed that a GR isoform, GR?, is located in the mitochondria and plays a role in regulating cell energy metabolism in a ligand independent manner (156).

GC THERAPY: DRAWBACKS AND OPTIMIZATION

GCs are therapeutically mainly used for their anti-inflammatory and immunosuppressive effects. These are a.o. the result of the transcriptional induction of several anti-inflammatory protein-coding genes such as *TSC22D3* (coding for glucocorticoid-induced leucine zipper, GILZ) and *DUSP1* (coding for Map Kinase Phosphatase 1, MKP1) and from the repression of pro-inflammatory TFs such as NF-κB and AP-1. GCs are used to treat inflammatory disorders such as asthma (157), skin rashes (158), rheumatoid arthritis (RA) (159), multiple sclerosis (160), and systemic lupus erythematosus (SLE) (161). In most cases, synthetic glucocorticoids are used but hydrocortisone is also a popular option.

Despite its strong anti-inflammatory capacity, GC therapy is limited by two major drawbacks. First, GCs are well-known to be associated with adverse effects, particularly when given in high doses for long time periods. **Figure 7** graphically presents GC-associated side effects, with osteoporosis, hyperglycemia, cardiovascular diseases, and infections as the four most worrisome adverse effects for clinicians (162). These side effect may be severe enough to affect the therapy or cause an increased risk to other negative effects. A recent study in RA patients showed a clearly increased risk of bone fractures correlated with the administration of GCs (osteoporosis) (163). Second, some patients are refractory to the therapy and are GC resistant (GCR). GCR can either be inherited, mostly via mutations in the *NR3C1* gene (52, 164), or acquired (165). The latter can be caused by ligand induced homologous downregulation of the GR, caused chronical GC treatment (166, 167), or by pathophysiological processes accompanying the inflammatory disease states [e.g., chronic obstructive pulmonary disease (COPD) (168), SLE (169)]. The pathophysiological processes provoking GCR are very heterogeneous, e.g., oxidative stress and inflammatory cytokines are known triggers of GCR and have multiple effects on GR biology (170–176). GCR occurs in 4–10% of the asthma patients, 30% of the RA patients and in almost all of the sepsis and COPD patients (177–179).

To achieve a positive benefit-to-risk ratio when using GCs, guideline recommendations regarding optimal dosing must be followed and potential adverse effects must be monitored, prevented and managed (180–183). Next to this, much research effort is put in developing innovative GCs or GR ligands that improve the therapeutic balance (184–186).

Currently available GCs in the clinic activate all GR activities. During the past 20 years intensive research for SEGRAMs, which promote a GR conformation favoring TR over TA, has been performed. This search for SEGRAMs is based on the central dogma in GR biology which states that GR monomer-mediated TR is sufficient to counteract inflammation, while GR dimer-mediated TA is responsible for most of the adverse effects of GCs, e.g., by the induction of genes encoding glucose-6-phosphatase (*G6P*) and phosphoenolpyruvate carboxykinase (*PCK1*). This long accepted dogma in GR biology originates from initial work with the GR^{Dim} mutant (187). This GR^{Dim} mutant carries a A465T mutation in the D-loop of the second zinc finger of the GR-DBD.

This D-loop is one of the primary dimerization interfaces, consequently this mutant shows impaired homodimerization and reduced functionality. Initial observation on the GR^{Dim} mutant showed a strongly impaired transactivation and retained capability to transcriptionally repress genes, particularly as a monomer (111). Follow-up work on the GR^{Dim} found that there was still transactivation of certain genes possible by these mutant receptors (129, 131). This raised the question again if the GR^{Dim} was still capable of some dimerization and or DNA binding. An *in vivo* imaging study with labeled GR showed that the GR^{Dim} is still capable of dimerization with endogenous and synthetic GCs, but with a lower efficiency than WT for endogenous GCs (188). The ability of the GR^{Dim} mutant to bind to the DNA has been a point of controversy since there is evidence against (111, 189) and pro DNA binding (131, 190, 191). Current evidence seems to suggest that the DNA binding capacity of the mutant is at least partially preserved. A second GR mutant was generated with an additional point mutation in the LBD of the receptor.

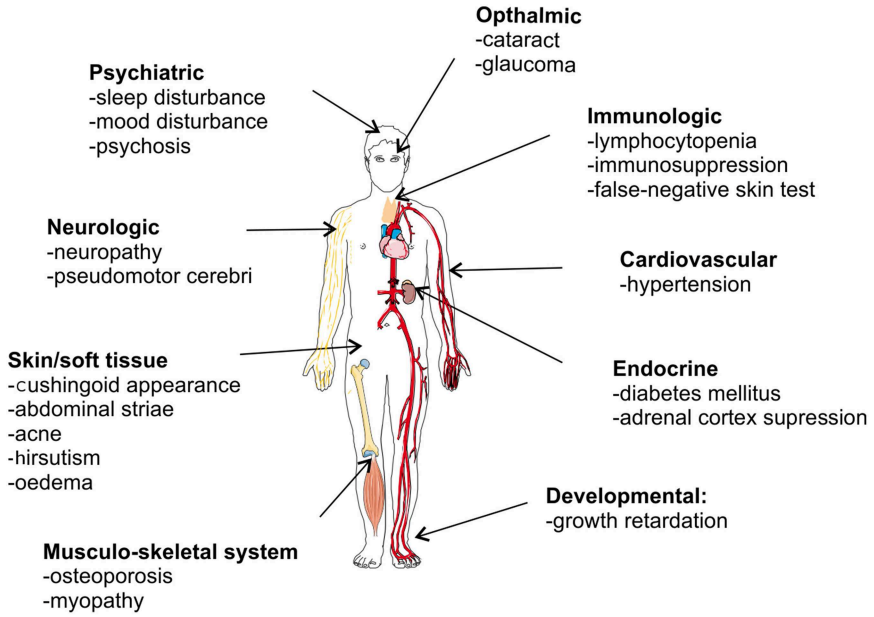

FIGURE 7 | Overview of glucocorticoid-associated side effects.

This mutation is believed to disrupt a secondary dimerization interface present in the LBD, leading to even poorer dimerization and function than the GR^{Dim} mutant (188). In addition, under normal physiological conditions, GR^{Dim} mice are healthy and show no obvious phenotypes, except that they express interferon genes in their intestinal epithelium (192), It has been shown that under physiological conditions, GR binds to the DNA as a monomer, exerting transcriptional functions related to cell-type-specific functions, and that only after acute stress or injection of GCs, GR dimers are formed leading to binding to full GRE elements (119). Also, elegant, NMR-based work by Watson et al. has shown that, depending on the DNA sequence where GR dimers bind, an intramolecular signal, via a lever arm, provides a dimer- and DNA-binding-stabilizing interaction between two DBD domains, precisely via the amino acid that was mutated in the GRDim version. The absence of this amino acid "weak binding" in the GR^{Dim} version was enough to cause less robust dimers and DNA binding (131).

It has been stated that the picture about the mechanisms of glucocorticoid actions (transactivation/transrepression) is still far from complete, especially for known GR mutants. In addition, the aforementioned functional PPI interfaces, recent structural biology work shows that the knowledge on GR dimerization and structural conformation may be incomplete based on structural homology and residue conservation between the NR transcription factor family, and new dimer interfaces that remain unexplored so far. In one study researchers have postulated that the conformation of the GR that is generally accepted as the dimeric conformation might not be correct and they propose different configurations (113). The fact most of the structural work so far was done on subdomains of the GR, as the whole protein is very hard to crystalize, may contribute to this limited knowledge of GR structure.

Many studies have investigated steroidal and non-steroidal SEGRAM in the hope to be able to dissociate the GC-induced anti-inflammatory effects from the GC-induced side effects (193–197). Several interesting SEGRAM have been characterized [e.g., Al-438, LGD-5552, ZK216348, Mapracorat and Compound A (CpdA)] and were shown to have dissociative profiles *in vivo* (198–206). Despite the intensive research, none of the SEGRAM have reached the market today. So far, only Fosdagrocorat (for RA) (207–209) and Mapracorat (for ocular inflammatory diseases and skin inflammation) have reached clinical trials.

To prevent GC-induced side effects, strategies other than shifting the balance between the monomeric and the dimeric GR are also followed (184–186). Some aim at cell-specific targeting of GCs via antibody- or peptide-GC conjugates (210) or via liposomes (211), thereby preventing systemic GC-effects. Other studies investigate the therapeutic use of GC-induced proteins (e.g., GILZ, the protein coded by the *TSC22D3* gene) without administrating GCs themselves. By this, steps are undertaken to develop therapies that stimulate only the wanted anti-inflammatory GC-functions without inducing the broad and also the unwanted GC-effects (212). Further, studies also invest in the therapeutic potential of combination therapies, such as the combination of GR and PPAR agonists (213, 214).

GC THERAPY IN ACUTE VS. CHRONIC INFLAMMATION: SIRS AND THE SEDIGRAM CONCEPT

During the recent years, it has become clear that the old idea in GC-research, that claims that GC anti-inflammatory effects can be separated from GC-induced side effects by simply dissociating GR TR from GR TA, because the former would

be mainly monomeric-driven GR functions and the latter GR homodimeric-driven functions. To date it is known that this separation cannot be made that strictly. In addition, GR^{Dim} mice studies showed that not all GC-induced side effects are GR dimer-driven and that thus also monomeric GR is involved in at least some side effects. Indeed, GR^{Dim} mice were observed to develop osteoporosis and muscle atrophy, despite their lack of GR dimer-dependent effects (215, 216). Next to this, the GR dimer was found to be indispensable for the GC-mediated protection in models of acute inflammation. GR^{Dim} mice are strongly sensitized in models of TNF- and LPS-induced Systemic Inflammatory Response Syndrome (SIRS) (217, 218) and these mice could furthermore no longer be protected by a prophylactic Dexamethasone administration (192). Additionally, GR dimer-induced GRE genes were found to be important in the protection against SIRS: this was shown for *DUSP1* (217) (encoding MKP-1) and *TSC22D3* (212) (encoding GILZ). Finally, skewing the GR toward the monomer by using CpdA sensitized mice for TNF-induced SIRS, suggesting that GR monomers are unable to protect in this model of acute inflammation and that GR monomers should rather be avoided in SIRS (219). Altogether these data illustrate the importance of the GR dimer in the protection against acute-inflammation.

As a consequence of the former observations in GR^{Dim} mice, the SEGRAM concept needed to be revised. Therefore, recently, it was proposed that chronic inflammatory diseases which require a long-term GC therapy would benefit from "Selective Monomer GR Agonists and Modulators" (SEMOGRAMs), since these SEMOGRAMs would avoid important side effects such as hyperglycemia that are detrimental for the patients. Recently, it was also observed that ligand-induced GR turnover leading to GCR is GR dimer dependent (220). The latter observation thus further supports the need for SEMOGRAMs for the treatment of chronic inflammation. On the other hand, in acute-inflammatory settings such as SIRS, where GR dimers are indispensable, the administration of GCs that increase the GR dimerizing potential, termed "Selective Dimer GR Agonists and Modulators" (SEDIGRAMs), would be the preferred strategy to follow (221).

There has been some doubt about the value of the GR^{Dim} mouse tool and its inability to form homodimers and bind DNA. Although *in vitro* experiments (making use of high GC-doses) showed very little effect of the Dim-mutation on GR dimerization and DNA binding (188, 191), *in vivo* research confirmed that GC-induced transcription is very broadly hampered in GR^{Dim} vs. GR^{WT} mice (192, 222). Moreover, the remaining GR^{Dim} transcription was observed to be especially the result of GR monomer functioning at half-sites (119). Although we are aware that a second interface in the GR LBD is also of relevance for dimerization and that remaining dimerization in the GR^{Dim} mutant is probably provided through this protein-protein contact, the latter studies confirm the value of the GR^{Dim} mouse-tool.

FUTURE PERSPECTIVES

Certain challenges and (new) questions remain to be answered or further investigated. GCR in patients is still largely an unresolved issue, especially in complex diseases such as sepsis but also in severe asthma. Understanding GC resistance, preventing or reverting it could mean a real breakthrough in current medical practice. Another avenue of research, aside from more selective dimer/monomer ligands, is GR structure and DNA binding conformation as some more recent research suggested that the GR can bind to DNA is a tetramer conformation instead of a dimer. Also, the non-genomic effects of GCs and GR are far from understood and need more research. Finally, a wealth of information has been published using a variety of GR ligands, some being endogenous ligands, others synthetic ligands, all of which may have very different effects on the canonical GR and non-canonical ones (splice variants, shorter proteins) and even different effects in different mammalian species or cell types. It is a big challenge for the community to try to streamline this information in a comprehensive way.

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Glucocorticoids Shape Macrophage Phenotype for Tissue Repair

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Inflammation is a complex process which is highly conserved among species. Inflammation occurs in response to injury, infection, and cancer, as an allostatic mechanism to return the tissue and to return the organism back to health and homeostasis. Excessive, or chronic inflammation is associated with numerous diseases, and thus strategies to combat run-away inflammation is required. Anti-inflammatory drugs were therefore developed to switch inflammation off. However, the inflammatory response may be beneficial for the organism, in particular in the case of sterile tissue injury. The inflammatory response can be divided into several parts. The first step is the mounting of the inflammatory reaction itself, characterized by the presence of pro-inflammatory cytokines, and the infiltration of immune cells into the injured area. The second step is the resolution phase, where immune cells move toward an anti-inflammatory phenotype and decrease the secretion of pro-inflammatory cytokines. The last stage of inflammation is the regeneration process, where the tissue is rebuilt. Innate immune cells are major actors in the inflammatory response, of which, macrophages play an important role. Macrophages are highly sensitive to a large number of environmental stimuli, and can adapt their phenotype and function on demand. This change in phenotype in response to the environment allow macrophages to be involved in all steps of inflammation, from the first mounting of the pro-inflammatory response to the post-damage tissue repair.

Keywords: glucocorticoids, macrophages, inflammation, tissue repair, phagocytosis glucocorticoid receptor

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Macrophages therefore, appear to be an ideal target of anti-inflammatory drugs due to their central role in inflammation. Glucocorticoids (GCs) are highly potent anti-inflammatory drugs, commonly used around the world. GCs have been used for decades to treat a variety of inflammatory diseases such as rheumatoid arthritis, contact allergy, or pulmonary diseases. Since the first GC therapies during the 1950s, various synthetic GCs have been developed to optimize their action, and new molecules are still under development to modulate therapeutic effects vs. the adverse effects of these drugs. Surprisingly, given the importance of macrophages in the inflammatory response, the direct effects of GCs on macrophages are less well-documented. The present review aims at summarizing the knowledge on macrophage functions during the post-injury inflammatory response, with a focus on sterile inflammation and tissue repair, discussing how GC signaling pathways operate in macrophages, and finally on the specific action of GCs on macrophages.

MACROPHAGES AND TISSUE REPAIR—EXAMPLE OF SKELETAL MUSCLE REGENERATION

Similar Macrophage Subtypes Are Found in Various Tissues During Repair

Macrophages belong to the innate immune system, however their role is far more than protecting against pathogens. In the late nineteenth century, Metchnikoff originally described and named these cells as "macro" (big) "phage" (eaters) due to their phagocytotic activity. In the following 100 years, scientists discovered that macrophages are not only phagocytic cells. Different macrophage subtypes were described, first in *in vitro* experiments, based on the main cytokinic activation of lymphocytes, allowing macrophages to be divided into different categories. "Classically activated" macrophages are induced by stimulation with the Th1 cytokine IFNγ and "alternatively activated" macrophages, involved in anti-inflammatory processes were observed when using the Th2 cytokine IL-4 (1). These two activation states were also called M1 (or pro-inflammatory macrophages) and M2 (or anti-inflammatory macrophages), respectively. However, this simplistic view of two potential statuses was quickly expanded on. Macrophages can adopt a very large panel of phenotypes depending on the inflammatory cues they encounter, even *in vitro* (2–4). *In vivo*, the situation is more complex. The terms M1 and M2, although widely used, are not appropriate to describe specific and dynamic inflammatory status that occurs in the inflammatory milieu of a living organism (5, 6). The Ly6C (Lymphocyte antigen 6 complex, a membrane protein expressed by monocytes, and macrophages) and CX3CR1 (chemokine (C-X3-C motif) receptor 1, another transmembrane protein involved in the adhesion and migration of leukocytes) antigens have been widely used to classify pro-inflammatory and anti-inflammatory macrophages in the context of post-injury inflammatory response (7). During sterile inflammation, pro-inflammatory $Ly6C^{pos}CX3CR1^{neg}(CCR2^{pos}F4/80^{low})$ cells infiltrate the injured tissue. After a rather undefined set of signaling events, a phenotypic switch occurs whereby macrophages lose Ly6C and CCR2 and gain CX3CR1 and F4/80 (forming $Ly6C^{neg}CX3CR1^{pos}CCR2^{low/neg}F4/80^{high}$ cells) corresponding to their anti-inflammatory status (8). This sequence of events from the infiltration of pro-inflammatory macrophages to the phenotypic switch toward anti-inflammatory activity appears to be universal. These events have been described after injury in heart (9), central nervous system (10, 11), liver (12), kidney (13, 14), and skeletal muscle (15–18).

Skeletal Muscle Regeneration

The core cell type within skeletal muscle is the myofiber—a multinucleated cell formed by fusion of precursor cells (19). Skeletal muscle has a high regenerative capacity, after injury, muscle regenerates *ad integrum*, where the old damaged cells are replaced by proliferation and differentiation of satellite cells, which are the muscle resident stem cells (MuSCs). Skeletal muscle regeneration, therefore, is an ideal paradigm to study the biological events involved in tissue repair/regeneration, helped by highly reproducible experimental models in mouse (20). Satellite cells are localized under the basal lamina surrounding each myofiber, in a quiescent state. After an injury, damaged myofibers undergo necrosis which triggers alteration of the satellite cell niche, in turn leading to their activation (19). Activated MuSCs proliferate, in order to produce a critical pool of cells necessary to repair muscle, after which MuSCs differentiate into myocytes, that eventually fuse to form new myofibers. While myogenesis takes place, multiple other biological processes occur simultaneously during muscle regeneration. Angiogenesis is required for efficient muscle regeneration. Endothelial cells and MuSCs communicate through secreted factors to mutually promote myogenesis and angiogenesis (21). Fibro-Adipogenic Precursors (FAPs) control the extracellular matrix remodeling during muscle regeneration, depending on the number and differentiation status of the FAPs (22). Thus, muscle regeneration is a complex process where multiple cell types interact and coordinate to reconstruct the tissue (**Figure 1**).

Each step of muscle regeneration is linked to the inflammatory response, which is mainly mediated by macrophages. Macrophages modulate myogenesis through MuSCs (17), as well as angiogenesis (21), and matrix remodeling (22) that occur concomitantly. Macrophages represent more than 75% of the leukocytes present in a regenerating muscle; however other immune cells are present in lower numbers (16) and are more prominent during the early steps of muscle regeneration. Neutrophils are transiently present during the very first days after injury, but their contribution to muscle regeneration has not been deciphered yet and may depend on the extent of the injury (23). Eosinophils participate in muscle regeneration through the secretion of IL-4 that activates FAP proliferation (24). Tregs secrete the growth factor amphiregulin that stimulates MuSC expansion and differentiation (25). Therefore, macrophages are major actors in the regulation of skeletal muscle regeneration through the establishment of various interactions with several cell types. While the above-mentioned studies clearly show how macrophagic populations impact on other cell types, the effect of those cells on macrophage phenotype and function has not been evidenced yet.

The Inflammatory Phase During Muscle Regeneration

Tissue injury triggers the release of chemoattractants into the bloodstream that recruit circulating leukocytes. Monocyte entry into the injured muscle is regulated through the CCL2 (MCP1)/CCR2 axis. In mouse models of CCR2 or CCL2 depletion, muscle regeneration is severely hindered (26, 27). Indeed, only circulating $Ly6C^{pos}CCR2^{pos}$ monocytes are recruited into the injured muscle (6, 15, 18). In the *nur77*KO mouse model where $CCR2^{neg}Ly6C^{neg}$ monocytes are absent from the circulation, muscle regeneration occurs normally, indicating that circulating $CCR2^{neg}Ly6C^{neg}$ monocytes are not recruited into the injured muscle (15, 18). Once in the tissue, macrophages clear debris from apoptotic and necrotic cells through efferocytosis. They also potentiate the survival and growth of MuSCs by establishing direct cell-cell contacts (28, 29).

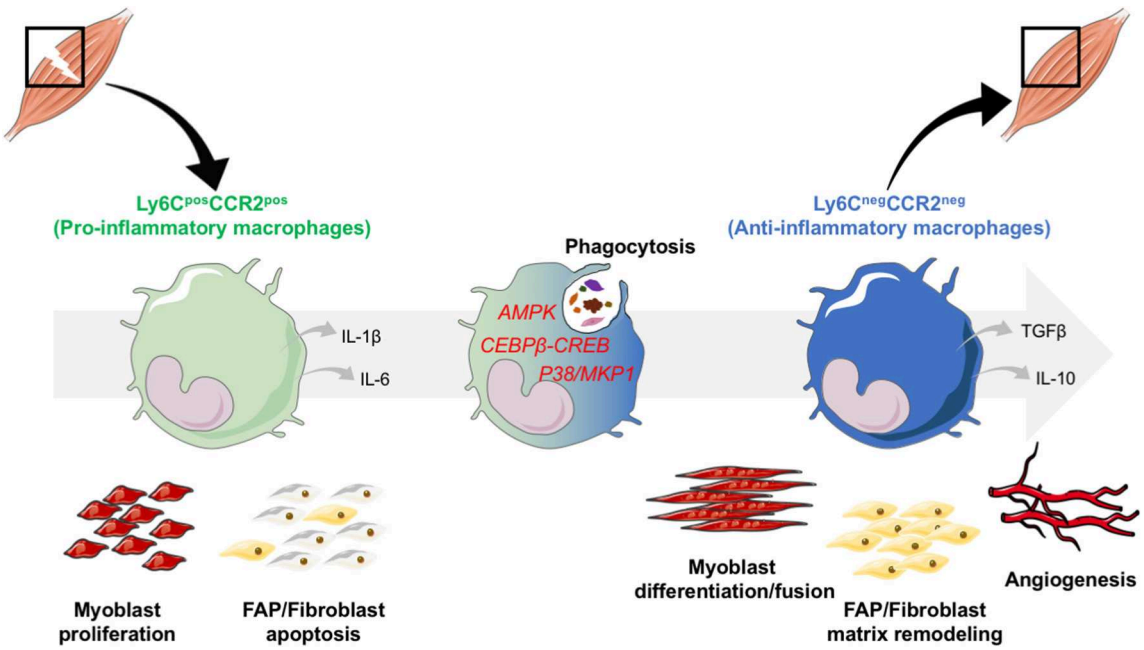

FIGURE 1 | Phenotype switch of macrophages regulates skeletal muscle regeneration. After an injury, monocytes are recruited from the bloodstream, and infiltrate the damaged area. In the tissue, monocytes acquire a damaged associated pro-inflammatory phenotype. They secrete inflammatory cytokines such as IL-1β and IL-6 and exert specific functions: they stimulate the proliferation of the myogenic precursors (myoblasts) and trigger fibroblast apoptosis to avoid excessive matrix deposition. Upon phagocytosis of cell debris that triggers the activation of AMPK, CEBPβ-CREB axis and P38/MKP1 pathways, pro-inflammatory macrophages switch their phenotype toward an anti-inflammatory restorative phenotype. Through the secretion of a variety of factors, among which anti-inflammatory cytokines IL-10 and TGFβ, anti-inflammatory macrophages are involved in tissue repair and regeneration through the stimulation of myoblast differentiation and fusion, of FAP/fibroblasts for matrix remodeling and of angiogenesis.

Moreover, pro-inflammatory macrophages secrete factors such as IL-6, IL-1β, or VEGF that stimulate MuSC proliferation (15, 17). Finally, pro-inflammatory macrophages control FAP apoptosis, preventing excess matrix deposition by fibroblastic cells (22, 30).

Macrophage Phagocytosis and the Resolution of Inflammation

At the time of resolution of inflammation, pro-inflammatory macrophages shift toward an anti-inflammatory phenotype (**Figure 1**). Signaling pathways involved in this switch are beginning to be documented in the literature. Currently, 3 main intracellular pathways have been described: AMPK, p38/MKP1, CREB-C/EBPβ (see below section "Time and space orchestration of the inflammatory response"). While the activation of these pathways is required, the activating upstream cues are still unknown. However, one likely candidate is the phagocytotic pathway that has been shown to be essential for the acquisition of an anti-inflammatory phenotype. Efferocytosis, that is the ingestion of apoptotic cells by macrophages, results in a reduction of pro-inflammatory markers, and an increase in the expression of anti-inflammatory markers, suggesting that the death signals of apoptotic cells may contribute to the generation of an anti-inflammatory phenotype (31–33). Anti-inflammatory macrophages act on several cell types in regenerating skeletal muscle, inducing both differentiation, and fusion of MuSCs as well as growth of the newly regenerated myofibers (15–17). Anti-inflammatory macrophages promote extracellular matrix remodeling by inducing fibroblast survival and collagen production through the secretion of TGF-β (30). *In vitro* experimentation has shown that anti-inflammatory macrophages stimulate endothelial cell sprouting and differentiation, inducing vessel formation concomitantly to myogenesis, through the secretion of specific effectors, such as the cytokine Oncostatin M (21). Accordingly, CCR2 KO mice exhibit defect of vascularization in the regenerating muscle, as macrophages are not efficiently recruited to the site of injury (34). Thus, anti-inflammatory macrophages are a key component of the regeneration phase. They act on multiple cell types within the muscle, promoting growth of newly formed muscle cells, remodeling of extracellular matrix and revascularization all simultaneously, allowing the full, and importantly functional, recovery of the muscle tissue.

Time and Space Orchestration of the Inflammatory Response

The inflammatory response needs to be tightly orchestrated to be efficient, and the regulation of macrophage activity is no exception. Resolution of inflammation is a key step in skeletal muscle regeneration, that must occur timely. Indeed, when the pro-inflammatory phase is blunted by the inhibition of the expression of the pro-inflammatory cytokine IFNγ (35) or reduced by the

early administration of anti-inflammatory cytokine IL-10 (36), muscle regeneration is impaired, resulting in the formation of smaller myofibers. Similarly, blunting the inflammatory phase by administrating anti-inflammatory drugs or icing the early injured muscle to prevent the entry of monocytes is detrimental for muscle regeneration [reviewed in (37)].

AMP-activated protein kinase (AMPK), a key metabolic regulator is also important for the generation of anti-inflammatory macrophages (16). Similarly, the p38/MKP1 pathway (MAP kinase pathway) modulates the phenotype of macrophages. Inhibition of the phosphatase MKP1 allows for an early activation of AKT, leading to a too early acquisition of the anti-inflammatory status in macrophages, resulting in to an impairment of muscle regeneration (36). Finally, blocking the CREB-C/EBPβ cascade prevents the acquisition of the anti-inflammatory phenotype of macrophages, that also impairs muscle regeneration (38). Given the importance of the process of the resolution of inflammation for tissue homeostasis, it is likely that other pathways are also involved in the switch of the inflammatory status of macrophages.

GLUCOCORTICOIDS: A GENERAL OVERVIEW

Origins of GCs

The hypothalamic-pituitary-adrenal axis is critical for the regulation of a variety of biological processes: stress, feeding, circadian rhythm, growth, and reproduction. GC production is regulated, via multiple hormonal inputs at all levels of the axis [reviewed in (39, 40)]. The hypothalamus secretes corticotropin releasing hormone (CRH), the first step in the regulation of GC secretion. CRH is controlled through input of the nervous system, such as exposure to stress, circulating hormones like progesterone and adrenaline, but also by GCs. CRH acts on the pituitary gland to induce the secretion of the Adreno Cortico Tropic Hormone (ACTH) into the bloodstream. ACTH binds to its receptor on cells of the adrenal cortex to regulate the secretion of a variety of hormones, especially the GC cortisol (in humans), and corticosterone (in mouse). The HPA axis, and therefore GC production is also under control of the inflammatory response. Using computational modeling and comparison to clinical data, it was demonstrated that after an inflammatory trigger, ACTH and cortisol rise within minutes to hours, slightly after cytokine release. However, this is not maintained for long, and returns to baseline after 10 h (41). The homeostatic release of GCs after an inflammatory challenge plays an important protective role, which without (e.g., through a disrupted HPA axis) results in relatively mild inflammation becoming deadly [reviewed in (42)]. Investigation into the potential medical use of GCs started in the 1930s, where Philip Hench, Edward Kendall, and Tadeusz Reichstein showed the incredible therapeutic potential of these molecules as anti-inflammatory drugs, and later received the Nobel prize for their work in 1950. From that point, GC therapies spread all around the world and are still used today to counter inflammation.

The GC Receptor

GCs act through the Glucocorticoid Receptor (GR), a member of the nuclear receptor superfamily, and first cloned in 1985 (43). The gene encoding GR is located on the locus 5q31.3 in the human genome comprised of 9 exons (43). GR expression gives rise to the expression of 2 major isoforms: GRα (777 amino acids) and GRβ (742 amino acids), along with other less well-expressed (and less well-studied) isoforms (43). GRα is the active isoform that binds GCs and that regulates target gene expression. GRβ isoform is a regulator of the α isoform, acting as a dominant negative (44, 45). A third isoform of the receptor, GRγ has also been characterized. This isoform only differs from GRα by one arginine in the DNA Binding Domain (DBD) that alters the capacity for the isoform to regulate gene expression, giving GRγ its own transcriptomic profile (46). This altered profile may play a role in GC resistant leukemia (47), however its action during inflammation has not yet been extensively studied.

The 3D structure of GR is comprised of several domains: the N-terminal domain, the DBD, the hinge region, the Ligand Binding Domain (LBD) and the C-terminal domain (48–50).

GR, like other nuclear receptors is a ligand regulated transcription factor, which regulates gene expression by binding either directly, or indirectly to the genome [review in (51)]:

- Activation: after ligand binding in the cytoplasm, GR translocates to the nucleus, and directly binds specific palindromic regions on DNA called Glucocorticoid Response Elements (GREs). GREs are present in the regulatory regions, such as the promoters, enhancers, and even within the exons or introns of target genes (such as *Gilz* and *Dusp1*) and binding of GR dimers induces the transcription of these genes (positive GRE) (51). Transactivation can also occur by a tethering mechanism, whereby GR associates with other transcription factors that positively drive gene expression. Transcription can also be induced by monomeric GR that binds DNA to a half-site motif (52).
- Repression: as with activation, nuclear GR can bind DNA and represses the transcription of genes. GR can directly act as a monomer in association with other transcription factors such as NFκB (53) or AP-1 (54) to transrepress gene expression by a tethering mechanism (51). GR monomer sequestrates transcription factors to prevent their binding to promoters and so to prevent transcription. Moreover, GR cis-repress genes by directly binding so called negative GREs or by directly binding the NFκB or AP-1 response elements (55). More mechanisms are currently emerging driven by genome wide studies that are reviewed in detail elsewhere in this Research Topic (Escoter-Torres et al., Submitted).

Thus, GR is a transcription factor that regulates gene expression through several pathways [reviewed in (45, 49, 56, 57)] and in a tissue dependent manner (58). Non-genomic effects of GCs, that is GC regulated actions that are independent from the regulation

of gene expression, have been described in several tissues and that were very recently reviewed in Panettieri et al. (59).

Adverse Effects of GCs

During the 1960s, it became clear that clinical use of GCs causes severe metabolic side effects. In 1970, David and colleagues reviewed 20 years of GC utilization (60). They discussed side effects that were observed in almost all tissues of the body. In 1970, it was already known that long exposure to GCs was responsible for several metabolic disturbances, but more recent studies have expanded on this, dramatically enhancing our knowledge about GC effects on metabolic organs. Chronic GC use results in the development of type 2 diabetes (due to increased gluconeogenesis, hepatosteatosis, decreased insulin sensitivity, and decreased glucose consumption) (61–63), skin (64, 65), and muscle atrophy (66), and bone mass reduction (both due to induction of catabolism and/or reduction of anabolism) (67). Moreover, free fatty acids are increased in the bloodstream and in clinical cases of GC excess—for example Cushing's Disease, this results in increased adipose tissue mass, but usually localized to the face and truck, resulting in a "Moon-Face" and "Buffalo Hump" (68, 69). Although literature documenting GC side effects is very abundant, the molecular mechanisms involved have not been completely elucidated, in part due to the complexity of the tissue specific effects of GCs.

Anti-inflammatory effects of GCs were historically associated with the monomeric form of GR, mainly due to the evidence that GR can bind and inhibit, and thus transrepresses the inflammatory transcription factor NFκB, downregulating the expression of pro-inflammatory cytokines (39, 70). The metabolic actions of GR were ascribed to the dimer, suggesting that drugs specific to monomeric, over dimeric GR would exhibit all beneficial anti-inflammatory effects without having negative side effects. A mouse model, in which GR dimerization is impaired (GR^{dim}), has allowed several laboratories to show that GR dimerization is also required for the anti-inflammatory properties of GCs in several contexts, such as rheumatoid arthritis (71, 72), septic shock (73, 74), or inflammatory bowel disease (75). Interestingly however, the metabolic side effects of GCs are enhanced in the GR^{dim} mice. The loss of dimerization can drive increased insulin resistance and obesity, suggesting that the classical view of monomeric GR only being associated with the anti-inflammatory actions is not entirely correct (76). Therefore, both inflammatory and metabolic regulation by GCs may be driven by both the dimer and the monomer, depending on the cell type, the tissue, and the pathology considered.

GLUCOCORTICOIDS, MACROPHAGES, AND TISSUE REPAIR

First investigations into the action of GCs on macrophages during tissue repair started a few decades ago. One of the side-effects of chronic GC exposure is the loss of bone mass (osteopenia/osteoporosis). Bone resorption, that is, the digestion of existing bone, is more efficient when highly specialized macrophages involved in bone remodeling, osteoclasts, are in direct contact with the bone. Resident tissue osteoclasts are derived from myeloid progenitor cells during development, however they are maintained throughout life by circulating blood monocytes fusing to existing osteoclasts in the bone (77). Osteoclasts treated with cortisol are more adherent to bone, more sensitive to RANKL, and release more calcium useable for bone resorption, enhancing the bone resorption process (78–80). GCs also increase osteoclastogenesis by driving the production of RANKL, the necessary factor for osteoclast differentiation, and downregulating osteoprotegerin, the decoy receptor for RANKL (81, 82). It was possible to prevent GC-induced osteoporosis by treating mice with a RANKL neutralizing antibody, further demonstrating that the effects of GCs on osteoclasts contribute to the bone loss that occurs during GC treatment (83). GCs can also have direct effects on osteoclasts. Using either mice deficient for GR in osteoclasts or 11BHSD2 overexpressing mice (where the GC inactivating enzyme is over-expressed in osteoclasts), it was confirmed that GCs act directly on osteoclasts to modulate bone density, in part by increasing the life span of osteoclasts (84, 85). Interestingly, chronic treatment with GCs decreases osteoclast life-span, suggesting a temporal effect (67, 86).

A mouse model based on the cre/loxP system was designed to specifically deplete GR in the myeloid lineage where the *cre* recombinase gene is located at the Lysozyme M locus. These so-called $LysM^{cre};GR^{fl/fl}$ mice, delete GR in monocytes, macrophages and neutrophils. In a mouse model of contact hypersensitivity, the anti-inflammatory effects of GCs were shown to be mediated through GR in macrophages, rather than other tissues. Treatment of $LysM^{cre};GR^{fl/fl}$ mice with GCs failed to repress the cytokines IL1-β, MCP1, MIP2, and IP10. In addition, GR^{dim} mice are also insensitive to GCs, indicating that GR dimerization, likely in macrophages, is required in this context (87). In a model of myocardial infarction, $LysM^{cre};GR^{fl/fl}$ mice die earlier after infarction than wild-type animals with full expression of macrophage GR, probably due to the persistence of $Ly6C^{pos}$ macrophages into the infarcted area, leading to a dysregulation of the resolution of inflammation and a defects in wound healing. This results in alteration of angiogenesis, abnormal production of TGFβ, decreased production of IL-1α and finally deregulation of myofibroblast differentiation leading to scar formation (88). Moreover, in a mouse model of inflammatory bowel disease, macrophages from $LysM^{cre};GR^{fl/fl}$ animals show a defect in the acquisition of the anti-inflammatory status. After 10 days, IL-1β, and IL-6 expression is not repressed and expression of anti-inflammatory genes (CD163, CD206, and IL-10) is not induced, leading to a defect in tissue repair (89). Local availability of GCs also plays an important role in inflammation. The enzyme 11-β-hydroxysteroid dehydrogenase (type-1) (11bHSD1) catalyzes the conversion of the inactive cortisone to cortisol, enabling binding to GR and signaling. Myeloid specific knockouts of 11bHSD1, preventing endogenous GC signaling in macrophages and neutrophils, result in a more severe arthritis phenotype (90). This is however not limited to macrophages, inhibition of 11bHSD1 increases neutrophil recruitment during peritonitis (91).

Expansion of GC research into zebrafish models is still in the early stages, and so appears somewhat contradictory. No effect of the GC beclomethasone has been observed on the migratory capacity of macrophages toward the wounding area in an amputation model in zebrafish (92). However in a separate model of wounding, prednisolone reduced macrophage accumulation in both larvae and adults (93). This may be due to the different ligands used, as different ligands have previously been shown to have different transcriptional effects (51). Thus, in most tissue injuries, GC-GR axis appears to be a central pathway in macrophages to regulate the resolution of inflammation and to proceed to tissue repair after injury.

GLUCOCORTICOIDS AND MACROPHAGES—CELLULAR ASPECTS

GCs Regulate Survival, Migration, and Proliferation of Macrophages

Maintenance of living immune cells in appropriate numbers is essential to modulate the inflammatory response, and GCs appear to play several roles in the regulation of macrophage life-span. GCs exert anti-apoptotic effects on macrophages: macrophages treated with dexamethasone are more resistant to lipopolysaccharide (LPS)-induced apoptosis (94). Similar results were obtained with other apoptotic stimuli (staurosporine, actinomycin D, or cyclohexine) where GC effects are mediated through ERK1/2 phosphorylation in an adenosine receptor A3-dependent-manner (95, 96). Moreover, macrophages treated with dexamethasone are smaller with less cytoplasmic extensions (97), which could be related to altered migratory capacity. The capacity of macrophages to move toward the injured area also shapes the inflammatory response. Macrophages treated with hydrocortisone (cortisol) show a decreased capacity to migrate *in vitro* (98, 99). *In vivo*, a similar effect was observed in a model of lung injury induced by bleomycin, where GCs inhibited macrophage infiltration into the lung (100). Studies using myeloid like cells and whole bone marrow preparations showed that GCs decrease proliferation of cells (including macrophages) *in vitro* (101, 102), but GC impact on proliferation has never been investigated on macrophage cultures. GR activation also has potent effects on nitric oxide (NO) production by macrophages. Initial studies in the J774a.1 macrophage cell line demonstrated that GCs suppress the induction of the NO-generating enzyme, nitric oxide synthase, thus controlling the level of NO produced by the cells in response to an inflammatory stimulus (103). Later studies however, showed that GCs are protective in a mouse model of stroke through increasing NO production in a non-genomic manner. By activating PI3K, GCs rapidly induce NO dependent vasodilation (104). The effects of GCs on NO production were further demonstrated to be dose dependent, with lower doses eliciting an increase in NO, while higher doses reducing the production of NO (105).

Thus, GCs promote macrophage survival in order to switch off inflammation and to sustain late phase of healing. In the following decades, studies have focused on the understanding of the molecular aspects of GC signaling pathways.

GCs and Phagocytosis

During inflammation, damaged tissue produces cell debris, and releases cytoplasmic proteins into the environment due to cell lysis (106). Before tissue repair can start, debris must be cleared up (106). The clearing process is mainly performed by neutrophils, then macrophages, through phagocytosis of tissue debris, i.e., efferocytosis (106). Since phagocytosis is a major function of macrophages and is an essential trigger of their inflammatory switch (see above section "Macrophage phagocytosis and the resolution of inflammation"), the action of anti-inflammatory treatments on this process is of importance. GCs were detected very early to have an impact on phagocytic activity of macrophages (107). Later on, studies showed in *in vitro* models using a variety of particles (zymosan, heat-kill yeast, apoptotic neutrophils, latex beads, bacteria) that dexamethasone increases the phagocytic activity of monocytes/macrophages (95, 102, 108–115). Some of these studies have also shown, using a GR antagonist (RU486), that GC-dependent phagocytosis is also GR dependent (109, 110). The increased macrophage phagocytic activity by dexamethasone is annexin 1-FRP1 dependent (116). Annexin 1 belongs to the superfamily of annexin protein, which bind acidic phospholipids in the presence of Ca^{2+} (116). Annexin A1 is described to be a pro-resolving molecule during inflammation (117). Indeed, when the annexin receptor FRP1 is antagonized by the Boc1 compound or in annexin 1-null macrophages, dexamethasone loses its effect on phagocytosis (118).

On closer examination of the phagocytic process, it became clear that GCs induce the up-regulation of several membrane receptors, such as the scavenger receptor CD163, required to detect and bind haptoglobin, a product from hemoglobin degradation (111, 113, 114, 119). The mannose receptor CD206, required for the detection of specific oligosaccharides on the bacterial wall, is also upregulated in macrophages treated by GCs (120). Moreover, GCs upregulate the membrane receptor Mer tyrosine kinase (MerTK) (121), in a C/EBPβ dependent-manner (122). When *mertk* is silenced, dexamethasone-induced phagocytosis is reduced (121). MerTK belongs to the Tyro3, Axl, MerTK (TAM) family of tyrosine kinase receptor. It binds to phosphatidyl serine exposed on the surface of apoptotic cells (121, 122). MerTK is also responsible for the phagocytosis of protein S-opsonized apoptotic neutrophils by GC-treated macrophages (123). The other members of the TAM family do not seem to be necessary for GC-induced phagocytosis, as Tyro3 deficient, or Axl deficient mice are able to successfully clear apoptotic cells in response to GCs (124). Interestingly, in a model of serum-transfer induced arthritis, Axl, MerTK, and CD163 upregulation in macrophages requires GR function on synovial fibroblasts, indicating their regulation through cross-talk between local cells (72). Finally, GCs regulate the C/EBPβ-dependent expression of nuclear receptors (liver X receptor [LXR], retinoid X receptor α [RXRα] and peroxisome proliferator-activated receptor δ [PPARδ]), which are required for prolonged phagocytosis of macrophages (122). Thus, GCs act on several steps of phagocytosis and their effects are mediated through various signaling pathways.

GLUCOCORTICOIDS AND GENE EXPRESSION IN MACROPHAGES—MOLECULAR ASPECTS

Although the first effects of GCs on macrophages were reported in 1950, the literature about their specific effects on this cell type is not abundant (see section GCs on macrophages: expression of anti-inflammatory effectors). In 1950, Dougherty and colleagues showed in a model of local inflammation in mice that cortisone treatment reduces the number of macrophages in the inflamed area (125). In another model of skin inflammation induced by injection of turpentine, Spain et al. showed that cortisone inhibits the formation of granulation in the inflamed area (granulations corresponding to macrophages according to the authors) and a decrease of carbon particle phagocytosis when administrated early during the inflammatory response (107). However, the experiments done by Gell and Hinde on intraperitoneal macrophages exposed to bacteria showed that cortisone does not alter either the number of macrophages or their phagocytic capacity (126).

GCs on Macrophages: Expression of Anti-inflammatory Effectors

It is well-known that macrophages can exert pleiotropic functions through the secretion of a variety of factors. Macrophages are highly versatile, and may secrete pro-inflammatory, anti-inflammatory, or other factors necessary at each step of the inflammatory response. GCs decrease the secretion of the pro-inflammatory cytokines TNFα (94, 127), IL-1, IL-6 in macrophages exposed to IFNγ (100, 113). Monocytes treated with GCs increase their secretion of IL-10 and TGFβ (128, 129) and express high levels of the anti-inflammatory membrane markers CD206 (120), CD163 (95, 111, 113, 114, 119, 130) and CD169 (95, 131). GC anti-inflammatory effects are partly mediated by Mitogen-activated protein kinase phosphatase-1 (MKP-1) in macrophages, as it was GC-driven inhibition of IL-6 expression was abrogated in MKP-1 deficient macrophages (132).

Furthermore, macrophages exposed to GCs secrete molecules which have direct functions on the extracellular matrix and therefore participate to matrix remodeling during the late phase of the inflammatory response. The production of elastase, collagenase and plasminogen activator (whose secretion is elevated in pro-inflammatory macrophages and which are required to degrade extracellular matrix) is reduced in macrophages treated with GCs (133, 134). On the contrary, macrophages exhibiting an alternatively activated status (i.e., IL-4 driven) secrete more fibronectin when treated with GCs, participating in matrix remodeling at the time of tissue repair (114, 135, 136).

GC Action on Macrophages: Regulation of Gene Expression

GCs act through either the GR dimer or GR monomer, entirely depending on the gene regulated. For example, in dermatitis, GR dimerization is required to shut down the expression of the pro-inflammatory cytokines IL-1β and MCP-1 whereas TNFα downregulation induced by GCs does not require GR dimerization (87). GCs also modulate chromatin architecture, mainly closing down access to genes involved in inflammation, preventing access to other transcription factors (137, 138).

Importantly, the gene regulatory actions of GCs depend on the activation state of macrophages. Indeed, more than 10,000 genomic GR binding sites are induced by dexamethasone in resting macrophages with more than 5,400 known GR target genes, while in macrophages pre-treated with GCs, then LPS, there is a rewiring of GR binding, with 13,000 binding sites and more than 6,400 GR target genes identified (139). Furthermore, GCs regulate a different set of genes in macrophages activated with LPS or IFNγ indicating that genes are regulated by GCs are also dependent on the inflammatory stimulus (130). LPS stimulation also increases the ability of GR to bind DNA indicating that pro-inflammatory stimulation potentiates GR DNA binding, likely through the generation of more potential binding loci (138, 139). Oh et al. also demonstrated that pre-treatment compared to post-treatment of GCs with LPS results in a differential effect on gene regulation. The number and location of GR binding sites and p65 binding sites were different between the GC pre-treated cells and the cells treated with LPS first, then GCs (138). Furthermore, another GR partner, the Glucocorticoid Receptor-Interacting Protein (GRIP) 1, also known as nuclear receptor co-activator 2 (NCOA2) is required for the acquisition of the anti-inflammatory phenotype of macrophages (140). GRIP1 can be phosphorylated by Cyclin-Dependent Kinase 9 (CDK9) in a GR dependent-manner. Phosphorylated GRIP1, in association with GR, binds GREs to induce the expression of anti-inflammatory genes. However, phosphorylated GRIP1 is not observed in GR repressed sites such as of *IL1a* or *IL1b*, indicating that phosphorylated GRIP1 only acts on positive transcription of anti-inflammatory genes, and it is likely that the phosphorylation status of GRIP1 can modulate GR transcriptional activity (141). Our understanding of the role of GR as an anti-inflammatory transcription factor is still evolving, and with new technologies, the actions of GR will become clearer with time.

The GC Effector GILZ in Macrophages

GC-mediated anti-inflammatory effects are known to be partly mediated through the regulation of the expression of specific proteins that in turn modulate inflammatory signaling. A very well-studied example is Glucocorticoid-Induced Leucine Zipper (GILZ). Originally found expressed in lymphoid tissues (thymocyte, spleen, lymph nodes) treated by dexamethasone (142), GILZ is a major regulator of GC effects in a variety of cells. GILZ was also found to be expressed by macrophages in liver and lung treated by dexamethasone (143). In the THP-1 macrophage cell line, dexamethasone induces *Gilz* mRNA expression after only 30 min of treatment (143). GILZ acts by binding the p65 subunit of the NFκB complex to shut down its activity (143). GILZ also inhibits the expression of the Toll like receptor 2 (TLR2), thus limiting the recognition of bacterial components and the associated inflammatory signaling (143).

GCs however also enhance the expression of TLR2 in a cell-type specific manner (144, 145), suggesting that GILZ may act as a homeostatic brake on GC enhanced TLR2 signaling. Furthermore, GC-induced GILZ expression is strongly reduced in annexin A1 deficient macrophages, therefore preventing the downregulation of the pro-inflammatory cytokines IL-1, IL-6, and TNFα (146, 147). This regulation is not dependent of the annexin receptor FRP (146), thus, the exact mechanism by which annexin regulates *Gilz* expression remains to be elucidated.

CONCLUSION

The effects of GCs on macrophages, especially in the broader context of resolution of inflammation during tissue repair, are not as well-understood as one would assume. GCs play key roles in the regulation of macrophage homeostatic functions, as well as the macrophage function as innate immunity cells. GR however, does not act alone. In association with several partners including other transcription factors (C/EBPβ, PPARs, NFκB) or proteins that modulate its activity (GRIP1), GR controls the functional properties of macrophages to resolve inflammation and tissue damage. Finally, GCs regulate the expression of a huge number of genes that are essential to relay their anti-inflammatory properties such as *Gilz* and *Annexin a*1. Despite 60 years of work on GCs, we are still discovering further molecular mechanisms that govern their actions. The role of the inflammatory context (138, 139) and species differences in GC mediated gene regulation (148) highlight that further investigation is necessary to decipher, for each situation, how GCs operate to regulate gene expression, and therefore control macrophage function.

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Cdk5 Deletion Enhances the Anti-inflammatory Potential of GC-Mediated GR Activation During Inflammation

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The suppression of activated pro-inflammatory macrophages during immune response has a major impact on the outcome of many inflammatory diseases including sepsis and rheumatoid arthritis. The pro- and anti-inflammatory functions of macrophages have been widely studied, whereas their regulation under immunosuppressive treatments such as glucocorticoid (GC) therapy is less well-understood. GC-mediated glucocorticoid receptor (GR) activation is crucial to mediate anti-inflammatory effects. In addition, the anti-cancer drug roscovitine, that is currently being tested in clinical trials, was recently described to regulate inflammatory processes by inhibiting different Cdks such as cyclin-dependent kinase 5 (*Cdk5*). *Cdk5* was identified as a modulator of inflammatory processes in different immune cells and furthermore described to influence GR gene expression in the brain. Whether roscovitine can enhance the immunosuppressive effects of GCs and if the inhibition of *Cdk5* affects GR gene regulatory function in innate immune cells, such as macrophages, has not yet been investigated. Here, we report that roscovitine enhances the immunosuppressive Dexamethasone (Dex) effect on the inducible nitric oxide synthase (iNos) expression, which is essential for immune regulation. *Cdk5* deletion in macrophages prevented iNos protein and nitric oxide (NO) generation after a combinatory treatment with inflammatory stimuli and Dex. *Cdk5* deletion in macrophages attenuated the GR phosphorylation on serine 211 after Dex treatment alone and in combination with inflammatory stimuli, but interestingly increased the GR-dependent anti-inflammatory target gene dual-specificity phosphatase 1 (Dusp1, Mkp1). Mkp1 phosphatase activity decreases the activation of its direct target p38Mapk, reduced iNos expression and NO production upon inflammatory stimuli and Dex treatment in the absence of *Cdk5*. Taken together, we identified *Cdk5* as a potential novel regulator of NO generation in inflammatory macrophages under GC treatment. Our data suggest that GC treatment in combination with specific Cdk5 inhibtior(s) provides a stronger suppression of inflammation and could thus replace high-dose GC therapy which has severe side effects in the treatment of inflammatory diseases.

Keywords: inflammation, glucocorticoid receptor, macrophage, Cdk5, Mkp1, p38Mapk, iNos

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INTRODUCTION

Acute and chronic inflammatory diseases characterized by excessive cytokine and nitric oxide (NO) generation are frequently treated with glucocorticoids (GCs) despite their negative effects including osteoporosis, insulin resistance, muscle atrophy, and depression (1). Adrenalectomized mice failed to survive endotoxic shock without the supplementation of exogenous GCs (2, 3). GCs act through the glucocorticoid receptor (GR or NR3C1), a ligand activated transcription factor, that translocates upon dissociation of accessory proteins (heat shock proteins and immunophilins) into the nucleus and acts either as a monomer or a homodimer to transrepress or transactivate target genes (4, 5). Both mechanisms are crucial to reduce the inflammatory processes either by the GR monomer interacting with pro-inflammatory transcription factors such as nuclear factor kappa B (NF-κB), activator protein 1 (AP-1) or interferon regulatory factor 3 (IRF3) or by the GR homodimer to induce genes that mediate anti-inflammatory effects like GC-induced leucine zipper (*Gilz*) or dual-specificity phosphatase 1 (*Dusp1*, also known as Map kinase phosphatase1, *Mkp1*). GC-induced Mkp1 inhibits inflammatory signaling pathways by dephosphorylation of p38Mapk or c-Jun N-terminal kinase (Jnk) (6, 7). Furthermore, *Gilz,* which is another GR target gene, inhibits NF-κB function in macrophages and T-cells (8–10). Macrophages are one of the first innate immune response cells, and are therefore important targets for the immunosuppressive effect of GC-mediated GR activation. Mice lacking the GR in macrophages show decreased survival during lipopolysaccharide (LPS)-induced endotoxic shock (11). Furthermore, the activation of the GR in macrophages is essential to limit pro-inflammatory cytokine production via Mkp1-mediated p38Mapk inhibition (7). In addition, GCs have been shown to mediate their anti-inflammatory effects during contact hypersensitivity and inflammatory lung injury through the GR in macrophages (12, 13).

Recently, inhibition of cyclin-dependent kinases (Cdks) was found to regulate inflammatory processes by inducing apoptosis in polymorphonuclear leukocytes (PMNs) (14). Roscovitine (Seliciclib, CYC202) is a potent Cdk inhibitor for Cdc2, Cdk2, Cdk5, Cdk7, and Cdk9 (15, 16). Inhibition with this small molecule inhibitor is known to promote apoptosis in cancer cell lines (17). *In vivo,* roscovitine has a potent anti-inflammatory effects during lung inflammation caused by either lipoteichoic acid (LTA) or *Streptococcus pneumoniae* and reduce PMN numbers in bronchoalveolar lavage fluid (18). In inflammatory models, such as bleomycin-induced lung injury and serum transfer-induced arthritis, roscovitine enhances the resolution of the inflammation by either decreasing the anti-apoptotic protein Mcl-1, promoting neutrophil apoptosis or reducing macrophages/monocyte numbers (19, 20). Moreover, the anti-inflammatory role of roscovitine was substantiated by studies with high doses in the RAW264.7 macrophage cell line, which identified a suppression of LPS-induced inducible nitric oxide synthase (*iNos*) expression and nitrite (NO_2^-) production, as well as Interleukin-1β (*Il-1β*), Interleukin-6 (*Il-6*), and Tumor necrosis factor-α (*Tnf-α*) mRNA levels (21, 22). Beyond roscovitine, GC-mediated GR activation in LPS-stimulated macrophages is known to reduce *Il-1β*, *Il-6*, *Tnf*-α, *iNos* mRNA levels, and nitrite (7, 11). Whether the inhibition of Cdks by roscovitine synergistically enhances the anti-inflammatory effects of GC-mediated GR activation in macrophages has not been investigated to date and could be a new therapeutic approach in the treatment of inflammatory diseases.

Roscovitine is likely to act predominantly immunosuppressive through inhibition of cyclin-dependent kinase 5 (*Cdk5)* as it has the highest affinity for this Cdk (23). *Cdk5* is a unique member of the *Cdk* family that was first described to play a pivotal role in the central nervous system (CNS), where it is involved in the regulation of brain development (24, 25), actin dynamics (26), microtubule stability (27, 28), axon guidance (29), and membrane transport (30–32). Beside its expression and function in the brain, *Cdk5* is expressed in immune cells such as neutrophils and T-cells and was shown to be involved in the regulation of neutrophil degranulation and T-cell activation (33, 34). Furthermore, the role of *Cdk5* and its activator p35 (*Cdk5r1*) was investigated in toll-like receptor (TLR)-stimulated primary macrophages. Either *Cdk5* knockdown or p35 knockout led to an increase of Interleukin-10 (Il-10) production by macrophages and resulted in immunosuppression (35). Furthermore, in a model of dextran sulfate sodium (DSS)-induced colitis and sepsis, p35-deficient mice were associated with an enhanced generation of Il-10 (35). The authors report that pro-inflammatory macrophages potentiate inflammation through *p35* and *Cdk5* activation, suggesting that Cdk5 inhibition in macrophages could lower their inflammatory potential (35).

Previous studies have shown that roscovitine also inhibits other Cdks, therefore we were interested in understanding whether the anti-inflammatory effect is mediated by specific inhibition of Cdk5. A link between Cdk5 and GR was reported by two studies in rat neuronal cells as well as in the prefrontal cortex and hippocampus of stress exposed mice showing that Cdk5 phosphorylates the GR at different serine residues and therefore modulates the GR transcriptional activity in the brain (36, 37). However, the role of *Cdk5* in combination with GCs mediated-GR activation in macrophages under inflammatory conditions has not been investigated to date.

Here, we report that roscovitine, a pan-Cdk inhibitor, as well as specific *Cdk5* deletion in macrophages enhance the anti-inflammatory effect of GCs. The treatment with Dexamethasone (Dex), a synthetic GC, in combination with roscovitine synergistically suppresses iNos mRNA and protein expression after LPS induction in bone marrow derived macrophages (BMDMs). *Cdk5* deletion confirmed a synergistic Dex-mediated suppression of iNos mRNA and protein as well as NO generation in inflammatory macrophages. However, roscovitine showed also in the absence of Cdk5 a synergistic effect with Dex to a certain degree mediated by the inhibition of other Cdks than Cdk5. This indicates that roscovitine enhances the anti-inflammatory Dex effect on iNos by inhibiting Cdk5 and other Cdks. However, Dex-mediated suppression of pro-inflammatory cytokines such as *Il-1β* and *Il-6* was not enhanced by roscovitine treatment or *Cdk5* deletion. In addition, the effect on iNos and NO production was associated with decreased phosphorylation of GR (Ser211), but interestingly induced expression of GR target gene Mkp1 and reduced p38Mapk activation in *Cdk5* deficient macrophages.

The reduction of p38Mapk activity further enhanced the Dex effect on iNos repression. These results show that inhibition of Cdk5, in combination with Dex treatment improves the suppression of iNos and NO in macrophages. Inhibiting Cdks by roscovitine and/or specific impairing Cdk5 activity could serve as a new treatment strategy in high-dose GC therapy of inflammatory diseases.

MATERIALS AND METHODS

Mice

Cdk5^{tm1Bibb} (C57BL/6) mice (hereafter named as *Cdk5*^{flox}) were kindly provided by Prof. Dr Johanna Pachmayr (Paracelsus Medical Private University, Austria) (38). *Cdk5*^{flox} mice were crossed with transgenic Lyz2$^{tm1(cre)Ifo/J}$ (C57BL/6) mice (hereafter named as LysMCre) to generate *Cdk5*^{LysMCre} mice. Male and female *Cdk5*^{LysMCre} mice and littermate controls (*Cdk5*^{flox}) at the age of 8–13 weeks were used for experiments. The mice were genotyped by PCR using genomic DNA isolated from the tails. All animals were housed under specific pathogen-free conditions at the Centre of Biomedical Research (ZBMF) at Ulm University. This study was carried out in accordance with the recommendations of Tierschutzgesetz and Tierschutz-Versuchstierordnung (Mitteilung nach § 4), Regierungspräsidium Tübingen, Baden-Württemberg. The protocol was approved by the Regierungspräsidium Tübingen, Baden-Württemberg.

Cell Culture

The primary BMDMs were isolated from humerus, femur and tibia of 8–13 weeks old mice as described previously (11). Briefly, cells were cultured until day 7 in DMEM (D5671, sigma) supplemented with 10% fetal bovine serum (FBS, F7524, sigma), 20% L929-cell conditioned medium, 1% Penicillin/Streptomycin (P0781, sigma), 1% L-Glutamine (G7513, sigma), 1% Sodium Pyruvate (S8636, sigma) at 37°C, and 5% CO_2. For roscovitine experiments, BMDMs from wildtype mice (C57BL/6) or from *Cdk5*^{flox} and *Cdk5*^{LysMCre} were pre-treated for 30 min with DMSO (as vehicle) or 10 μM roscovitine (Seliciclib, CYC202) (Selleckchem). For p38Mapk inhibition, BMDMs from wildtype mice (C57BL/6) were pre-treated for 1 h with DMSO or 5 μM SB203580 (sigma). BMDMs were isolated from littermate wildtype (*Cdk5*^{flox}) and *Cdk5*^{LysMCre} mice. All BMDMs were treated with PBS as control, LPS (100 ng/ml, L6529, sigma), Dex (10^{-6} M, D2915, sigma), or LPS + Dex (100 ng/ml LPS and 10^{-6} M Dex) for the indicated durations. For alternative macrophage (M2-like) polarization as well as for TAM and phagocytic receptor expression analysis, cells were treated 24 h as indicated with PBS as control, Il-4 (20 ng/ml, Immunotools), Il-13 (20 ng/ml, Immunotools), Il-4 + Il-13 (20 ng/ml Il-4 and 20 ng/ml Il-13), Il-10 (20 ng/ml, Immunotools), Il-10 + Dex (20 ng/ml Il-10 and 10^{-7} M Dex), Dex (10^{-7} M), or LPS + Dex (100 ng/ml LPS and 10^{-7} M Dex).

NO Measurement

Bone marrow derived macrophages were isolated from *Cdk5*^{flox} and *Cdk5*^{LysMCre} mice and grown until day 6. Afterwards, cells were seeded in a 96-well plate (150'000 cells/well) with DMEM media without phenolred (D1145, sigma) supplemented with 10% fetal bovine serum (FBS, F7524, sigma), 20 ng/ml M-CSF (R&D system), 1% Penicillin/Streptomycin (P0781, sigma), 1% L-Glutamine (G7513, sigma), 1% Sodium Pyruvate (S8636, sigma), and incubated at 37°C and 5% CO_2. At day 7, BMDMs were treated for the indicated time points. Supernatant was collected after 48 h, centrifuged (13'000 rpm, 5 min) and nitrite was measured as a stable metabolite of NO with Griess reagent (Molecular Probes; G7921) according to the manufacturer's protocol.

ELISA

For the determination of Il-6 secretion, the medium of BMDMs from littermate wildtype (*Cdk5*^{flox}) and *Cdk5*^{LysMCre} mice after 4 h treatment with PBS as control, LPS (100 ng/ml), Dex (10^{-6} M) or LPS + Dex (100 ng/ml LPS and 10^{-6} M Dex) was collected, sterile filtered (0.2 μm) and stored at −80°C until measurement was performed. The Il-6 ELISA was performed with the Mouse Il-6 ELISA set (BD OptEIA™) according to the manufacturer's protocol. The absorption was measured using the Dynex Opsys MR 96-Well Microplate Reader at 405 nm with a correction wavelength of 650 nm.

RNA Isolation and Quantitative RT-PCR

Primary macrophages were washed with 1x PBS and then scraped in RLT (Qiagen) + 10 μl β-mercaptoethanol/ml buffer. RNA was isolated using the RNeasy® Mini Kit (Qiagen) according to the manufacturer's protocol. Next, 1,000 ng RNA was reversed transcribed to cDNA by using Superscript II® (Superscript® Reverse Transcriptase, Invitrogen). Quantitative RT-PCR (qRT-PCR) was performed with the ViiA™ 7 Realtime PCR System (Life technologies) using Platinum SYBR Green (Invitrogen). For analysis the QuantStudio Realtime-PCR software and the ΔΔCT method was used. *β-Actin* and *Ribosomal protein L* (*Rpl*) served as housekeeping genes. The specific primers were obtained from Sigma with the sequences as listed below:

Primer Sequences

Gene	Forward Primer (5′ → 3′)	Reverse Primer (3′ → 5′)
β-Actin	GCACCAGGGTGTGATGGTG	CCAGATCTTCTCCATGTCGTCC
Anxa1	AAGGTGTGGATGAAGCAACC	AGGGCTTTCCATTCTCCTGT
Axl	AGCCTTCCTGTGCCCCTA	GAGGTGGGGGTTCACTCA
Cd36	TGGCAAAGAACAGCAGCAAA	CACAGTGTGGTCCTCGGG
Cd163	GGCTAGACGAAGTCATCTGCAC	CTTCGTTGGTCAGCCTCAGAGA
Cd206	CCACAGCATTGAGGAGTTTG	ACAGCTCATCATTTGGCTCA
Cdk5	TGGACCCTGAGATTGTGAAGT	GACAGAATCCCAGGCCTTTC
Gilz	ACCAGACCATGCTCTCCATT	GGCCTGCTCAATCTTGTTGT
Il-1β	GGCTGTGGAGAAGCTGTGGCA	GGGTCCGACAGCACGAGGCT
Il-6	AAACCGCTATGAAGTTCCTCTCTGC	AGCCTCCGACTTGTGAAGTGGT
Il-10	CAGAGCCACATGCTCCTAGA	TGTCCAGCTGGTCCTTTGTT
iNos	CTGCTTTGTGCGAAGTGTCAGT	GGCACCCAAACACCAAGCTC
Mertk	GCTGGCATTTCATGGTGGAA	CATTGTCTGAGCGCTGCAC
Mkp1	GTGCCTGACAGTGCAGAATC	CACTGCCAGGTACAGGAAG
Rpl	CCTGCTGCTCTCAAGGTT	TGGCTGTCACTGCCTGGTACTT
Tyro3	TGGAGCCATCCTAGAGTTCC	GAGGGGCCTGACTTCCTG
Ym1	CTGGGTCTCGAGGAAGCC	AGTGAGTAGCAGCCTTGGAA

Western Blot Analysis

Bone marrow derived macrophages were washed with 1x PBS and lysed directly on the dishes with ice-cold 1x Lysis Buffer (Cell Signaling) or 1x RIPA buffer. PhosphoStop (Roche) and protease inhibitor cocktail (Roche) were added to both buffers. The lysates were centrifuged at 14'000 rpm at 4°C for 10 min. The protein concentration was determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. For Western blot analysis, protein samples were adjusted to 15–35 μg protein with Lysis or RIPA buffer and boiled in 5x Laemmli buffer (with 10 μl/ml β-mercaptoethanol) at 95°C for 5 min. Equal protein amounts were separated on 7.5–10 % SDS—PAGE gels and subsequently electrotransferred onto nitrocellulose membranes (Biorad) using the Tank Blot System (Biorad). The membranes were blocked with 5% skim milk powder (Fluka Analytical) or BSA (Sigma) in Tris-buffered saline with Tween20 (TBS-T) for 1 h at RT and probed over night at 4°C with primary antibodies against β-Actin (Sigma Aldrich), Cdk5 (Cell Signaling #2506), GR (Cell Signaling #12041), phospho-GR Ser211 (Cell Signaling #4161), iNos (Santa Cruz Biotechnology sc-650 or Cell Signaling #13120), p38Mapk (Cell Signaling #9218), phospho-p38Mapk Thr180/Tyr182 (Cell Signaling #4511), Mkp1 (Santa Cruz Biotechnology sc-871684). After washing with TBS-T for 30 min, membranes were incubated with horseradish peroxidase-coupled goat anti-mouse (Dako) or goat anti-rabbit (Life technologies) antibodies for 1 h at RT. For visualization the LuminataTM Forte Western HRP Substrate (Milipore) and the ChemiDocTM MP Imaging System (Biorad) was used. If membranes were stripped, blots were incubated with stripping buffer (with 0.5 μl β-mercaptoethanol/ml) at 60°C for 30 min. Phospho-proteins were always first detected and total protein after stripping. Quantification was performed with Photoshop software. Cdk5, iNos, and Mkp1 were normalized to β-Actin as loading controls. Phospho-p38Mapk was normalized to p38Mapk as loading control. pGR was normalized to β-Actin on the same gel and total GR was normalized to β-Actin on the same gel and afterwards p-GR/GR ratio was calculated.

Multiplex-Assay

Phospho-Erk1/2 (Thr202/Tyr204) protein was detected with the Bio-Plex Pro™ cell signaling MAPK-Panel (#LQ00000S6KL81S, Biorad). The Bio-Plex Assay was conducted according to the manufacturer's protocol. The median fluorescence intensity (MFI) was detected with the Bio-Plex 200 machine (Biorad) and analyzed with the Bio-Plex Manager™ 6.1 software (Biorad).

Statistical Analysis

Statistical analysis was carried out with GraphPad Prism 7 software. All data are shown as mean ± SEM. Outlying sample exclusion criteria were done with GraphPad Prism Outlier Calculator. All data were tested using the Wilcoxon-Mann-Whitney test (two-tailed). In comparison, mean values which show significance are indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.001$; ns: not significant.

RESULTS

Roscovitine Enhances the Dex-Mediated Suppression of iNos in Inflammatory Macrophages

To examine whether roscovitine enhances the immunosuppressive effects of GCs we first determined the expression levels of pro-inflammatory cytokines in BMDMs isolated from wildtype mice. Since iNos expression and ultimately NO production in macrophages are essential for immune regulation during inflammation, we further examined iNos expression. As expected from previous studies (7, 11), 4 h LPS stimulation upregulated the expression of *Il-1β*, *Il-6*, and *iNos* mRNA and iNos protein, whereas Dex treatment during LPS stimulation significantly reduced their expression, and Il-6 protein showed a trend in reduction (**Figures 1A–E**). Moreover, as previously shown (19), the LPS-mediated induction of inflammatory mediators *Il-1β*, *Il-6*, and iNos was significantly decreased upon solely roscovitine treatment both on the mRNA and protein levels (**Figures 1A–E**). Interestingly, the combinatorial treatment of Dex with roscovitine during LPS stimulation further reduced the *Il-1β*, *Il-6*, and *iNos* mRNA and Il-6 and iNos protein levels (**Figures 1A–E**). To investigate whether the strong reduction in inflammatory mediator expression is mediated by either additive or synergistic anti-inflammatory effects of roscovitine and Dex, we calculated the degree of the suppressive Dex effect with and without roscovitine by setting LPS treatment to 100%. We found that the Dex-mediated suppression of the pro-inflammatory cytokines *Il-1β* and *Il-6* was not enhanced by roscovitine treatment in inflammatory macrophages, suggesting that the immunosuppressive roscovitine effect is mediated by independent pathways (**Figures 1A,B** and **Supplementary Figures 1A,B**). However, roscovitine increased the anti-inflammatory potential of Dex on LPS-induced *iNos* expression (**Figures 1C,E** and **Supplementary Figure 1C**). These results suggest that roscovitine has a strong immunosuppressive effect that is enhanced when given in combination with Dex, further reducing inflammatory mediator expression in macrophages. Interestingly, in the case of iNos expression, roscovitine enhances the anti-inflammatory potential of Dex in a synergistic manner.

Specific Cdk5 Deletion Enhances the Suppressive Dex-Effect on iNos in Inflammatory Macrophages

Roscovitine shows highest affinity for *Cdk5* (15, 16) therefore we assumed that the immunosuppressive effects of roscovitine are mainly due to the inhibition of *Cdk5*. *Cdk5* has primarily been implicated in brain development and is particularly important in neuronal maturation and migration (24–31). It also has been reported to be expressed in immune cells like macrophages with a functional relevance *in vitro* and *in vivo* (33–35). Since we observed a difference on the anti-inflammatory Dex

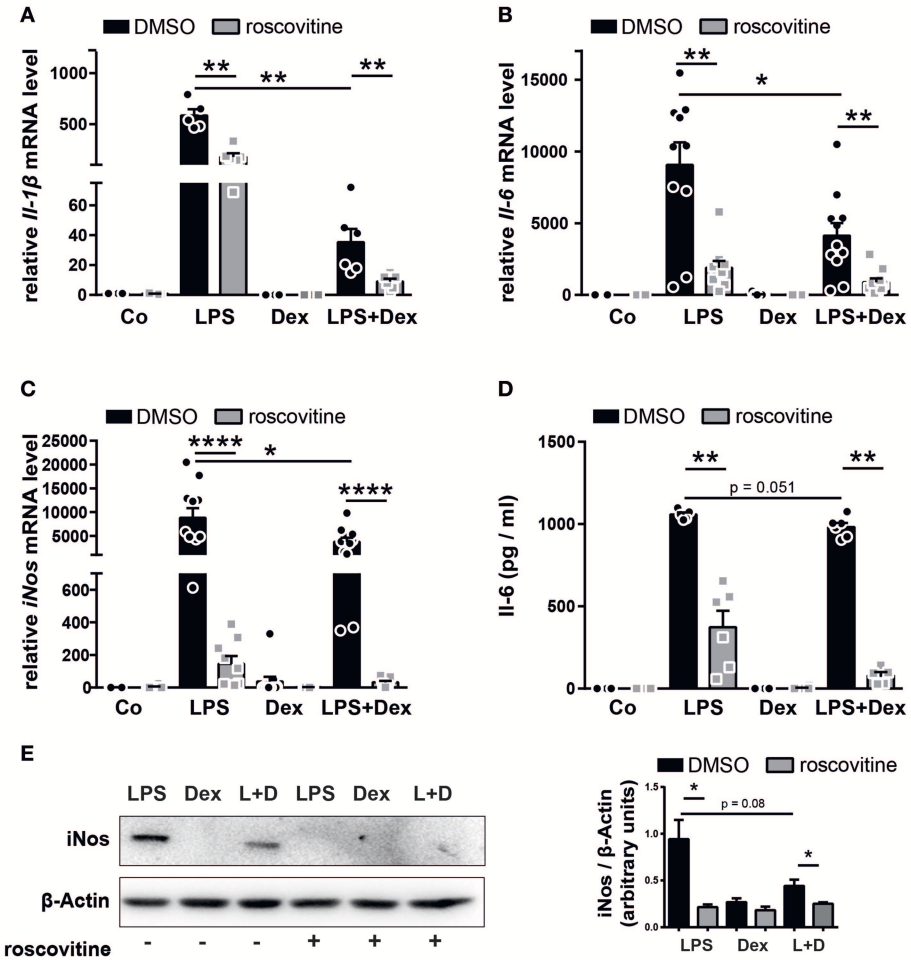

FIGURE 1 | The combination of roscovitine and Dex shows additive and synergistic anti-inflammatory effects in inflammatory macrophages. **(A–E)** BMDMs derived from wildtype mice were stimulated 4 h with PBS (Co), LPS (100 ng/ml), Dex (10^{-6} M), or a combination of LPS + Dex (L + D) with 30 min pre-treatment of either DMSO or 10 μM roscovitine. **(A)** Relative *Il-1β* mRNA expression, **(B)** relative *Il-6* mRNA expression, and **(C)** relative *iNos* mRNA expression were analyzed by qRT-PCR after 4 h. **(D)** Il-6 protein concentration was determined in the BMDM supernatant by ELISA after 4 h. **(E)** BMDMs derived from wildtype mice were stimulated 4 h with LPS (100 ng/ml), Dex (10^{-6} M), or a combination of LPS + Dex (L + D) with 30 min pre-treatment of either DMSO or 10 μM roscovitine and iNos protein (130 kDa) was detected by western blot after 4 h and quantified. β-Actin (43 kDa) served as loading control. Data shown in **(A)**: $n = 5$–6; **(B)**: $n = 11$–12; **(C)**: $n = 9$–11; **(D)**: $n = 5$–6; and **(E)**: $n = 3$. Results are depicted as mean ± SEM. Statistical analysis was performed by Wilcoxon-Mann-Whitney test (two-tailed) $^*p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$.

effect upon combinatorial treatment with roscovitine in LPS-stimulated BMDMs, we further investigated under the same conditions the impact of *Cdk5* deficiency on inflammatory processes in macrophages. Thus, we used the Cre/loxP-system and crossed $Cdk5^{flox}$ mice with myeloid specific lysozyme MCre mice ($Lyz2^{tm1(cre)Ifo}$, hereafter named as LysMCre) (39). The BMDMs isolated from the mutant mice ($Cdk5^{LysMCre}$) showed a significant decrease in *Cdk5* at the mRNA and protein levels (**Supplementary Figures 2A,B**). Thus, $Cdk5^{LysMCre}$ mice serve as a suitable model to study specific *Cdk5* effects in macrophages.

As expected, the mRNA expression of *Il-1β*, *Il-6* and *iNos* as well as Il-6 and iNos protein expression were increased after 4 h of LPS stimulation and significantly reduced by Dex after LPS induction in $Cdk5^{flox}$ BMDMs (**Figures 2A–E**). However, *Cdk5* deletion did not reduce *Il-1β*, *Il-6*, and *iNos* mRNA as well as Il-6 and iNos protein expression after single LPS stimulation (**Figures 2A–E**). This is in contrast to our observation upon roscovitine treatment (**Figures 1A–E**), suggesting that the strong immunosuppressive effect of roscovitine on inflammatory mediators is not solely mediated by the inhibition of Cdk5 but by the inhibition of several Cdks. To determine whether the observed synergistic effect of roscovitine and Dex on *iNos* suppression, but not on *Il-1β* and *Il-6*, is mediated by inhibition of Cdk5 we investigated the *Il-1β*, *Il-6*, and *iNos* expression in BMDMs isolated from wildtype ($Cdk5^{flox}$) and $Cdk5^{LysMCre}$ mice. The *Cdk5* deletion had no effect on the anti-inflammatory potential of Dex on *Il-1β* an *Il-6* expression (**Figures 2A,B** and **Supplementary Figures 1D,E**). However, *Cdk5* deletion

enhanced significantly the Dex-mediated downregulation of *iNos* expression after 4 h of LPS induction in comparison to wildtype BMDMs (**Figure 2C** and **Supplementary Figure 1F**). Furthermore, we found that *Cdk5* deletion significantly enhanced the suppressive Dex effect also on the iNos protein level after 4 h of LPS + Dex stimulation (**Figure 2E**), followed by a stronger reduction of NO production in the supernatant of $Cdk5^{LysMCre}$ BMDMs (**Figure 2F**). This suggests that the enhanced Dex effect in the presence of roscovitine may be mediated by the inhibition of Cdk5. To further confirm the enhanced anti-inflammatory Dex effect by roscovitine and *Cdk5* deletion, we investigated the effect of roscovitine in $Cdk5^{LysMCre}$ macrophages. Roscovitine reduced *Il-1β*, *Il-6*, and *iNos* expression in LPS treated macrophages in the absence of Cdk5 (**Supplementary Figures 3A–C**), showing that the anti-inflammatory potential of roscovitine is mediated mainly by the inhibition of other kinases than Cdk5. Moreover, we observed that roscovitine treatment of LPS + Dex treated $Cdk5^{LysMCre}$ macrophages reduced the expression of *Il-1β* and *Il-6,* suggesting that these effects are mediated by Cdk5-independent pathways (**Supplementary Figures 3A,B,D,E**). Interestingly, *iNos* expression was also reduced in LPS + Dex treated $Cdk5^{LysMCre}$ macrophages when roscovitine was present (**Supplementary Figures 3C,F**). This suggests that roscovitine enhances the anti-inflammatory Dex effect on iNos mainly by a Cdk5-independent mechanism.

However, deletion of Cdk5 is sufficient to increase the Dex effect on *iNos,* indicating Cdk5 as an important target to increase anti-inflammatory efficacy of GCs.

Specific *Cdk5* Deletion Has no Effect on Anti-inflammatory Markers in Macrophages

GCs mediate their anti-inflammatory effects not only by suppressing pro-inflammatory mediators in M1-like macrophages, but also by promoting alternative anti-inflammatory M2-like macrophage polarization. Since we observed a synergistic anti-inflammatory effect on the M1 marker iNos upon *Cdk5* deletion and Dex treatment, we next examined if a combination of *Cdk5* deletion and Dex treatment has an effect on M2 macrophage polarization *in vitro*. Therefore, BMDMs from $Cdk5^{flox}$ and $Cdk5^{LysMCre}$ were stimulated for 24 h with the M2 stimuli Il-4, Il-10, Il-13, Dex, Il-4 + Il-13, and Il-10 + Dex. We found that *Cdk5* deletion alone had no impact on known typical M2-like markers (*Cd163*, *Cd206*, *Ym1*, and *Il-10*) (**Supplementary Figure 4**). When *Cdk5* deleted BMDMs were treated with Il-10, a trend toward a reduction in expression of *Cd163* was observed (**Supplementary Figure 4A**). Similarly, no significant changes were observed in the expression of *Cd206* (**Supplementary Figure 4B**), *Ym1* (**Supplementary Figure 4C**), and *Il-10* (**Supplementary Figure 4D**). Furthermore, no *Cdk5* specific effects on M2 marker expression were determined in combination with Dex (**Supplementary Figures 4A–D**). However, the combination of *Cdk5* deletion and Dex treatment led to an enhanced induction of *Mertk* expression (**Supplementary Figure 4E**). *Mertk* is a member of the TAM receptor family, which includes Tyro3, Axl, and Mer. These receptors are important for macrophage phagocytic function (40). Therefore, we examined whether *Cdk5* deletion regulates TAM and phagocytic receptor expression in inflammatory macrophages treated with Dex. Our findings revealed that the expression of other phagocytic receptors such as *Tyro3* (**Supplementary Figure 4F**), *Axl* (**Supplementary Figure 4G**), *Cd36* (**Supplementary Figure 4H**), and *Anxa1* (**Supplementary Figure 4I**) were not altered upon *Cdk5* deletion. Therefore, we concluded that Cdk5 alone as well as in combination with Dex does not play a major role in regulating M2-like markers and TAM receptor expression in macrophages *in vitro*. We interpret these results to mean that *Cdk5* deletion potentiates the suppressive Dex effect on the pro-inflammatory marker iNos and NO production in LPS stimulated BMDMs (**Figures 2C,E,F**).

Cdk5 Deletion Is Associated With a Reduced GR Phosphorylation at Ser211, but Interestingly Increased Induction of the GR Target Gene Mkp1

The Cdk5-regulated pathways beyond the brain have not been well studied; therefore, we further examined how *Cdk5* deletion enhances the Dex effect on iNos and NO. There are reports showing that Cdk5 can directly interact with the GR by changing the phosphorylation status and thereby influencing GR transcriptional activity (36, 41). It was reported that GR phosphorylation by Cdk5, in particular at serine 211 (Ser211), reduces GR transcriptional activity in the context of neurons (41). We therefore investigated the GR phosphorylation at Ser211 in $Cdk5^{flox}$ and $Cdk5^{LysMCre}$ BMDMs. Our results demonstrated that Dex and LPS + Dex treatment increased GR phosphorylation at Ser211 in $Cdk5^{flox}$ BMDMs after 4 h, whereas deletion of *Cdk5* led to a significant decrease in GR phosphorylation after Dex and LPS + Dex treatment (**Figure 3A** and **Supplementary Figure 5A**). In line with this, roscovitine treatment of wildtype macrophages also confirmed a tendency toward a reduced GR Ser211 phosphorylation after Dex and LPS + Dex treatment (**Supplementary Figure 5B**). Thus, our findings show for the first time that deletion of *Cdk5* diminishes GR phosphorylation at Ser211 in macrophages.

Since GR phosphorylation at Ser211 has been described as an activating phosphorylation site (42), but is known to act also as a suppressive phosphorylation site in neurons (36), thus, we further analyzed GR transcriptional activity upon *Cdk5* deletion. To this end, we tested the expression of the anti-inflammatory target *Gilz* and we did not observe differences irrespective of genotype upon 4 h of Dex stimulation (**Figure 3B**). *Gilz* is a negative regulator of Raf-Mek1/2-Erk1/2 activation (43) and in line, we observed no changes in Erk1/2 phosphorylation in $Cdk5^{flox}$ and $Cdk5^{LysMCre}$ BMDMs (**Supplementary Figure 5C**). Moreover, we detected strikingly reduced GR phosphorylation at Ser211 after 4 h of Dex and LPS + Dex treatment in $Cdk5^{LysMCre}$ BMDMs (**Figure 3A** and **Supplementary Figure 5A**). However,

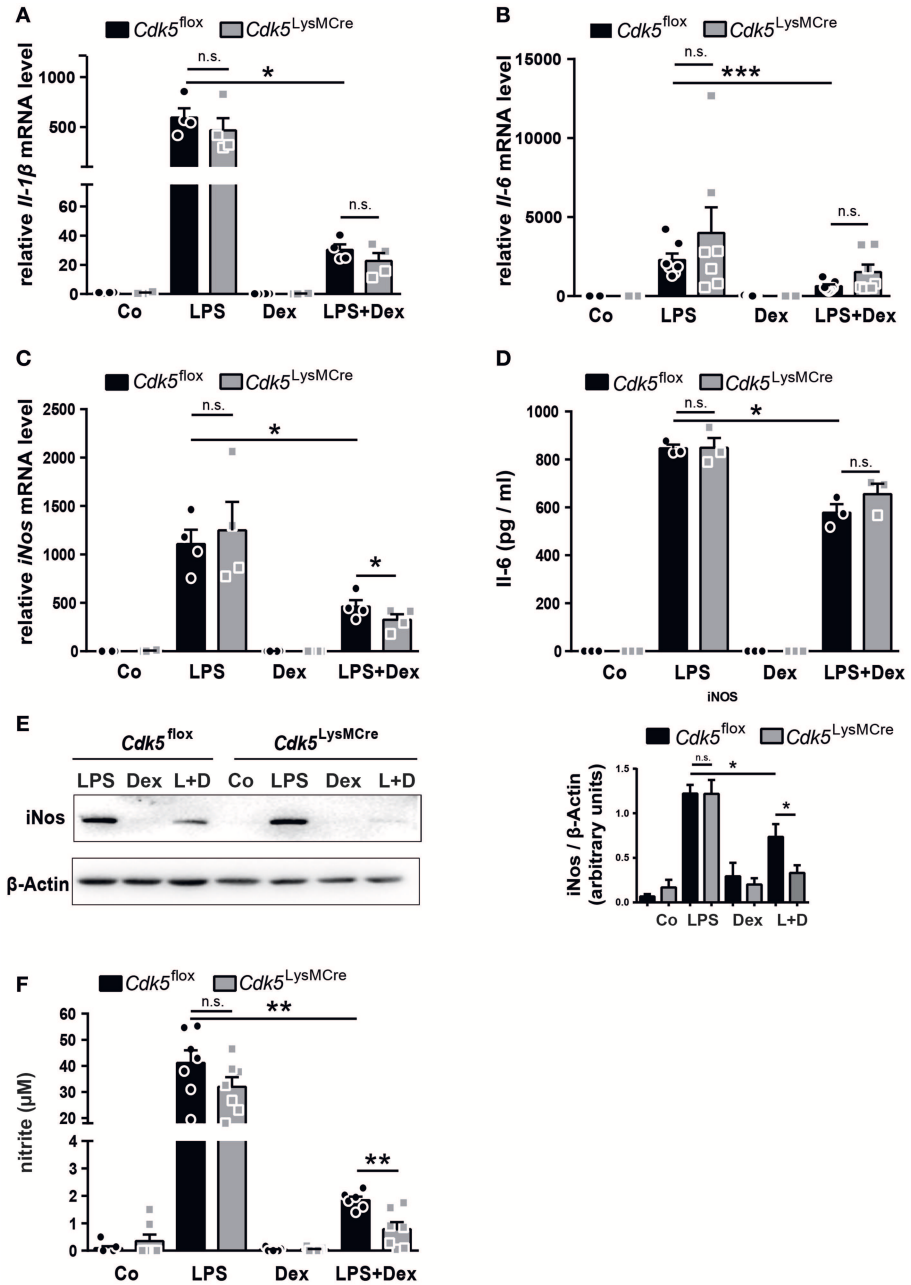

FIGURE 2 | *Cdk5* deletion has no impact on inflammatory mediators but potentiates the Dex effect on iNos and NO production in macrophages. **(A–E)** BMDMs from *Cdk5*^{flox} and *Cdk5*^{LysMCre} mice were stimulated with PBS (Co), LPS (100 ng/ml), Dex (10^{-6} M), or a combination of LPS + Dex (L + D) for 4 h. **(A)** Relative *Il-1β* mRNA expression, **(B)** relative *Il-6* mRNA expression, and **(C)** relative *iNos* mRNA expression were analyzed by qRT-PCR after 4 h. **(D)** Il-6 protein concentration was determined by ELISA in the supernatant of *Cdk5*^{flox} and *Cdk5*^{LysMCre} BMDMs after 4 h. **(E)** BMDMs from *Cdk5*^{flox} and *Cdk5*^{LysMCre} mice were stimulated with LPS (100 ng/ml), Dex (10^{-6} M) or a combination of LPS + Dex (L+D) for 4 h and iNos protein (130 kDa) was detected by western blot after 4 h and quantified. β-Actin (43 kDa) served as loading control. **(F)** BMDMs were treated as described in A and nitrite (a stable NO metabolite) was measured in the supernatant of *Cdk5*^{flox} and *Cdk5*^{LysMCre} BMDMs after 48 h. Data shown in **(A)**: $n = 4$; **(B)**: $n = 7$; **(C)**: $n = 4$; **(D)**: $n = 3$; **(E)**: $n = 5$–8; **(F)**: $n = 6$–7. Results are depicted as mean ± SEM. Statistical analysis was performed by Wilcoxon-Mann-Whitney test (two-tailed) $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s. not significant.

the anti-inflammatory GR target *Mkp1* was significantly induced in *Cdk5*-deficient BMDMs after 4 h of Dex and LPS + Dex stimulation (**Figure 3C**). In addition, Mkp1 protein was increased in *Cdk5* deleted BMDMs upon 4 h Dex and LPS + Dex stimulation (**Figure 3D** and **Supplementary Figure 5D**). This suggests that also for certain GR target genes in macrophages the phosphorylation at Ser211 is associated with diminished GR transcriptional activity.

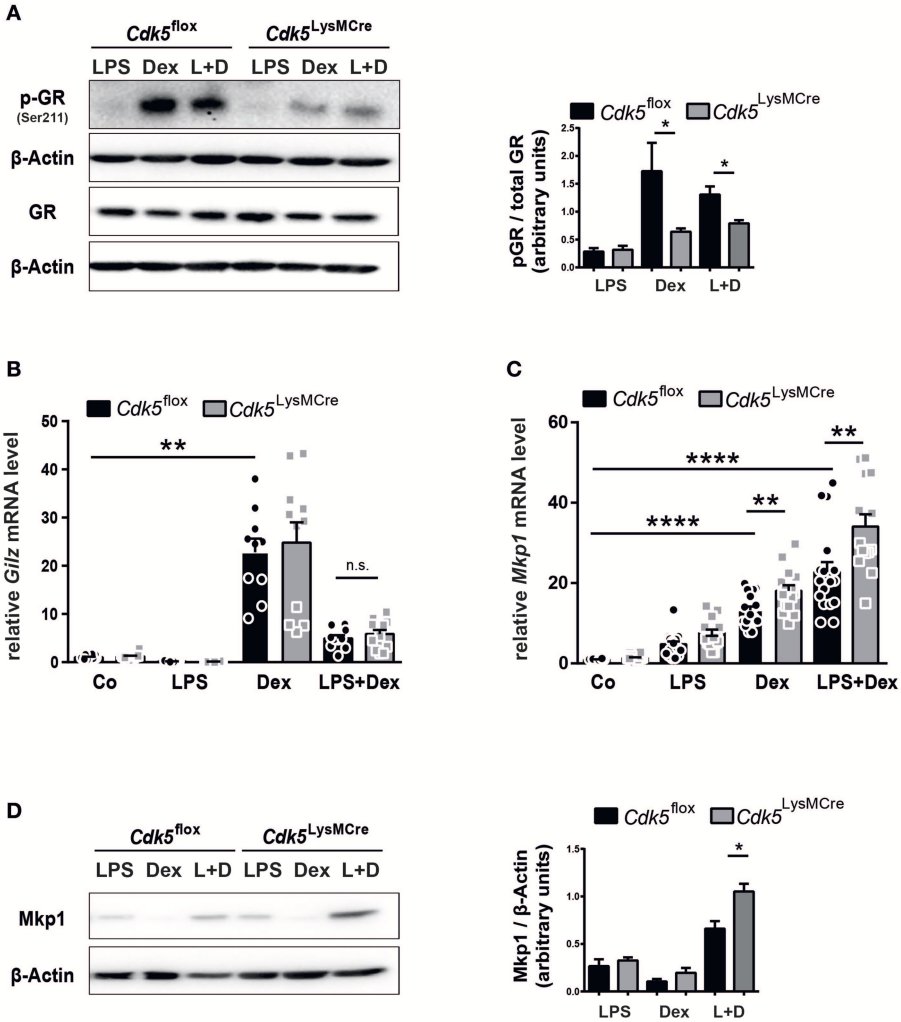

FIGURE 3 | *Cdk5* deletion diminish GR phosphorylation, but increases the GR target gene Mkp1. **(A)** BMDMs derived from *Cdk5*^{flox} and *Cdk5*^{LysMCre} mice were stimulated with LPS (100 ng/ml), Dex (10^{-6} M) or a combination of LPS + Dex (L + D) for 4 h and phosphorylated GR (Ser211) protein (95 kDa) and total GR protein (94 kDa) was detected by western blot on two separate gels and quantified. β-Actin (43 kDa) served as loading control on the individual gels. **(B,C)** BMDMs from *Cdk5*^{flox} and *Cdk5*^{LysMCre} mice were stimulated with PBS (Co), LPS (100 ng/ml), Dex (10^{-6} M), or a combination of LPS + Dex for 4 h and **(B)** relative *Gilz* mRNA expression and **(C)** relative *Mkp1* mRNA expression were measured with qRT-PCR after 4 h. **(D)** BMDMs were treated as described in **(A)** Mkp1 protein (40 kDa) was detected by western blot after 4 h. β-Actin (43 kDa) served as loading control. Data shown in **(A)**: $n = 3$–4; **(B)**: $n = 10$–12; $n = 14$–15; and **(D)**: $n = 5$. Results are depicted as mean ± SEM. Statistical analysis was performed by Wilcoxon-Mann-Whitney test (two-tailed) $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$; n.s. not significant.

Cdk5 Deletion Reduces Phospho-p38Mapk and Hence iNos and NO Production During LPS Stimulation and Dex Exposure

Our experimental data revealed that *Cdk5* deletion synergistically reduces LPS-induced iNos expression and NO production in combination with Dex treatment (**Figures 2C,E,F**). Previous studies have shown that iNos expression is also regulated by Mapk pathways, such as p38Mapk (44), whose activating phosphorylation levels are reduced by an increased Mkp1 expression in response to LPS induction (45). Therefore, we further investigated p38Mapk as a potential link between *Cdk5* regulating iNos and NO production via Mkp1. We demonstrated that 4 h of LPS stimulation increased p38Mapk phosphorylation, whereas Dex treatment attenuated its phosphorylation after LPS stimulation in *Cdk5*^{flox} BMDMs (**Figure 4A**), as expected (7). Interestingly, *Cdk5* deletion in combination with Dex treatment during inflammatory stimuli attenuated p38Mapk phosphorylation to a greater extent compared to *Cdk5*^{flox} macrophages (**Figure 4A**). However, similar a previous publication (21), roscovitine treatment of inflammatory wildtype macrophages show unchanged levels of p38Mapk phosphorylation (**Supplementary Figure 5E**).

To prove whether *Cdk5* deletion enhances the Dex effect on NO production after inflammatory stimuli via the GR-Mkp1-p38Mapk axis, we examined the NO production in the supernatants of *Cdk5*^{flox} and *Cdk5*^{LysMCre} BMDMs after

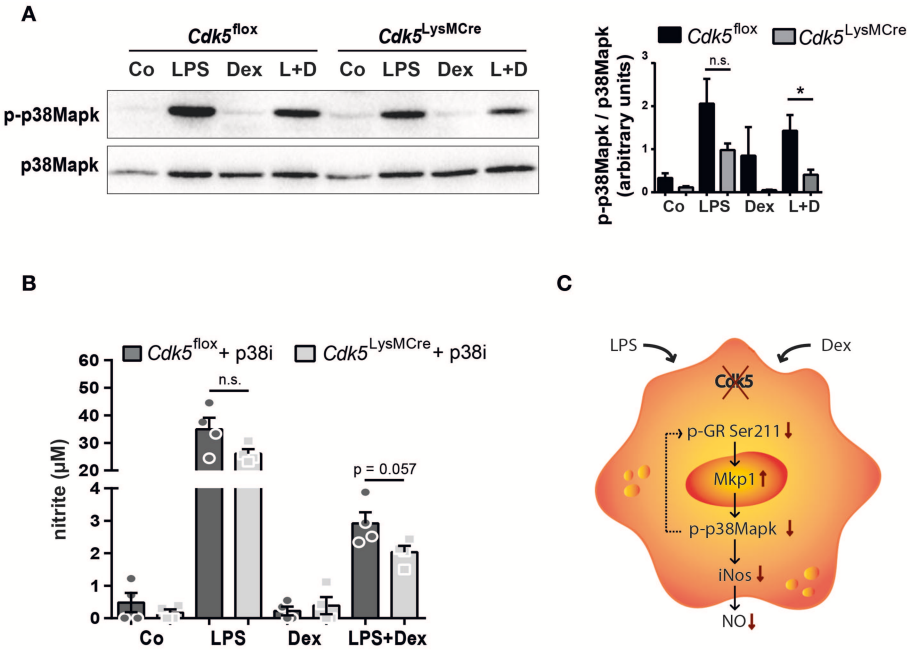

FIGURE 4 | *Cdk5* deletion reduces phospho-p38Mapk leading to decreased NO production. **(A)** BMDMs derived from *Cdk5*^{flox} and *Cdk5*^{LysMCre} mice were stimulated with PBS (Co), LPS (100 ng/ml), Dex (10^{-6} M), or a combination of LPS + Dex (L + D) and phospho-p38Mapk (43 kDa) and p38Mapk (40 kDa) protein were detected by western blot after 4 h and quantified. **(B)** BMDMs were treated as described in **(A)** with 1 h pre-treatment of either DMSO or 5 µM SB203580 (p38Mapk inhibitor = p38i) and nitrite (a stable NO metabolite) was measured in the supernatant after 48 h. **(C)** Scheme showing NO regulation in the absence of *Cdk5* via the GR-Mkp1-p38Mapk axis in macrophages under inflammatory (LPS) conditions and GC (Dex) treatment. Possible reduction of GR (Ser211) phosphorylation by p38Mapk is shown with a dotted arrow. Data shown in **(A)**: $n = 5$–6 and **(B)**: $n = 4$. Results are depicted as mean ± SEM. Statistical analysis was performed by Wilcoxon-Mann-Whitney test (two-tailed) $^*p < 0.05$; n.s. not significant.

inhibition of p38Mapk. Indeed, we observed a trend toward a potentiated repressive Dex effect on NO production after 48 h of p38Mapk inhibition in LPS + Dex treated *Cdk5*^{LysMCre} BMDMs (**Figure 4B**). This suggests that a lack of *Cdk5* enhances the suppressive Dex effect through a reduced GR Ser211 phosphorylation and increased *Mkp1* expression that in turn attenuates p38Mapk activation and thus iNos and NO production in inflammatory macrophages (**Figure 4C**).

DISCUSSION

In this study we showed that roscovitine exert its function, in contrast to the general view, rather independent of Cdk5. Moreover we presented for the first time that roscovitine as well as *Cdk5* deletion potentiated the anti-inflammatory effect of Dex on iNos and NO production in LPS-stimulated macrophages by mainly two independent mechanisms. We further demonstrated that under inflammatory conditions and Dex treatment GR Ser211 phosphorylation is stronger reduced upon *Cdk5* deletion, whereas the GR transcriptional target gene Mkp1 was induced. An increased expression of Mkp1 phosphatase led to an increased dephosphorylation of p38Mapk, which in turn resulted in a decreased iNos and NO production. Collectively, our findings showed for the first time that macrophage specific *Cdk5* deletion in combination with Dex potentiates the anti-inflammatory effect of GCs on iNos.

Roscovitine Enhances the Immunosuppressive Dex Effect on iNos Independent of Cdk5

Roscovitine is a small molecule inhibitor that is currently in phase II clinical trials for cancer treatments like Cushing syndrome and non-small cell lung cancer (Clinical trials NCT03774446, NCT00372073). Furthermore, roscovitine treatment reduced lung inflammation and enhanced the resolution of inflammation during arthritis by enhancing apoptosis of neutrophils and favoring phagocytosis by macrophages (18, 20). However, in macrophages, only limited *in vitro* studies have been performed using cell lines (RAW264.7), showing that high concentrations (20 µM) of roscovitine inhibit cell viability after 24 h of treatment (22). In the current study 10 µM roscovitine (for a duration of 4 h) had no phenotypic effect on proliferation and apoptosis in primary macrophages.

We analyzed the inflammatory response of primary macrophages after roscovitine treatment. In line with Du et al. and Jhou et al. roscovitine treatment led to a significant reduction of *Il-1β*, *Il-6*, and *iNos* mRNA expression as well as Il-6 and iNos protein expression after LPS stimulation (21, 22). Cdk5 is a high affinity roscovitine target, and so has been the

focus of studies investigating inflammatory diseases such as DSS-induced colitis, LPS-induced endotoxic shock and experimental autoimmune encephalomyelitis (34, 35). However, macrophage specific *Cdk5* deletion did not reduce *Il-1β, Il-6, iNos* mRNA, and Il-6 and iNos protein expression after LPS induction, showing that the anti-inflammatory effect of roscovitine is not mediated by Cdk5 inhibition. At high concentrations (10 μM) roscovitine also inhibits other kinases including Cdk1 (IC_{50} = 0.65 μM), Cdk2 (IC_{50} = 0.7 μM), Cdk7 (IC_{50} = 0.46 μM), Cdk9 (IC_{50} = 0.6 μM), and Erk (IC_{50} = 14–34 μM). Therefore, it is likely that the observed effect is mediated by the inhibition of several Cdks (15, 16). A previous study demonstrated that the anti-inflammatory effect of roscovitine on *Il-1β*, *Il-6*, *TNFα*, and iNos was observed only at high concentrations, between 10 and 25 μM, but not at 1 μM in RAW264.7 macrophages (21). This suggests that the inhibition of Erk, a kinase known to be involved in the regulation of cytokine expression (46, 47), could mediate this effect. In addition, inhibition of Cdk7 by a specific inhibitor (BS-181) and siRNA-Cdk7 knockdown has already been demonstrated to reduce *IL-1β, IL-6, IL-8* transcript levels, and IL-1β/IL-6 secretion in LPS-induced MH7A cells (48), suggesting that the inhibition of Cdk7 may also be involved.

Since GCs are one of the most potent immunosuppressants and roscovitine was shown to be a potent anti-inflammatory drug, we further investigated whether a combination of roscovitine and Dex enhances the immunosuppressive effects on inflammatory mediator production in macrophages. Indeed, roscovitine in combination with Dex lead to stronger reduction of *Il-6, Il-1β* and *iNos* mRNA, and Il-6 and iNos protein expression after LPS induction. This finding suggests that a combinatorial treatment of roscovitine and Dex may be most beneficial for the treatment of inflammatory diseases. Furthermore, we demonstrate that the effect on inflammatory cytokines (*Il-6, Il-1β*) is additive and Cdk5 independent, suggesting that the immunosuppressive effect is mediated by independent pathways. Interestingly, for the iNos suppression roscovitine increased the anti-inflammatory potential of Dex after LPS induction. In addition, we showed that *Cdk5* deletion also led to a stronger reduction of iNos expression and NO production after LPS + Dex treatment. We still observed an albeit reduced roscovitine effect on *iNos* expression in *Cdk5* deficient LPS + Dex treated macrophages, suggesting an inhibition of additional kinases mediating the roscovitine effect. This suggests that roscovitine enhances the anti-inflammatory Dex effect on iNos mainly by Cdk5- independent mechanisms, which is in contrast to the general view where Cdk5 was shown to be a high-affinity target of roscovitine. Taken together, our results showed that the loss of Cdk5 potentiates the anti-inflammatory Dex effect on iNos and NO generation in inflammatory macrophages, a finding that has been not described so far.

Specific *Cdk5* Deletion Has no Effect on Anti-inflammatory Markers in Macrophages

We also found, that *Cdk5* deletion had no major impact on the polarization of alternative (M2-like) macrophages as shown for example for *Il-10* expression after 24 h of stimulation. Seok et al. examined in detail the knockdown and knockout of *p35* (the *Cdk5* activator) and knockdown of *Cdk5* in LPS stimulated macrophages and showed that this enhances *Il-10* mRNA and Il-10 protein expression after 24 h (35). Moreover, we did not observe differences in the Dex-mediated induction of the M2 markers (*Cd163, Cd206*, *Ym1, Il-10*) upon Cdk5 deletion, except for *Mertk*, a phagocytosis marker, that was upregulated by Dex in the absence of *Cdk5*. Expression of other TAM receptors, such as *Tyro3*, *Axl*, and other phagocytosis receptors (*Cd36, Anxa1*) (49, 50) were not affected after *Cdk5* deletion in macrophages. Whether the deletion of *Cdk5* in macrophages increases the Dex-induced phagocytic capacity due to *Mertk* upregulation remains to be elucidated.

Cdk5 Regulates the Dex Effect on NO Production Through GR Phosphorylation, Mkp1, and p38Mapk During Inflammation

It is known that LPS increased iNos through p38Mapk in macrophages (44) and Dex reduced iNos expression and NO through destabilization of mRNA and increased iNos protein degradation by calpain (51–55). We therefore investigated the mechanism by which *Cdk5* deletion enhances the anti-inflammatory potential of Dex and suppresses iNos and NO production in pro-inflammatory macrophages.

The phosphorylation of GR at Ser211 was reduced in macrophages upon *Cdk5* deletion. *In vitro* kinase assays showed that Cdk5 phosphorylates the human GR at multiple serine residues (Ser203, Ser211, and Ser226) (41). In addition, Cdk5 phosphorylates GR at Ser211 and Ser203 in HCT116, Cos7, and rat cortical neuronal cells (41). This is in line with our observation showing reduced GR phosphorylation (Ser211) in macrophages lacking *Cdk5*. More recent *in vivo* studies suggested that Cdk5 is a crucial component of GR-dependent stress response in the brain by regulating GR phosphorylation (36, 56). To our knowledge, our data is showing for the first time that Cdk5-mediated GR phosphorylation (Ser211) is not restricted to the nervous system, but might also play an important role in innate immune cells like macrophages. Furthermore, the Cdk5-GR interaction seems to reduce GR activity especially for the target gene Mkp1.

Kino et al. reported an enhancement of mRNA expression for protein phosphatase 1 regulatory subunit 10 (*Ppp1r10*), the neuropeptide Y receptor (*Npy1r*), and serum and glucocorticoid-induced kinase (*Sgk*) in rat cortical neuronal cells, regardless of reduced GR phosphorylation upon Cdk5 inhibition (41). This is consistent with our results, which showed an induced Mkp1 expression but reduced GR Ser211 phosphorylation in the absence of *Cdk5* during Dex and LPS + Dex stimulation. Cdk5 was reported to contribute to an impaired GC-induced recruitment of the coactivators p300/CBP and SNF2 to the GRE-containing MMTV and endogenous Sgk promotors resulting in a reduced transcriptional activity (41). In addition, p300 was shown to act as an activator for Mkp1 expression (57). However, if coactivator recruitment is increased upon *Cdk5* deletion in macrophages remains to be addressed.

Mkp1 was shown to control Erk activation (58) and increased Mkp1 protein levels correlate with reduced Erk phosphorylation after 16 h of Dex treatment in mast cells (58). However, we did not observe genotype differences in Erk activation, but we did observe increased Mkp1 expression upon *Cdk5* deletion in macrophages after 4 h Dex treatment.

Mkp1 has also been reported to dephosphorylate p38Mapk and therefore contribute to the reduction of pro-inflammatory mediators (6, 58–63). In macrophage cell lines Mkp1 has already been described to negatively regulate iNos and NO production by inhibiting p38Mapk activity (45). Since we observed an upregulation of Mkp1 upon *Cdk5* deletion this could explain the lower levels of phosphorylated p38Mapk observed in LPS + Dex treated $Cdk5^{LysMCre}$ macrophages. In line with this, $Cdk5^{LysMCre}$ macrophages showed a stronger reduction in iNos and NO production during LPS + Dex treatment compared to $Cdk5^{flox}$ macrophages. Furthermore, inhibition of p38Mapk confirmed a trend toward a potentiated repressive Dex effect on NO production after 48 h LPS + Dex treated $Cdk5^{LysMCre}$ BMDMs. Thus, we conclude that upon *Cdk5* deletion the induction of Mkp1 leads to a stronger dephosphorylation of p38Mapk resulting in the reduced iNos and NO production in inflammatory macrophages. Our findings are further supported by the fact, that $Mkp1^{-/-}$ mice are more sensitive to models of inflammatory diseases, such as sepsis and endotoxemia (6, 64), furthermore higher iNos expression was observed in the liver of $Mkp1^{-/-}$ mice during sepsis (65). It should be mentioned, that roscovitine treatment did not reduce p38Mapk phosphorylation in wildtype macrophages during LPS treatment regardless of inflammatory cytokine inhibition. This is in line with previous work by Du et al. which showed enhanced p38Mapk phosphorylation upon roscovitine and LPS stimulation but reduced cytokine expression (21). Beyond this, p38Mapk was shown to phosphorylate GR at Ser211 (42, 51, 52, 66), therefore the reduced levels of phosphorylated p38Mapk upon Cdk5 deletion could be involved in the reduction of GR phosphorylation. Whether Cdk5 also influences metabolic GR target genes and therefore reduce or enhance severe side effects has not been investigated.

Here we report that roscovitine, a Cdk inhibitor, is a potent anti-inflammatory drug and combinatorial treatment with Dex leads to an additive suppression of pro-inflammatory mediator expression such as *IL-1β* and *Il-6*. However, we have shown, that roscovitine synergistically with Dex suppresses iNos induction in inflammatory macrophages. Furthermore, we have demonstrated by generating Cdk5 conditional knockout mice that *Cdk5* deletion is sufficient to enhance the anti-inflammatory effect of Dex on iNos. Since roscovitine also exerts its immunosupressive effect in *Cdk5* deficient macrophages albeit to a lesser degree, the effects of roscovitine inhibition is mainly mediated by the inhibition of other Cdks than Cdk5. Macrophage specific *Cdk5* deletion reduced Dex-dependent GR Ser211 phosphorylation, but induced Mkp1 expression and reduced p38Mapk phosphorylation hence resulting in a decrease of iNos and NO production. Here we have shown a novel mechanism of Cdk5 involved in the anti-inflammatory effects of GCs. In summary, this study supports the use of combinatorial treatment of inflammatory diseases with specific Cdk5 inhibitor(s) and GCs to potentiate the anti-inflammatory effect on iNos and NO. Furthermore, combinatorial treatment may be a possible therapeutic objective to lower GC doses and therefore avoid negative side effects.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Tierschutzgesetz and Tierschutz-Versuchstierordnung (Mitteilung nach § 4), Regierungspräsidium Tübingen, Baden-Wurrtemberg. The protocol was approved by the Regierungspräsidium Tübingen, Baden-Wurrtemberg.

AUTHOR CONTRIBUTIONS

PP, MF, UB, LL, and SV performed experiments. MF revised the manuscript. PP and SV designed the study, performed experiments, processed the data, and wrote the 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.2019.01554/full#supplementary-material

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REVIEW
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GR Dimerization and the Impact of GR Dimerization on GR Protein Stability and Half-Life

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Pharmacologically, glucocorticoids, which mediate their effects via the glucocorticoid receptor (GR), are a most effective therapy for inflammatory diseases despite the fact that chronic use causes side-effects and acquired GC resistance. The design of drugs with fewer side-effects and less potential for the development of resistance is therefore considered crucial for improved therapy. Dimerization of the GR is an integral step in glucocorticoid signaling and has been identified as a possible molecular site to target for drug development of anti-inflammatory drugs with an improved therapeutic index. Most of the current understanding regarding the role of GR dimerization in GC signaling derives for dimerization deficient mutants, although the role of ligands biased toward monomerization has also been described. Even though designing for loss of dimerization has mostly been applied for reduction of side-effect profile, designing for loss of dimerization may also be a fruitful strategy for the development of GC drugs with less potential to develop GC resistance. GC-induced resistance affects up to 30% of users and is due to a reduction in the GR functional pool. Several molecular mechanisms of GC-mediated reductions in GR pool have been described, one of which is the autologous down-regulation of GR density by the ubiquitin-proteasome-system (UPS). Loss of GR dimerization prevents autologous down-regulation of the receptor through modulation of interactions with components of the UPS and post-translational modifications (PTMs), such as phosphorylation, which prime the GR for degradation. Rational design of conformationally biased ligands that select for a monomeric GR conformation, which increases GC sensitivity through improving GR protein stability and increasing half-life, may be a productive avenue to explore. However, potential drawbacks to this approach should be considered as well as the advantages and disadvantages in chronic vs. acute treatment regimes.

Keywords: glucocorticoid receptor dimerization, acquired glucocorticoid resistance, Compound A, GR^{dim} mutant, GR^{mon} mutant, ubiquitin proteasomal system, biased ligands, half-life

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INTRODUCTION

Pharmacologically, glucocorticoids are a cost-effective effective therapy for inflammatory and autoimmune diseases and are widely prescribed (1–3). Despite the effectiveness of glucocorticoids in treating inflammation chronic use causes side-effects (4) and acquired glucocorticoid resistance (5, 6). The design of drugs with fewer side-effects and less potential for the development of resistance is therefore considered crucial for improved therapy (7).

Glucocorticoids mediate their effects via the glucocorticoid receptor (GR) a ligand activated transcription factor. The GR has a domain structure that consists of an N-terminal domain (NTD), a DNA-binding domain (DBD) separated from the ligand binding domain (LBD) by a hinge region (**Figure 1A**) (10). The DBD contains two zinc fingers both of which are involved in DNA-binding, while the second zinc finger is also involved in dimerization. Binding of ligand to the LBD induces the cytoplasmic GR to dimerize and translocate to the nucleus where it can enhance transcription by binding cooperatively as a homodimer to glucocorticoid response elements (GREs), a consensus DNA sequence consisting of two hexameric half-sites separated by a 3-bp spacer. The monomeric GR can also repress transcription by binding directly to negative glucocorticoid response elements (nGREs) or GRE half-sites or by tethering to DNA-bound transcription factors such as NFκB or AP-1 (11–15).

The ability of the GR monomer to repress pro-inflammatory genes activated by NFκB or AP-1, while activating genes that result in the metabolic side-effects of glucocorticoids via the dimer binding to GREs suggested that separation of the transrepression and transactivation functions of the GR could give rise to safer drugs and resulted in the development of selective GR agonists (SEGRAs) or modulators (SEGRMs), collectively referred to as SEGRAMS (16–21). Despite the fact that the usefulness of this paradigm has been challenged as being outdated and oversimplifying the complexity of GR-signaling by negating the role of GR dimers in curbing inflammation and the role of GR monomers in eliciting side-effects (19, 22), it may still hold promise for drugs tailored to specific diseases phenotypes (18, 23, 24).

Although dimerization of the GR is an integral step in glucocorticoid signaling and fundamental to the concept of SEGRAMs it has only relatively recently been explicitly identified as a possible molecular site to target for drug development of anti-inflammatory drugs with an improved therapeutic index (23). In this review we thus discuss the identification of the GR dimerization interfaces, the use of GR dimerization mutants and conformationally biased ligands to further our understanding of the role of GR dimerization in GC signaling and the implications of loss of GR dimerization for reduction of side-effects, while highlighting the recent finding that loss of dimerization may also be a fruitful strategy for the development of drugs with less potential to develop glucocorticoid resistance.

GR DIMERIZATION

Although the ability of GR to form dimers in solution has been debated (8, 25–31) several studies have shown that the GR, liganded or unliganded, can dimerize in solution (32–36) and that dimerization may already be present in the cytoplasm (35, 37–39).

X-Ray Crystallography of GR Domains Identifies Amino Acids Involved in Dimerization

Two interfaces in the GR have been identified that mediate receptor dimerization, the DBD and the LBD dimerization interfaces. Although no crystal structure of the full-length GR has been reported to date, separate crystal structures of the DBD and LBD have been reported, which identified specific amino acids involved in the dimerization interfaces and for the orientation of binding to DNA.

The first crystal structure of the rat GR DBD (amino acid residues 440–525) complexed to a canonical GR-binding element (GRE) identified a dimerization interface (**Figure 1A**) in the second zinc finger of the GR consisting of 7 amino acids (rat residues L475, A477, R479, D481, I483, I487, N491, which corresponds to the human residues L456, A458, R460, D481, I483, I487, N491) with three of the inter-subunit contacts in a region referred to as the D-box (C476–C482) (8). The two molecules of the DBD bind cooperatively to one face of the DNA (**Figure 1B**) when the two hexameric sites are separated by a 3-base pair spacer in a head-to-head fashion so that their dimerization loops (D-box) are aligned and contacting each other (8, 25). Furthermore, crystal structures of the DBD bound to different GREs were virtually super-imposable except for the lever arm, a loop region in the DBD between the DNA recognition helix (first zinc finger) and the dimerization loop, where different GREs dictate discrete alternate conformations (40). In addition, human residue H472 in the lever arm adopts one of two conformations: packed in the first monomer, which binds to the initial conserved half-site, and flipped in the second monomer, which binds to the second variable site in the GRE.

In contrast to the head-to-head binding of the DBD to GREs, crystal structures indicate that at a nGRE (**Figure 1B**), in the *TSLP* gene, which is like the canonical IR-GBS sequence: CTCC(n)$_{0-2}$GGAGA (41), GR binds as two monomers orientated tail-to-tail in an everted repeat orientation on opposite sides of the DNA (42). This prevents DNA-mediated dimerization as the D-loops are directed away from each other and results in binding that is characterized by strong negative cooperativity, where binding of the first GR monomer to the high affinity site hampers binding of the second monomer to the low affinity site. The two-site binding event (**Table 1**) characterized by two non-identical, monomeric binding events has a lower binding affinity (363 nM and 63 μM) than positive cooperative binding to a GRE site (73 nM) (42). This suggests that the nGRE sequence not only preferentially binds GR monomers but that it contributes to a repressive conformation, which may involve a distinct lever arm conformation where H472 (rat residue) is flipped in both monomers (42). Crystal structures of GR DBD bound to AP-1 response elements (TREs: TGA(G/C)TC) (46) (**Figure 1B**) suggest a similar binding orientation and comparable binding affinities (**Table 1**). In contrast, crystal structures of GR DBD bound to NF-κB response (κBRE) elements (45) (**Figure 1B**) indicate that binding is head-to-head as for binding to the GREs but resembles those of the nGRE in that it presents with a two site-binding curve which, like for the nGRE (44), is abolished by the S425G human mutant. Although only one monomer binds to the conserved AATTY sequence (Y represents a pyrimidine base), it binds as a "D-loop" engaged dimer with high and low binding affinities in the same range as binding of the DBD to nGREs (**Table 1**). Collectively, the negative cooperativity of DNA binding as well as results with GR dimerization deficient mutants suggest that monomeric GR

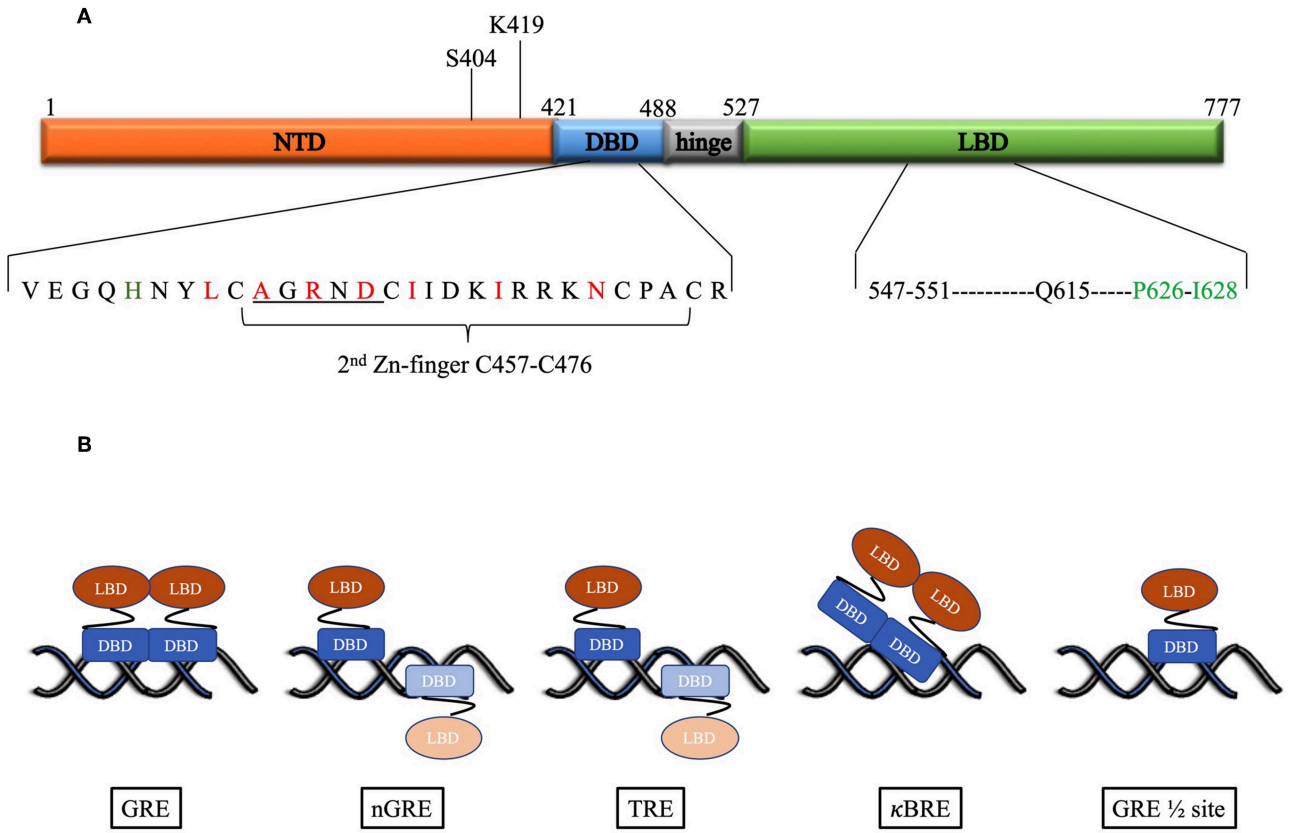

FIGURE 1 | (A) Domain structure of the human GR. Above the figure is indicated the position of the post-translational modifications required for proteasomal degradation. Below the figure the DBD and LBD residues involved in the dimer interface are expanded. For the DBD, the underlined residues indicate the D-box, while red residues are those identified as important for the dimerization interface by Luisi et al. (8). In addition, in green is H472 in the lever arm that adopts one of two conformations: packed or flipped depending on whether binding to GREs or nGREs occur. For the LBD black residues are those involved in hydrogen bonds, while the green residues form hydrophobic interactions to stabilize the dimer interface as identified by Bledsoe et al. (9). **(B)** DNA-binding motifs determine orientation and GR monomer vs. dimer binding. Faded monomer indicates binding to low affinity site.

is likely sufficient at repressive GR binding elements (nGRE, TRE, and κBRE) *in vivo*. Occupancy of GR monomers at GRE half-sites has also been confirmed *in vivo* (14).

Comparison of initial structural studies of the free GR DBD solved by NMR (48–51) with that of the crystal structure of DNA bound GR DBD (8) suggested that the largest difference occurred in the D-box and led to the assumption that DNA binding was required for dimerization. However, comparison of a recent crystal structure of the free human GR DBD (residues 418–517) (52) with that of previously determined crystal structures of the GR DBD bound to a GRE or a nGRE reveal a very similar core structure with a similar D-loop conformation and indicates that the largest difference is located in the lever arm. Molecular dynamic simulations of the lever arm suggest that it is most mobile in the free state sampling the most diverse number conformations, while in the nGRE-bound state an intermediate number of conformations are present, which is further reduced in the GRE-bound state. Thus, binding to DNA constrains the number of conformations that the lever arm can sample, which is further reduced upon dimerization, however, the D-loop is accessible in solution for dimerization via the DBD.

The crystal structure of the GR LBD lagged behind because of solubility problems, however introduction of a single mutation (human residue F602S) significantly improved solubility without affecting function and allowed for crystallization of the LBD (human residues 521–777) in the presence of ligand dexamethasone (DEX) and TIF2, a coactivator peptide (9). This led to the identification of a dimerization interface (**Figure 1A**) stabilized by hydrophobic interactions, specifically reciprocal interactions between P625 and I628 in the H5–H6 loop, and hydrogen bonds, from particularly residues between 547 and 551 (extended strand between helices 1 and 3) and Q615 (last residue in helix 5) from each LBD, that allows formation of four hydrogen bonds (9). Subsequent GR LBD crystal structures (53–58) in the presence of agonist or antagonist, focused mainly on the ligand-binding pocket rather than on the dimerization interface and generally conform to the crystal structure of the Bledsoe group (9), besides identifying differences in the ligand-binding

TABLE 1 | DNA-binding affinity (K_d^a) of domains and full-length wild-type and GR^{dim} dimerization deficient mutant (Hill-slope added in brackets).

		GR^{wt}	DBD mutant: $^bGR^{dim}$
DBD	GRE	• 73 nM (42) • 1.6 – 5.7 nM (1.8 – 2.1) (43) • 80–890 nM (40) • 73 nM (44) • 5.7 nM (25) • 7.14 – 25.7 nM (37)	• 370 nM (42) • 16 – 28 nM (1.3 – 1.4) (43)
	nGRE	• 360 nM and 63 μM (42) • 363 nM and 63.2 μM (44)	• 1.1 μM (42)
	κBRE	• 215 – 239 nM and 17 – >50 μM (45)	
	TRE	• 12 – 402 nM and 1 – 12 μM (46)	
Full-length	GRE	• 50 nM (36) • 0.5 nM (25) • 1.2 – 2.56 nM (37) • 34 nM (46) • 35 nM (45) • 32 – 490 nM (47) • 140 nM (2.5) (30)	• 300 nM (36)
	κBRE	• 51 nM (45)	
	TRE	• 42 nM (46)	
	GRE ½ sites	• 1,210 nM (36) • 185 nM (1.08) (14)	• 1,260 nM (36)

[a] (K_{app}), determined using the Langmuir binding model, is given as only some investigators (30, 47) determined K_{tot}, the total affinity for assembling two GR monomers at the palindromic GRE.
[b] GR^{dim} = human GR^{A458T}, mouse GR^{A465T}, and rat GR^{A477T}.

pocket and helix 12. Recently Bianchetti et al. (59) evaluated the physiological relevance of the GR LBD dimerization interface by analyzing 20 published GR LBD crystal structures using estimates of dimer stability (surface area in $Å^2$ buried upon dimerization and estimated free energy variation (Δ^iG) upon formation of the interface) coupled to evolutionary sequence conservation analysis of the interface. One GRα LBD homodimer structure, the apH9 dimer, consistently stood out as being more stable, by having the largest contact surface area (850$Å^2$) and the lowest binding free energy variation upon formation of the interface (Δ^iG: −42.9 kcal/mol), and as having highly (82%) conserved residues at the interface (27 of the 33 residues that contributed to binding were conserved), however, this structure was formed by only one of the crystal structures investigated (PDB ID:4P6W) (53). In contrast, the other dimerization structures observed in GR LBD crystals were less stable and not significantly conserved, with the bat-like structure for the GR LBD, suggested by Bledsoe et al. (9), which was observed in 6 PDB entries (28%) (9, 53–55, 57, 58), being amongst the least stable (surface area buried is 288$Å^2$ and Δ^iG: −20 kcal/ mol) and conserved (7/16 = 44%), while the most frequent H1 structure, observed in 9 entries (43%) (9, 53–58), had a slightly higher stability (332$Å^2$ and Δ^iG −30 kcal/ mol) and lower number of conserved residues (2/5) (59). In summary, this suggests that the GR LBD dimers are generally weaker and less conserved than the nuclear receptor LBD dimer through H9-H10-H11 (also called the butter-fly like structure with 1494$Å^2$ and Δ^iG: −77.5 kcal/ mol and 73% of conserved residues at the interface), which is found in the ER LBD, a sentiment supported by Billas and Moras (60). Despite the fact that the bat-like dimer structure was found to be physiologically the least stable by Bianchetti et al., of the residues suggested to be important for stabilization of the dimer interface, three residues involved in the hGR hydrophobic interface core (Y545 in H1-loop-H3, P625 in S1-turn-S2 and I628 in S2) and one (Gln 630 in H5) identified as part of the hydrogen-bond network, were previously identified by Bledsoe et al. (9). Interestingly, the surface area buried originally reported for the bat-like structure (1623$Å^2$) by Bledsoe et al. (9) is much higher than that reported by Bianchetti et al. (59) (288$Å^2$) for this structure.

GR Dimerization Mutants Confirm Role of GR Dimerization Interfaces

Genetic strategies have also been used to verify the GR interfaces involved in dimerization and the relevance of specific amino acids identified from crystal structures. Although, these GR dimerization deficient mutants have been studied extensively for their role in the regulation of gene expression (12, 61–63), here mainly effects on dimerization will be discussed.

Mutants That Target the DBD

Most of the GR dimerization mutation studies focused of the DBD dimerization interface (64), specifically the three amino acids in the D-loop (**Figure 1A**), with the GR^{dim} mutant (human GR^{A458T}, mouse GR^{A465T}, and rat GR^{A477T}) the most widely characterized and extensively studied (64–66). A backbone hydrogen bond is formed between the carbonyl of A777 and the amide of I483 on the associated dimer partner (8) and mutation of the Ala to Thr has been shown disrupt this interaction (43, 65, 66).

Effects on dimerization

There has been much controversy surrounding the dimerization potential of the GR^{dim} mutant with several publications suggesting that dimerization equal to that of GR^{wt} occurs. Most of the studies showing similar dimerization as the GR^{wt} were semiquantitative: co-immunoprecipitation (62) and Numbers & Brightness (N&B) assay (31).

However, quantitative studies at the single-cell level, using fluorescence correlation spectroscopy (FCS) combined with a microwell system, have shown that GR^{dim} has a dissociation constant (K_d) of dimerization (**Table 2**) in the presence of DEX that is only slightly lower than that of the GR^{wt} in the absence of ligand [370 nM for $GR^{dim(+DEX)}$ vs. 410 nM $GR^{wt(-DEX)}$ *in vitro* (36) and 6.11 μM for $GR^{dim(+DEX)}$ vs. 7.4 μM for $GR^{wt(-DEX)}$

TABLE 2 | Dimerization dissociation constants (K_d) of domains and full-length wild-type and select mutant GRs ([a]Method used and DEX concentration in brackets).

	GR^{wt}	DBD mutant: [b]GR^{dim}	LBD mutant: [c]GR^{I628A}
DBD	• 13 – 21 nM (EMSA) (37)		
LBD	**Liganded:** • 1.5 μM (AU; 10 μM) (9)		**Liganded:** • 15 μM (AU; 10 μM) (9)
Full-length	**Unliganded:** • 410 nM (FCS) (36) • 3.9 nM (EMSA) (37) • 100 μM (AU) (30) • 416 nM (FCS) (35) • 7.4 μM (FCS*) (35)	**Unliganded:** • 390 nM (FCS) (36)	
	Liganded: • 140 nM (FCS; 500 nM) (36) • 139 nM (FCS; 100 nM) (35) • 3 μM (FCS*; 100 nM) (35) • 107 nM (FCS; 500 nM) (67)	**Liganded:** • 370 nM (FCS; 500 nM) (36) • 379 nM (FCS; 100 nM) (35) • 6.11 μM (FCS*; 100 nM) (35)	

[a]*Methods to determine dimerization:*
- *EMSA, electrophoretic mobility shift assay*
- *AU, analytic ultracentrifugation*
- *FCS, fluorescence correlation spectroscopy (only method also done in intact live cells and indicated as FCS*).*

[b]GR^{dim} = *human* GR^{A458T}, *mouse* GR^{A465T}, *and rat* GR^{A477T}.
[c]*human* GR^{I628A}, *mouse* GR^{I634A}, *and rat* GR^{I646A}.

in vivo (35)], but significantly higher than that of GR^{wt} in the presence of DEX [370 nM for $GR^{dim(+DEX)}$ vs. 140 nM $GR^{wt(+DEX)}$ *in vitro* (36) and 6.11 μM for $GR^{dim(+DEX)}$ vs. 3 μM for $GR^{wt(+DEX)}$ *in vivo* (35)]. This indicates that the dimerization potential of the mutant GR^{dim} is substantially lower than that of the GR^{wt} in the presence of DEX and closer to the dimerization potential of GR^{wt} in the absence of ligand. Although it is evident that the GR^{dim} can form dimers, it is also clear that the monomer-dimer equilibrium of the mutant is shifted in the direction of monomers and it is clearly deficient in dimerization potential when compared to GR^{wt}.

The dimerization equilibrium may also be influenced by receptor concentration. At low concentrations of GR (335 fmol/mg protein or 26200 GR/cell) the extent of DEX-induced dimerization of GR^{dim} (37%) is much less than that of the GR^{wt} (100%), but similar to that of uninduced GR^{wt} (43%), while at about a 4-fold higher receptor concentration (1,420 fmol/mg protein or 111,000 GR/cell), the extent of DEX-induced dimerization of GR^{dim} (90%) approaches that of the induced GR^{wt} (100%) and uninduced GR^{wt} (102%) (38).

Effects on DNA binding

Binding to diverse GR binding motifs could also support dimer vs. monomer GR conformations especially if the Hill-slope[1] is reported as a measure of cooperativity (**Table 1**). Positive cooperative DNA-binding requires binding of a GR dimer, where binding of the first monomer facilitates binding of the second monomer, and exhibits an increased binding affinity with a Hill-slope larger than 1. Although it was initially reported that the GR^{dim} could not bind to DNA (65, 66) it is now clear that maximal DNA-binding of the GR^{dim} mutant, both as DBD and as full-length receptor, to a GRE is not affected (43). However, the mutant binds with a lower affinity (**Table 1**) (36, 42, 43). Furthermore, the A477T mutant dissociates faster that the wild type receptor (5–12x faster *in vitro* for DBD with a dissociation half-life (t½) of 23–55 s for GR^{wt} vs. 4.7–4.8 s for the GR^{dim} (43) and 10x faster *in vivo* for the full-length receptor with a residence time for GR^{wt} that is 1.45 s vs. 0.15 s for GR^{dim} (68) due to a reduction, but not abrogation, in positive cooperative DNA binding (Hill-slope for GR^{wt} 1.8–2.1 and for GR^{dim} 1.3–1.4) (43). Interestingly, in addition to GR^{dim}, other salt bridge mutations (rat GR^{R479D} or GR^{D481R}) disrupting the DBD dimer interface also result in lower binding to a single GRE but higher binding to paired GREs and thus enhanced transcriptional synergy at reiterated GREs (69–71).

Comparison of binding affinities of the GR^{wt} to that of GR^{dim} to other GR DNA-binding motifs (**Table 1**) is also informative in terms of probing a more monomeric binding configuration for GR^{dim}. Thus, although GR^{dim} substantially decreases the overall affinity of the DBD for a GRE, for a nGRE, it binds with a similar affinity as the GR^{wt} binding to a nGRE (42). Furthermore, the full-length receptor GR^{dim} mutant binds to a GRE half-site with an equivalent affinity as that of the GR^{wt} (36). Additionally, ChIP-exo in liver and in primary bone marrow–derived macrophages (15) or human U2OS osteosarcoma cell lines (14, 72) indicates that GR^{wt}, but not GR^{dim}, binds to GRE sequences as a dimer, while both receptors bind to tethered and half-site motifs as monomers.

Mutants That Target the LBD

There is a paucity of GR dimerization mutation studies focusing on the LBD dimerization interface, most probably as this dimerization interface was characterized (9) almost 10-years later than that of the DBD interface (8). Although the dimerization affinity of the liganded human GR LBD (1.5 μM) is already low in comparison to that of the DBD or the full-length receptor (**Table 2**), it was reduced 10-fold by the LBD mutant, hGR^{I628A}, which displays a phenotype very similar to that of the GR^{dim} mutant (9). However, in contrast, using the N&B assay it was shown that the mouse GR^{I634A} mutant displayed reduced dimerization relative to GR^{wt} and GR^{dim} at equivalent DEX concentrations, suggesting that the LBD plays a potentially larger role than the DBD in GR dimerization (31). Furthermore, a combination mutant involving both the DBD and LBD domains ($mGR^{A465T/I634A}$ called GR^{mon}) has recently been described and comparison of the dimerization potential with that of liganded GR^{wt} and single mutants using N&B assays indicate that the order of DEX dimerization efficiency is $GR^{wt} = GR^{dim} > GR^{I634A} > GR^{mon}$, however, at higher DEX concentration (1 μM) significant dimerization of the GR^{mon} is still seen (31).

[1]If the Hill slope is = 1, binding is additive, if >1, binding displays positive cooperativity, while if >1, binding displays negative cooperativity.

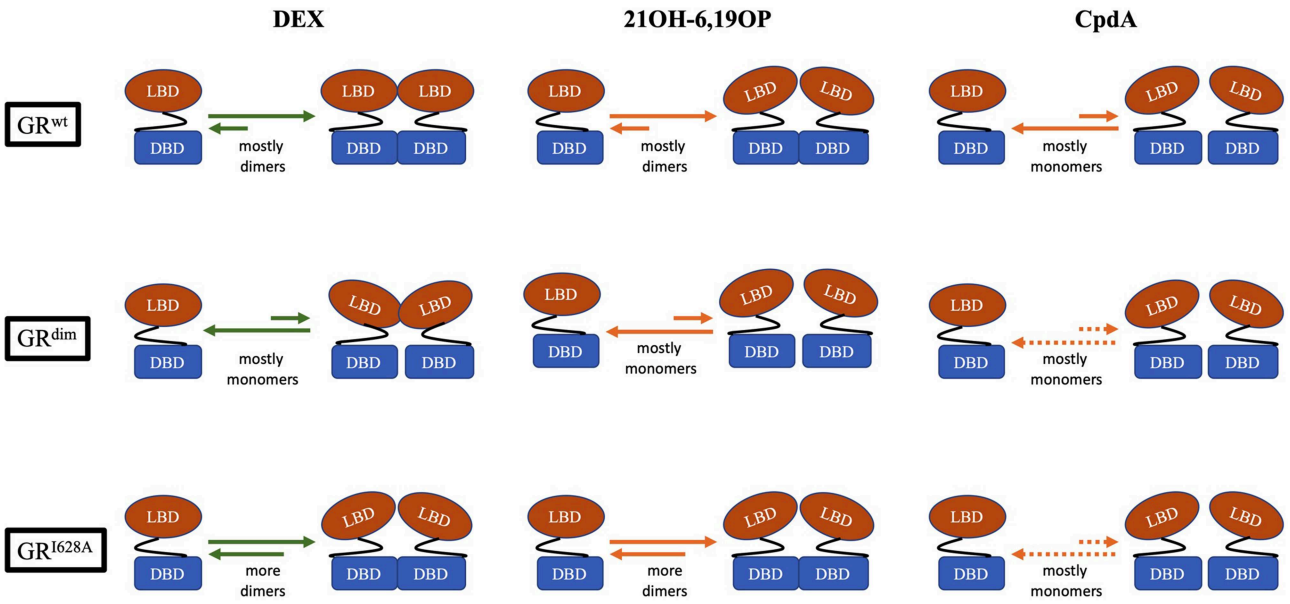

FIGURE 2 | Schematic representation of the monomer-dimer equilibrium for GR^{wt}, the DBD-dimerization deficient mutant, GR^{dim}, and the LBD-dimerization deficient mutant, GR^{I628A}, bound to either, DEX, 21OH-6,19OP, or CpdA. In the equilibrium, green arrows represents quantitative data, while orange arrows represents semiquantitative or qualitative data (see **Table 2**). Dotted orange arrows represents hypothesized equilibria not yet determined.

Small Molecules Displaying Loss of GR Dimerization (Conformationally Biased Ligands)

Despite the fact that one would assume that the search for SEGRAMs would have yielded several small molecule ligands that perturb the GR monomer-dimer equilibrium as the concept is underpinned by the idea that targeting for loss of GR dimerization would reduce the side-effect profile (23), it appears that the guiding principle in this search has rather been to assay for a preference to induce transrepression rather than transactivation and that very few SEGRAMs have been evaluated for their effects on GR dimerization (18, 73–77). Two conformationally biased ligands that perturb the GR monomer-dimer equilibrium have, however, been identified: CpdA (Compound A: 2-(4acetoxyphenyl)-2- chloro-*N*-methylethylammonium chloride), an analog of a naturally occurring compound found in the Namibian shrub *Salsola tuberculatiformis* Botsch (78), and 21-hydroxy-6,19-epoxyprogesterone (21OH-6,19OP), a progesterone derivative (79, 80).

CpdA not only prevents dimerization of the full-length GR^{wt} receptor *in vitro* and *in vivo* (**Figure 2**), but abrogates basal (uninduced) GR dimerization (31, 38, 81, 82). In contrast, 21OH-6,19OP does not prevent dimerization of the full-length GR or the LBD dimerization mutant, GR^{I634A} (**Figure 2**), but does prevent dimerization of the DBD GR^{dim} mutant, suggesting that it prevents dimerization via the LBD (31), which is supported by molecular dynamics simulations that suggests this ligand triggers a conformational change in the H1–H3 loop dimerization interface that differs substantially from that induced by DEX (83).

Despite the fact that it is clear that the GR monomer-dimer equilibrium may be modulated by changes in receptor and ligand concentrations (31, 38), by dimerization deficient mutants (31, 66) and by conformationally biased ligands (80, 81), there is still a controversy regarding the relative contributions of the DBD (60, 84) and LBD (31) to dimerization of the full-length receptor and whether other regions, such as the hinge region (39) and the N-terminal-domain (37), play a substantial role in dimerization. In addition, it seems unlikely that a single point mutation in either the DBD or the LBD would fully abrogate the ability of the GR to dimerize. Quantitative analysis in live cells (29) comparing the dimerization affinity of different GR dimerization mutants, such as done for GR^{dim} (35, 36), could, however, help to resolve the relative contributions of point mutations to the dimerization potential of the GR. Dimerization assays in intact live cells clearly deliver dimerization affinity constants that differ significantly from those obtained in cell lysates as seen in the study of Tiwari et al. (35), where for example, the K_d of dimerization of the liganded GR^{wt} is significantly lower *in vitro* (139 nM) than *in vivo* (3 μM) (**Table 2**). The most parsimonious explanation for this phenomenon entails that an increase in free GR monomer concentration or a decrease in free dimer concentration occurs *in vivo* after ligand-binding, which would be sufficient to favor a higher K_d[2]. In support of this, it has recently been suggested that in mouse livers the GR binds predominantly as a monomer under physiological conditions but that after addition of exogenous glucocorticoid there is a ligand-dependent redistribution of GR from monomer to dimer

[2]($K_d = \frac{[GR\ monomer]x[GR\ monomer]}{[GR\ dimer]}$).

at GR binding sites (15), thus effectively decreasing free dimer and increasing free monomer concentrations in the nucleus. Furthermore, the implications of higher order GR tetramers bound to DNA, that are produced from GR dimers preformed in the nucleoplasm, recently described (29, 85), in terms of the GR monomer-dimer equilibrium still remains to be elucidated as do the individual amino acids involved in this interaction.

IMPACT OF GR DIMERIZATION ON THE THERAPEUTIC INDEX OF GLUCOCORTICOIDS

Despite their wide-spread use the therapeutic index (TI[3]) of glucocorticoids remains low (86), especially in the chronic long-term (>6 months), high-dose (>2.5–10 mg/day) scenario (87, 88), with side-effects (4, 89, 90) and loss of glucocorticoid sensitivity or glucocorticoid resistance (5, 91), respectively, affecting the numerator and denominator of the TI.

The discussion in this section will focus on *in vivo* studies of loss of GR dimerization achieved using either the GR^{dim} mutation or CpdA. 21OH-6,19OP, which affects dimerization of only the LBD and as such does not affect dimerization of the full-length GR^{wt} receptor (31), was originally described as a specific passive antiglucocorticoid (92, 93) but displays dissociated activity *in vivo* (94), However, as very few *in vivo* studies (79, 80) have been conducted this molecule will not be discussed further.

Glucocorticoid-Induced Side-Effects

Evaluation of the impact of GR dimerization on glucocorticoid signaling has focused mainly on the modulation of the side-effect profile elicited by glucocorticoids (23, 95).

Generally, loss of GR dimerization, whether through the use of the GR^{dim} mutant and/or the $GR^{dim/dim}$ mouse model (66), or the monomeric favoring ligand, CpdA, has resulted in effective inflammatory control with a reduction in side-effects (96–98). For example, in a recent systemic review comparing the efficacy and safety of SGRMs to that of glucocorticoids in arthritis it was found that CpdA generally displays an improved TI with a similar efficacy but a better safety profile than glucocorticoids (17).

To illustrate, the effect of loss of GR dimerization on two side-effects of systemic use of glucocorticoids for severe asthma in the UK with an increased hazard ratio (HR), namely diabetes (HR:1.20) and osteoporosis (HR: 1.64) (99), will be discussed. Diabetogenic effects, which include increased blood glucose levels, gluconeogenesis, glycogen storage, insulin secretion and/or liver metabolic enzyme transcription are mediated by GR transactivation and requires GR dimerization, were not observed with GR^{dim} (63, 100, 101) or with CpdA (82, 97, 102–104). While, osteoporosis, mediated by both transrepression (osteocalcin transcription) and transactivation (osteoblast differentiation) and thus requiring both GR monomers and dimers (95), was not induced by CpdA, either *in vitro* or *in vivo* (105–109), while the GR^{dim} mice still developed osteoporosis concomitant with a potent suppression of osteoblast differentiation both *in vitro* and *in vivo* (110–112).

Interestingly, loss of GR dimerization through use of GR^{dim} mice also appears to limit gastrointestinal side-effects of DEX such as enhanced glucose transport in the small intestine (63) and an increase in gastroparesis (delayed stomach emptying) and gastric acid secretion (113). However, some side-effects of glucocorticoids still occur in GR^{dim} mice (95, 114). For example, DEX induced a similar degree of atrophy in the tibilialis anterior and gastrocnemius muscles of GR^{wt} and GR^{dim} mice (115). Investigation involving a key regulator of muscle atrophy, the E3-ubiquitin ligase, MuRF1, suggests that GR-binding is stabilized by the binding of an adjacent FOXO1 on a composite DNA-binding element in the proximal promotor of the gene, as GR^{dim} alone, in contrast to GR^{wt}, did not induce the MuRF1 promoter but did result in a modest induction in the presence of FOXO1, which itself is upregulated by DEX via GR^{wt} (116), but not GR^{dim} (115). CpdA has not been evaluated in this model and it would be interesting to establish if, like for osteoporosis, loss of dimerization through CpdA administration has a more favorable outcome than seen with GR^{dim}. Tantalizingly, in the *mdx* mouse model of Duchenne muscular dystrophy CpdA, unlike prednisolone, did not reduce gastrocnemius muscle mass (117).

However, as an important caveat it should be noted that loss of GR dimerization through the GR^{dim} mutation can impair the effect of glucocorticoid treatment in some inflammatory conditions and as discussed may still display some DEX-induced side-effects (95, 114). For example, in skin, inhibition of the swelling response during the challenge phase, upon re-exposure to the hapten, 2,4-dinitrofluorobenzene, by exogenous intraperitoneal or oral DEX administration in contact dermatitis, a T cell–dependent delayed-type hypersensitivity reaction, is not observed in GR^{dim} mice (118), yet in phorbol ester-induced inflammation, a classic model of acute irritant inflammation and epidermal hyperplasia, topical DEX-treatment was as effective in GR^{dim} mice (96). For CpdA, results in acute irritant inflammation of the skin are conflicting and may depend on the topical dose used. At low doses [μg range (119, 120)] CpdA not only inhibited irritant-induced skin inflammation and hyperplasia but also did not induce skin atrophy, an important side-effect of topical glucocorticoid treatment. However, at higher doses (mg range) CpdA increased, rather than decreased, epidermal thickness (121).

In two models of arthritis in mice, antigen-induced arthritis (AIA), a mouse model of human rheumatoid arthritis, and glucose-6-phosphate isomerase-induced arthritis, a severe form of polyarthritis, GR^{dim} mice were, respectively, fully or partly resistant to intravenous Micromethason (liposomal encapsulated DEX) treatment (122). In contrast, CpdA administered intraperitoneally showed similar or slightly reduced efficacy compared to DEX in attenuating collagen-induced arthritis (82, 123, 124) and repressed the inflammatory response as effectively as glucocorticoids in *ex-vivo* models using fibroblast-like synoviocytes (FLS) from rheumatoid arthritis or osteoarthritis patients (108, 123, 125, 126), while displaying less side-effects, such as hyperinsulinemia (82), bone-loss

[3] $TI = \frac{TD50\ (dose\ of\ drug\ that\ causes\ severe\ side\ effects\ in\ 50\%\ of\ subjects)}{EC50\ (dose\ of\ drug\ that\ has\ desired\ pharmacologivcal\ effect\ in\ 50\%\ of\ subjects)}$

(108, 124) and homologous down-regulation of the GR (123), than glucocorticoids.

Both GR^{dim} (127) and CpdA (104, 128) was as effective as DEX treatment in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis, while CpdA, unlike DEX, did not elicit hyperinsulinemia or hypothalamic-pituitary-adrenal axis suppression (104). However, in allergic airway inflammation (AAI), a mouse model of allergic asthma, GR^{dim} mice, unlike GR^{wt} mice, did not respond to intraperitoneal injection of DEX (129), while CpdA was as effective as DEX in this model (130).

In acute systemic inflammatory settings GR^{dim} mice are highly vulnerable and resistant to glucocorticoid treatment. For example, in two mouse models of sepsis, cecal ligation and puncture and lipopolysaccharide (LPS)-induced septic shock, GR^{dim} mice are highly susceptible to sepsis and their bone marrow-derived macrophages are resistant to DEX treatment *in vitro* (131). Interestingly, even low dose LPS treatment resulted in GR^{dim} mice displaying exaggerated sickness behavior compared to GR^{wt} mice (132). Furthermore, in TNF-induced acute lethal inflammation GR^{dim} mice displayed increased TNF sensitivity and resistance to DEX treatment (133, 134). Acute graft- vs.-host disease, a severe complication of hematopoietic stem cell transplantation, is another severe inflammatory disease characterized by a cytokine storm in which GR^{dim} mice presented with exacerbated clinical symptoms and increased mortality relative to GR^{wt} (135). To our knowledge CpdA has not been evaluated in these acute inflammatory models although it has been suggested that it would be as ineffective as the GR^{dim} mice as for full resolution of the inflammatory response dimerization of the GR is required (22, 23).

In addition, concerns regarding specifically the use of CpdA as a therapeutic agent have been raised (102, 124, 128, 130) as it degrades to an aziridine in solution (78) thus mediating cytotoxic effects independent of the GR that may severely narrow its therapeutic window.

Glucocorticoid-Induced Resistance

Glucocorticoid resistance is characterized by impaired sensitivity to glucocorticoid treatment and may be inherited (136) or acquired, which is more common and may result from disease progression or chronic high-dose glucocorticoid treatment (5, 91). One of the main drivers of acquired glucocorticoid resistance is homologous down-regulation of the GR (5, 137, 138).

Mechanism-based pharmacodynamic models use the term drug tolerance to describe the decrease in expected pharmacological response after repeated or continuous drug exposure (139) and modeling of the pharmacogenomic responses of glucocorticoid-induced leucine zipper (GILZ) (140) and tyrosine aminotransferase (TAT) (141) mRNA induction by both acute and chronic glucocorticoid regimes in diverse rat tissues indicate that drug tolerance is primarily controlled by the cytosolic free receptor density, which is substantially down-regulated.

Receptor density is modulated by *de novo* receptor synthesis and receptor degradation, which may be described by a simple "push" vs. "pull" mechanism (5), where the "push" mechanism includes transcription initiation and mRNA stability, while the "pull" mechanism involves degradation of the receptor.

Already 30 years ago, it was established that ligand-mediated down-regulation of the GR occurs at the level of both transcription initiation and GR protein degradation, but not at the level of mRNA stability (142). Further elucidation of the process has established that inhibition of transcription is mediated through binding of the liganded-GR to a nGRE in exon 6 of the GR gene and assembly of a repressive complex, consisting of the GR, the coregulator NCoR1, and histone deacetylase 3 (HDAC3), at the transcriptional start site through DNA-looping (143), while ligand-dependent GR protein degradation has been localized to the ubiquitin-proteasome system (UPS) through the use of the proteasome inhibitors (144). Proteasomal degradation requires ligand-induced phosphorylation of the human GR at S404 (**Figure 1A**) by glycogen synthase kinase 3β (GSK3β) (145), which is required for ubiquitination of the human GR at the upstream K419 (mouse GR K426) in a PEST sequence (144, 146). Ubiquitin is attached to the GR in a three step pathway involving ubiquitin activating (E1), conjugating (E2), and ligase (E3) enzymes to produce a polyubiquitylated receptor for targeting to the 26S proteasome (147). Several E2-conjugating enzymes, such as ubiquitin-conjugating enzyme 7 (UbcH7) (148), susceptibility gene 101 (TSG101) (149), and Ubc9 (150–152) and E3-ligases, such as E6-AP (encoded by the *Ube3a* gene) (153, 154), carboxy-terminus of heat shock protein 70-interacting protein (CHIP)(155–157), murine (Mdm2), or human (Hdm2) double minute (158–160), UBR1 (161), and F-box/WD repeat-containing protein 7 (FBXW7α) (162), have been shown to interact with the GR. Recently, however, micoRNAs (miRNAs), upregulated by glucocorticoids (163, 164), have been implicated in the ligand-induced reduction of the GR mRNA pool (5, 10), suggesting that the initial study indicating that receptor density is not regulated by the stability of mRNA levels has to be re-examined.

The relative contributions of GR mRNA and protein down-regulation may be dependent on the dose of glucocorticoid and/or the duration of treatment. For example, in podocytes GR protein, but not RNA, is down-regulated during both short (1 h) high (100 μM) dose and long-term (5 days) low (1 μM) dose DEX regimes (165), while in HeLa S3 cells, 24 h, 2 weeks or a 2-year low (1 μM) dose DEX regime suggests that at 24 h, GR protein is more profoundly down-regulated than mRNA, while at 2 weeks both protein and mRNA is down-regulated, while by 2-years no detectable protein or RNA was observed (166). Furthermore, in FLS derived from patients with rheumatoid arthritis a short (7 h) vs. long (30 h) protocol of low (1 μM) dose DEX indicates substantially more GR protein down-regulation at the longer time point (123).

Although little to no work has been done on the implications of GR dimerization for GR resistance, some tantalizing results with GR ligands have been noted. For example, RU486 (mifepristone), a GR antagonist shown to cause significantly less dimerization than DEX (167), was unable to down-regulate nascent GR RNA (143) and was less effective than DEX at down-regulating GR protein levels (168), while ZK216348, a SEGRA (169) for which no data on GR dimerization is available, did not

down-regulate GR protein levels (102). CpdA, which abrogates GR dimerization (31, 81, 82, 170), does not result in GR down-regulation at either protein (102, 123, 171–175) or RNA (123, 172) level.

Recently, our laboratory investigated the hypothesis that GR dimerization may be required for homologous down-regulation of the GR by employing conditions that either promote or reduce GR dimerization (176). Promotion of GR dimerization through the use of dimerization promoting ligands, such as DEX and cortisol, induced significant down-regulation of GR^{wt}, both transiently transfected and endogenous in HepG2 cells, while reduction of dimerization, through the use of either CpdA or GR^{dim}, severely restricted GR turn-over. Receptor down-regulation was primarily mediated by increasing the rate of receptor protein turnover by the proteasome as (1) promotion of GR dimerization significantly increased the rate of turnover and decreased receptor half-life relative to the unliganded receptor and (2) inhibition of the proteasome by MG132, but not protein synthesis by cycloheximide, abolished GR turn-over. Interestingly, the GR^{wt} half-life with CpdA was very similar to that of the half-life of the unliganded receptor, a finding previously reported (171). Mechanistically, degradation of the GR by the proteasome requires hyperphosphorylation of the GR at S404 by GSK3β (145), which enables binding of the E3 ligase FBXW7α (162). Loss of GR dimerization restricted hyperphosphorylation at S404 and interaction with FBXW7α. Furthermore, inhibition of DEX-mediated S404 hyperphosphorylation through the use of the pharmacological GSK3β inhibitor, BIO, restored GR levels. In summary, GR dimerization is required for ligand-induced post-translational processing and downregulation of the receptor via the UPS system. Subsequently, the requirement of GR dimerization for autologous down-regulation of the GR was confirmed in a study in arthritic mice indicating that DEX does not down-regulate the GR in GR^{dim} mice, in contrast to GR^{wt} mice (164).

Although, loss of GR dimerization has been generated by using either dimerization deficient mutants such as GR^{dim}, or monomerization biased ligands such as CpdA, and it has been suggested that the behavior of DEX-induced GR^{dim} equates to that of CpdA-induced GR^{wt} (81), results show that the two scenarios do not always produce exactly the same results. At a molecular level, for example, although both GR^{dim} and CpdA prevent homologous down-regulation of the GR the two conditions differ in terms of the extent of the repression of the post-translational modifications (PTMs) required for the process, with CpdA reducing S404 phosphorylation, while no discernible, not even basal, phosphorylation is observed with GR^{dim} (176). Nuclear translocation of the GR is another area of potential difference as some studies show that CpdA does not allow for nuclear translocation of the GR^{dim} (176), while others suggest that both GR^{dim} and CpdA can cause nuclear translocation albeit with diminished maximal import (81, 170). Furthermore, in disease models, although glucocorticoid-induced metabolic side-effects may be attenuated under both conditions, GR^{dim} can still induce osteoporosis, while CpdA does not, which has been ascribed to the ability of GR^{dim}, but not CpdA, to suppress interleukin-11 via interaction with AP-1 (108, 111, 177). Additionally, in terms of efficacy in disease models loss of dimerization through CpdA administration often had a more favorable outcome than seen with GR^{dim} mice, in for example, arthritis (82, 108, 122–126) and allergic asthma (128–130) models. Although it may be tempting to ascribe these differences to the extent of GR dimerization elicited, with total abrogation of dimerization by CpdA (31, 81) and no (31, 62), to partial (38), to almost full (35, 36) loss of dimerization via GR^{dim}, this would probably be an oversimplification. More likely is that CpdA, in contrast to GR^{dim} that impacts only the DBD (65), also elicits a differential conformation of the LBD upon binding (97), which could impact on GR PTMs (97, 171, 176) and interaction with cofactors (178, 179). Despite the fact that both CpdA and GR^{dim} modulate GR dimerization there are few comparative studies directly comparing implications for molecular aspects of GR signaling or the impact on the therapeutic index in mouse models of disease.

CONCLUSION

Monomeric GR, like the dimer, binds to DNA and is transcriptionally functional (101), thus these two receptor species may represent distinct drug targets to tailor for improved glucocorticoid treatments. Rational design of conformationally biased ligands that select for a monomeric GR conformation, may be a productive avenue to explore in the pursuit of drugs that lessen the side-effect profile and increase glucocorticoid sensitivity through improving GR protein stability and increasing half-life, yet the optimal conformational and gene expression signatures to either drive the monomer-dimer equilibrium toward a particular state or evaluate its implications remain elusive, as does the question of whether this would be feasible or even desirable in the clinic.

For rational structure-based drug optimization strategies the field needs to look at both methods to accurately measure and quantify GR dimerization bias and an updated theoretical framework or model to evaluate the implications of GR dimerization.

Biased signaling is well-developed in the field of GPCR signaling (180) and offers quantification approaches (181) that yield useful empirical parameters, such as the transduction coefficient (τ/K_A) that incorporates ligand efficacy and potency as well as receptor density, to compare extent of bias relative to a reference ligand, usually the endogenous ligand (182). However, in the GR field there have been only isolated reports that harnessed classical analytical pharmacology approaches to generate quantitative information about the pharmacodynamic properties of GR ligands (183, 184). In addition, although mechanistic pharmacokinetic and pharmacodynamic models for the GR (140, 185, 186) and mathematical models to increase drug specificity (187–189) are being developed their uptake by most investigators has been slow. This is unfortunate as they provide a much-needed new perspective and are an essential component for understanding the quantitative behavior of biased GR ligands and to provide tractable design strategies such as functional selectivity fingerprints for drug development.

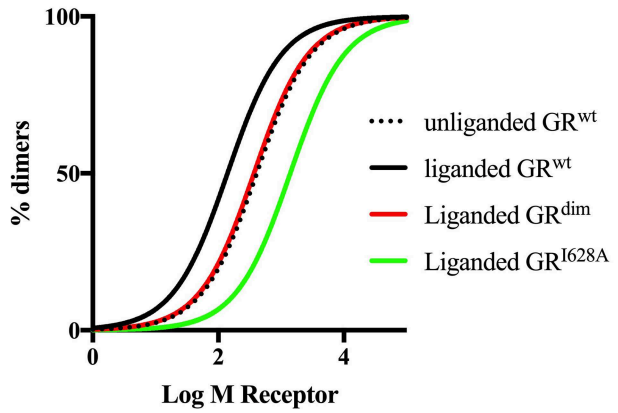

FIGURE 3 | Simulated dimerization curves for unliganded and liganded GR^{wt} and liganded GR^{dim} and GR^{I628A}. Simulations were done using GraphPad Prism version 7. K_d values from Oasa et al. (36) were used, except for liganded GR^{I628A}, where a 10-fold increase in the K_d of the unliganded GR^{wt} was used as per Bledsoe et al. (9). The figure clearly shows that ligand-binding to the GR^{wt} results in a left shift of the dimerization curve, while mutations in either the DBD or the LBD dimerization interfaces result a right shift of the curve relative to GR^{wt}, with a more pronounced shift in the case of the mutation to the LBD dimerization interface.

The importance of quantitative, rather than semiquantitative analysis is illustrated by the recent commotion around the usefulness of the GR^{dim} model to investigate effects of loss of dimerization. The initial study by Presman et al. (31) using the N&B assay that demonstrated dimerization by the GR^{dim} was semiquantitative yet several reviews since then have given this evidence underserved prominence. Mass action dictates that increasing GR levels would force the steady state to dimerization even in the case of a GR species poorly able to elicit dimerization, such as the GR^{dim}. Thus, a valid evaluation and comparison of the dimerization potential of the GR^{dim} requires a quantitative approach that measures dimerization affinity such as done by the group of Kinjo (35, 67). Furthermore, it has recently been pointed out that the N&B assay may suffer from drawbacks, which could be avoided by using the two-detector number and brightness analysis (TD-N&B) (190), whereby it was shown that the GR^{dim} is poorly dimerized in the nucleus, with a concentration ratio between monomers and dimers of 1:0.66 as compared to GR^{wt} that has a concentration ratio between monomers and dimers of 1:19.1. Finally, simulated dimerization curves using the K_d values obtained from the literature (**Figure 3**) clearly shows that the GR^{dim} is indeed poor at eliciting dimerization in comparison to GR^{wt}.

Despite optimism regarding the potential of biased ligands such as SEGRMs to improve on the therapeutic potential of glucocorticoids, to date none have entered the market (191). For biased ligands promoting GR monomers there are indeed legitimate concerns raised that for full resolution of inflammation transactivation by GR-dimers of genes such as mitogen-activated protein kinase phosphatase-1 (MKP-1), GC-induced leucine zipper (GILZ), and IL10 are required (22). Notwithstanding these concerns a strong argument has been made for the tailoring of ligands that favor GR monomer formation for chronic long-term use (23), a scenario where the additional ability of these ligands to prevent resistance would be most relevant.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Could GILZ Be the Answer to Glucocorticoid Toxicity in Lupus?

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Glucocorticoids (GC) are used globally to treat autoimmune and inflammatory disorders. Their anti-inflammatory actions are mainly mediated via binding to the glucocorticoid receptor (GR), creating a GC/GR complex, which acts in both the cytoplasm and nucleus to regulate the transcription of a host of target genes. As a result, signaling pathways such as NF-κB and AP-1 are inhibited, and cell activation, differentiation and survival and cytokine and chemokine production are suppressed. However, the gene regulation by GC can also cause severe side effects in patients. Systemic lupus erythematosus (SLE or lupus) is a multisystem autoimmune disease, characterized by a poorly regulated immune response leading to chronic inflammation and dysfunction of multiple organs, for which GC is the major current therapy. Long-term GC use, however, can cause debilitating adverse consequences for patients including diabetes, cardiovascular disease and osteoporosis and contributes to irreversible organ damage. To date, there is no alternative treatment which can replicate the rapid effects of GC across multiple immune cell functions, effecting disease control during disease flares. Research efforts have focused on finding alternatives to GC, which display similar immunoregulatory actions, without the devastating adverse metabolic effects. One potential candidate is the glucocorticoid-induced leucine zipper (GILZ). GILZ is induced by low concentrations of GC and is shown to mimic the action of GC in several inflammatory processes, reducing immunity and inflammation in *in vitro* and *in vivo* studies. Additionally, GILZ has, similar to the GC-GR complex, the ability to bind to both NF-κB and AP-1 as well as DNA directly, to regulate immune cell function, while potentially lacking the GC-related side effects. Importantly, in SLE patients GILZ is under-expressed and correlates negatively with disease activity, suggesting an important regulatory role of GILZ in SLE. Here we provide an overview of the actions and use of GC in lupus, and discuss whether the regulatory mechanisms of GILZ could lead to the development of a novel therapeutic for lupus. Increased understanding of the mechanisms of action of GILZ, and its ability to regulate immune events leading to lupus disease activity has important clinical implications for the development of safer anti-inflammatory therapies.

Keywords: GILZ, glucorticoids, lupus (SLE), transcription factor, treatment, regulation

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SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)—A DIVERSE CHRONIC AUTOIMMUNE DISEASE

Systemic lupus erythematosus (SLE) is an incurable chronic disease, affecting ∼1 in 1,000 people world-wide (1, 2), resulting in a marked loss of life expectancy and quality of life. The prevalence of SLE is higher in women, particularly of child bearing age, and in African American, Asian and indigenous Australian populations (1). SLE is one of the top 10 causes of death in young adult women (3), but the current treatment consists mainly of glucocorticoids (GC) which cause severe adverse effects (4).

SLE is characterized by multisystemic inflammation occurring in periodic disease flares, which can affect multiple organs such as the kidneys, lungs, brain, heart, blood, and skin. The most common symptoms include rash, arthritis, and fatigue. The heterogeneity of the disease makes clinical diagnosis and measurement very challenging, resulting in the use of clinical criteria to categorize patients with autoimmune disease as having SLE. For example, the American College of Rheumatology (ACR) classification criteria, which comprise 11 disease features, identifies patients who have at least four features as SLE (5). The requirement for multiple features highlights the heterogeneity of the disease. The majority of SLE patients are positive for antinuclear antibodies (ANA) which target the nuclear components of cells. The most commonly detected specific autoantibodies are dsDNA, anti-Ro and anti-Sm antibodies which also can act as markers for kidney disease (6). Furthermore, the presence of autoantibodies especially to Ro, La, Sm, and RNP is strongly associated with detection of interferon (IFN)-induced gene expression in peripheral blood (7). This profile of IFN-induced genes is termed the IFN signature, and is found in approximately 95% of children and 50–70% of adults with SLE (8–10).

Although the initiating cause of SLE is unknown, it is thought to result from a failure in tolerance checkpoints in several components of the immune system. This includes the escape and proliferation of autoreactive B cells, the development of autoantibodies, and the formation of immune complexes which can initiate organ inflammation and/or directly damage cells (8, 11).

Production of Autoantibodies and Immune Cell Dysfunction

One theory regarding the initiation of SLE is impaired clearance of apoptotic cells. Phagocytes from SLE patients are less effective in clearing apoptotic cells, and the uncleared apoptotic cells present apoptotic bodies and nucleic antigens to the extracellular space (12). These nucleic antigens, if internalized via Fc receptor binding of nucleic acid immune complexes, activate toll like receptors (TLRs). Particularly TLR 7 whose ligand is ssRNA [associated with the production of anti-Sm antibodies in SLE (13)], and TLR 9 whose ligand is unmethylated CpG-rich DNA. Downstream signaling resulting in the production of multiple cytokines, including interleukin (IL)-6, IL-1, and tumor necrosis factor (TNF)-α, which play a pivotal role in immune cell dysfunction and chronic inflammation (14).

Importantly, this pathway also leads to production of type I IFN (IFN-α and IFN-β), an important hallmark of SLE (15). Type I IFNs can also be induced via TLR-independent pathways such as retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation-associated protein 5 (MDA-5) and cyclic GMP-AMP synthase (cGAS) receptors which activate innate immune cells through the detection of cytoplasmic nucleic acids (16). The main producers of type I IFN are plasmacytoid DCs (pDC). Despite a reduction in the number of circulating pDC in SLE patients, these cells accumulate at inflamed sites, particularly the skin and kidneys, and secrete large amounts of type I IFNs (17, 18).

One of the actions of IFN-α is to prime mature neutrophils and assist the formation of neutrophil extracellular traps (NETs) (19, 20). NETs are mesh-like structures composed of chromatin fibers and nuclear components, designed to trap and kill microbes (21). However, when inappropriately cleared, these NETs are also a source of auto-antigens. Thereby, they contribute to the development of auto-antibodies, particularly against dsDNA, and the immune complexes that cause organ damage in SLE (22). Finally, by exposing intracellular nucleic acid antigens, NETs activate pDCs and further exacerbate the production of type I IFN, creating a cycle of type I IFN production and NET formation in SLE patients (20).

Components of apoptotic cells can also be taken up by antigen-presenting cells and will activate T and B cells through the normal antigen presentation pathway. $CD4^+$ T cells from SLE patients display a high expression of CD40 ligand (CD40L) compared to healthy donors, which also assists in activation and differentiation of B cells due to its role as co-stimulatory molecule (23). T follicular helper cells are expanded in SLE patients and promote the differentiation of autoantibody producing B cells (24). Additionally, Th17 cells, which promote inflammation, are increased in SLE, whilst T regulatory cells (Tregs) are suppressed (25). Furthermore, increased T cell numbers in SLE provides more T cell help for B cell differentiation, survival and proliferation (25), as does an elevated level of B cell activating factor (BAFF; also known as B Lymphocyte Stimulator, BLyS or TNF like ligand, *TNFSF13B*). Overexpression of BAFF is associated with increased survival of activated autoreactive B cells and a decrease in self-tolerance which leads to lupus-like autoimmune disease in mouse models (26). BAFF also assists in B cell survival during differentiation and is associated with SLE disease activity (14). B cells, which are activated by $CD4^+$ T cells, contribute to disease both via antibody production and antigen presentation to T cells. B cells in SLE are hyperactive and contribute significantly to the production of autoantibodies, cytokines and augmented antigen presentation to T cells (25). Naïve B cells are reduced in number in the blood of SLE patients, whilst there is an increase in plasmablasts leading to an increase in antibody production. The cycle of excess antibody production perpetuates inflammation via immune complexes, as noted above.

Thus, SLE is a diverse autoimmune disease mediated by the disordered activation of multiple immune cells causing widespread chronic inflammation, resulting in multi-system

morbidity and making management an enormous challenge. To combat the diverse nature of the systemic inflammation, the broad effects of GC on immune cell function has led to them being used widely to elicit broad suppression of autoimmunity and its inflammatory consequences.

Glucocorticoid Treatment of SLE

GC have been used for decades for the treatment of inflammatory and autoimmune diseases such as rheumatoid arthritis and SLE (27, 28). GC have a substantial impact on the immune system via the ability to regulate 1,000 of genes. Gene regulation occurs via GC binding to the GC receptor (GR), which is expressed in almost every cell in the body. Thus, the effects of GC are not tissue- or organ-specific and treatment with GC has the ability to target inflammation in multiple parts of the immune system, and in multiple organs, simultaneously. This is valuable in a disease like SLE, in which cell types are implicated and multiple organs are affected. In SLE, dosing of GC varies according to the severity of inflammation and the nature of the organs affected (28). Unfortunately, adverse effects of GC, chiefly metabolic, are also dose-dependent, and are seen almost universally in patients with SLE treated with GC long term (28, 29).

To reduce reliance on GC, and hence dose-dependent adverse effects, patients with SLE are usually also treated with additional immunomodulatory or immunosuppressive drugs, such as anti-malarials, mycophenolate mofetil, or azathioprine. These drugs are also broad spectrum in their effects, and associated with significant adverse effects. The only targeted therapy approved for the treatment of SLE is belimumab, approved by the FDA for use in 2011. Belimumab is a neutralizing human monoclonal antibody to BAFF, which is elevated in SLE, suggesting that a BAFF inhibitor has the potential to control B cell dysfunction in SLE (14, 26). Belimumab has been approved for treatment of patients with active autoantibody positive SLE who have not responded to conventional therapies (30). Belimumab does not induce a rapid clinical benefit and though its use is associated with reduced GC dosing (31), GC remains the treatment for the majority of SLE patients. Additional targeted therapies directed against key inflammatory cytokines for SLE, such as IL-6, have been found to be ineffective in Phase II trials in SLE, despite their efficacy in rheumatoid arthritis (32). This suggests upstream targeting of inflammatory signaling pathways is important for development of a GC replacement therapy.

That GC are the mainstay treatment for SLE despite its use being plagued with severe side effects, such as increased cardiovascular disease, osteoporosis and diabetes (28, 29, 33), highlights the lack of a viable alternative. Thus, there is a critical need for the development of new therapeutics with similar potent immune actions but without the detrimental metabolic effects.

THE MECHANISM OF GC ACTION IN THE TREATMENT OF SLE

The main mechanism via which GC act on the immune system is through binding to the glucocorticoid receptor (GR). The GR is encoded by the *NR3C1* gene and has two major forms, GRα and GRβ, which are alternative splicing isoforms from N3C1 (34). GRα is the form that resides in the cytoplasm and is dependent on GC binding for function. The GR contains an N-terminal regulatory domain, central DNA binding domain, hinge region and C-terminal ligand binding domain (35–37). The GRα is located in the cytoplasm and forms a complex with several proteins including heat shock protein 70 and 90 (38). Upon ligand binding, GRα is released from this complex and can interact with cytoplasmic signal transduction molecules or translocate to the nucleus.

The main mechanisms via which GC drive the transcription and regulation of multiple genes are direct DNA binding, tethering and composite binding (38–40) (**Figure 1**). In the nucleus, GRα is able to modulate gene transcription through binding to target sequences termed GC-response elements (GREs), largely in the cis-regulatory region of target genes (41). This results in either induction or repression of target gene expression. The binding of the GRα to the GRE can also cause conformational changes in the GR which causes the recruitment of cofactors and coregulators to the site with the ability to modulate and alter the transcriptional rate of many target genes (35, 37). Direct DNA binding of the GRα to GREs results in the induction of gene expression and causes the transcription of multiple genes, including anti-inflammatory genes such as IL-10 and IL-1 receptor antagonist (38, 40) as well as GC-induced leucine zipper (GILZ). GRα can also directly bind to negative GREs (nGRE), which results in suppression of the transcription of several proinflammatory modulators and cytokines including interleukin (IL)-1β (38, 40, 42). Another form of gene repression, termed tethering, is mediated by the ability of GRs to tether to pro-inflammatory transcription factors such as the p65 subunit of nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1), antagonizing their function (35, 43–45). Finally, composite binding encompasses the binding of GRα to a gene locus containing a GRE as well as a binding site for another transcription factor (**Figure 1**).

Additional factors also influence the ability of GRα to regulate gene transcription such as chromatin structure, epigenetic regulators, proximity to the TATA box, and indirect activation of target genes (38, 41). Next to the genomic actions of GRα, it also affects cellular function through non-genomic mechanisms, including suppression of mitogen activated protein kinases (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways and activation of proteins with SRC homology 3 [SH3] domains (46) (**Figure 1**).

Other factors influencing GC regulation of gene expression include tissue-specific differences in GR expression, and the level of GC dosing (47, 48). Whereas, a single bolus GC dose highly activates GC-mediated biological responses, chronic GC dosing can be associated with GC resistance. Studies in acute adrenalectomized rat models have demonstrated that this resistance is correlated with a down-regulation of GR mRNA and cytosolic receptor density (49, 50). A study by Ayyar et al. found a similar effect of chronic dosing when studying GILZ as a pharmacodynamic marker of GC action in different rat tissues (47). Similar to Ramakrishnan et al. (50) this study also demonstrated drug-induced tolerance during chronic dosing,

where GC receptor down-regulation was the main mechanism of regulation (47). Taken together these studies highlight that tissue-specific, and GC-induced, differences in GR levels are important physiological factors to consider in GC responses.

The molecular mechanisms of GRα-mediated effects have been extensively studied, however, much less is known about the GRβ isoform. GRβ results from alternative splicing in exon 9 of NR3C1 and is a truncated isoform that lacks helix 11 and 12 from the ligand-binding domain (51). Since these helices are important for ligand binding, GRβ cannot bind GC and does not directly affect GC-sensitive genes (52). In fact, it is thought that GRβ acts as a dominant negative regulator for GRα. Overexpression of GRβ suppresses GRα-mediated gene transcription, and cells which have higher endogenous levels of GRβ are less responsive to the effects of GC (53–55). How this inhibition occurs is not well-understood, but various mechanisms have been proposed. These include heterodimerization of GRβ with GRα to inactivate GRα, competition for binding at GREs and competition for binding to coactivators such as GRIP1 (53, 56, 57). Furthermore, GRβ also has transcriptional activity independent of GRα and controls its own set of genes (58, 59). Interestingly, GRβ expression is induced by a variety of pro-inflammatory cytokines, such as IL-2, IL-4, IL-17A, IL-17F, IL-23, and TNF-α (60–62). Given their prominent role in inflammatory diseases and the dominant-negative role of GRβ, it is not surprising that high GRβ levels seem to be related to GC resistance in diseases such as asthma, ankylosing spondylitis and SLE (63–65). Furthermore, a polymorphism in *NR3C1* which enhances the stability of GRβ is associated with rheumatoid arthritis (66). Therefore, a better understanding of GRβ biology and how to suppress its function may be an important step toward improving GC-based therapies.

It should be noted that GRα and GRβ are not the only forms of GR. In fact, 27 splice variants of the *NR3C1* gene have currently been identified (67), and hundreds of single nucleotide polymorphisms (SNPs), insertions and deletions which potentially also lead to different variants of the GR proteins have been cataloged (67). Although the physiological role of these variants is currently unknown, they may play a role in individual GC response and therefore warrant further study.

In the context of SLE, GC elicit rapid and potent anti-inflammatory effects upon multiple organs and immune cells. Many of the immune regulatory effects of GC are through direct binding to transcription factors including NF-κB, AP-1, nuclear factor of activated T cells (NFAT) and T-bet (38, 68, 69). This causes a myriad of effects upon immune cells, described in more detail below, and important for the treatment of SLE, the suppression of key mediators of inflammation TNF-α and type I IFNs (9, 14, 70).

Effect of GC Treatment on Immune Cells Operative in SLE

Thymocytes, particularly double positive $CD4^+CD8^+TCR^{low}$ thymocytes, are sensitive to GC-induced apoptosis (71). Cell death can also be induced in mature T cells indirectly by GC-mediated inhibition of IL-2 activation and production (72, 73). GC have been described to affect T cell polarization, shifting the phenotype from Th1 to Th2 (74, 75), however it should be noted that GC affect both T-bet and GATA-3 transcriptional activity, with long-term GC treatment favoring Th2 expansion (75). Further support for polarization toward a Th2 phenotype comes from GC increasing expression of Itk, a Tec kinase able to induce Th2 differentiation through the negative regulation of T-bet (76, 77). GC also increases Treg number and activity, promoting IL-10 producing T cells. This is through several mechanisms: inhibition of activation of T effector cells, GC-mediated Foxp3 induction, and Tregs being more resistant to GC (76).

GC treatment also affects B cells. Firstly, GC induce apoptosis in B cells at all developmental stages (78–80). In addition, GC suppress plasma cell differentiation potentially via down-regulation of Blimp1 and Bcl6 (81, 82). Finally, GC may also directly affect the production of IgG antibodies through inhibition of activation-induced cytidine deaminase (AICDA), an enzyme required for class switch recombination and somatic hypermutation (83). As a result of these GC-induced changes, GC reduce the number of plasma cell precursors and plasma cells and the level of anti-nuclear antibodies demonstrated in the murine MRL/lpr model for SLE (82). Importantly, the number of circulating B cells in human blood is also reduced upon GC treatment (84).

GC also impair dendritic cell (DC) maturation and function (85). DCs treated with GC increase their antigen uptake, decrease their expression of maturation markers (CD80, CD86) and decrease TNF-α, IL-6, and IL-12 production which in turn decreases the induction of T cell responses (85, 86). GC treated DCs have also been described as tolerogenic with the ability to drive T cells toward a Treg phenotype, creating an increase in IL-10 production (87, 88). As a result of these effects, GC enhance the clearance of dead cells and toxins, and increase scavenger function and phagocytosis. GC treatment also decreases the number of pDCs in the peripheral blood, which is important in SLE since they are key IFN-α producers (89). Following GC treatment, levels of IFN-α have been reported to be reduced to levels approximately 25-fold below those seen in untreated healthy donors (89). As type I IFN is implicated in disease severity and activity in SLE, control of pDC number and IFN-α production by GC could be of benefit for the treatment of SLE. Of note, however, pDC IFN production has been reported to be resistant to GC inhibition in SLE, because of TLR-induced NF-κB overcoming GC inhibitory effects (90); it is noteworthy that the IFN signature recognized as associated with SLE is still present in GC-treated patients. Were it to be proven that SLE-related IFN activity was resistant to GC, this would provide a novel target for "assisting" the effects of GC in SLE, for example by reversing factors associated with their inability to suppress IFN in this disease.

Treatment with GC increases the phagocytic ability of macrophages, a finding demonstrated in human macrophages and mouse models (91, 92). This process is assisted by up regulation of mannose receptor (CD206) and scavenger receptors (CD163) on macrophages and enhancement of IL-10 production (93, 94) in response to GC. Significantly for SLE, GC treatment thus also increases the phagocytosis of apoptotic neutrophils by macrophages

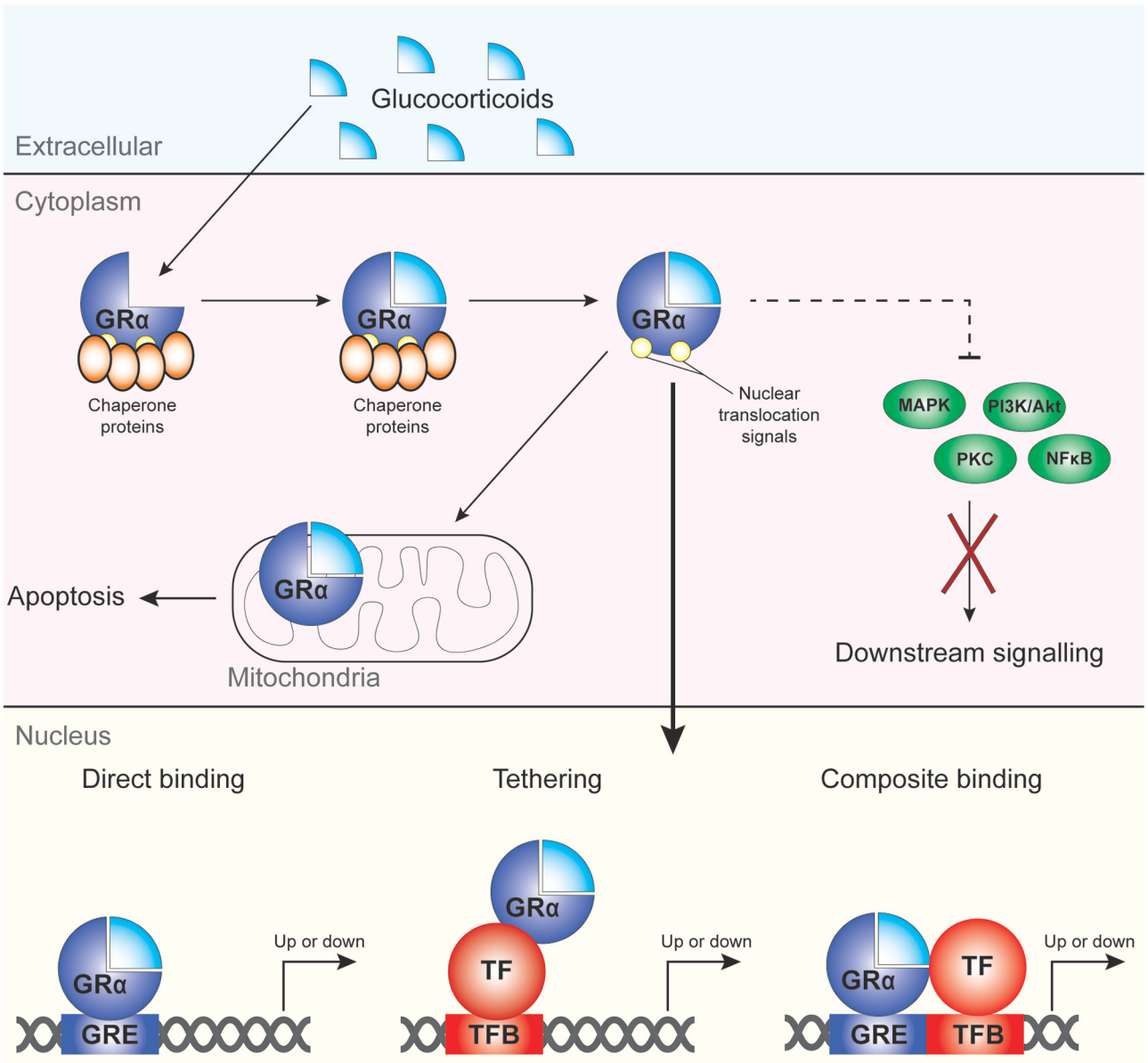

FIGURE 1 | Mechanisms of cellular regulation by GC and GRα. GC bind to GRα, which then dissociates from its chaperone proteins and can regulate target gene expression upon migration to the nucleus. Non-genomic effects of GRα include modulation of apoptotic processes in the mitochondria and direct and indirect (dotted line) regulation of cytoplasmic kinases and NF-κB.

(95), thus reducing the number of NETs and reducing their pro-inflammatory impact. GC also have direct immune modulating effects on neutrophils, dampening their activation through several mechanisms including anti-apoptotic effects (96), inducing detachment via effects on cell surface CD62L (97) and reducing expression of pro-inflammatory cytokines (98).

This wide array of immunomodulation entrained by GC underpins reliance on GC treatment in SLE. Thus, it is imperative that any replacement for GC is able to target multiple immune pathways and provide potent anti-inflammatory immune cell regulation (**Figure 2**). The ability to achieve these effects without causing the devastating metabolic adverse effects of GC has been described as the "holy grail" of inflammatory pharmacology (99).

COULD GC-INDUCED LEUCINE ZIPPER (GILZ) TARGETING BE A REPLACEMENT FOR GC TREATMENT IN SLE?

GILZ mRNA expression negatively correlates with SLE disease activity (100) and we have demonstrated that active SLE is associated with lower intracellular GILZ protein levels across multiple leukocyte subsets (11). These findings

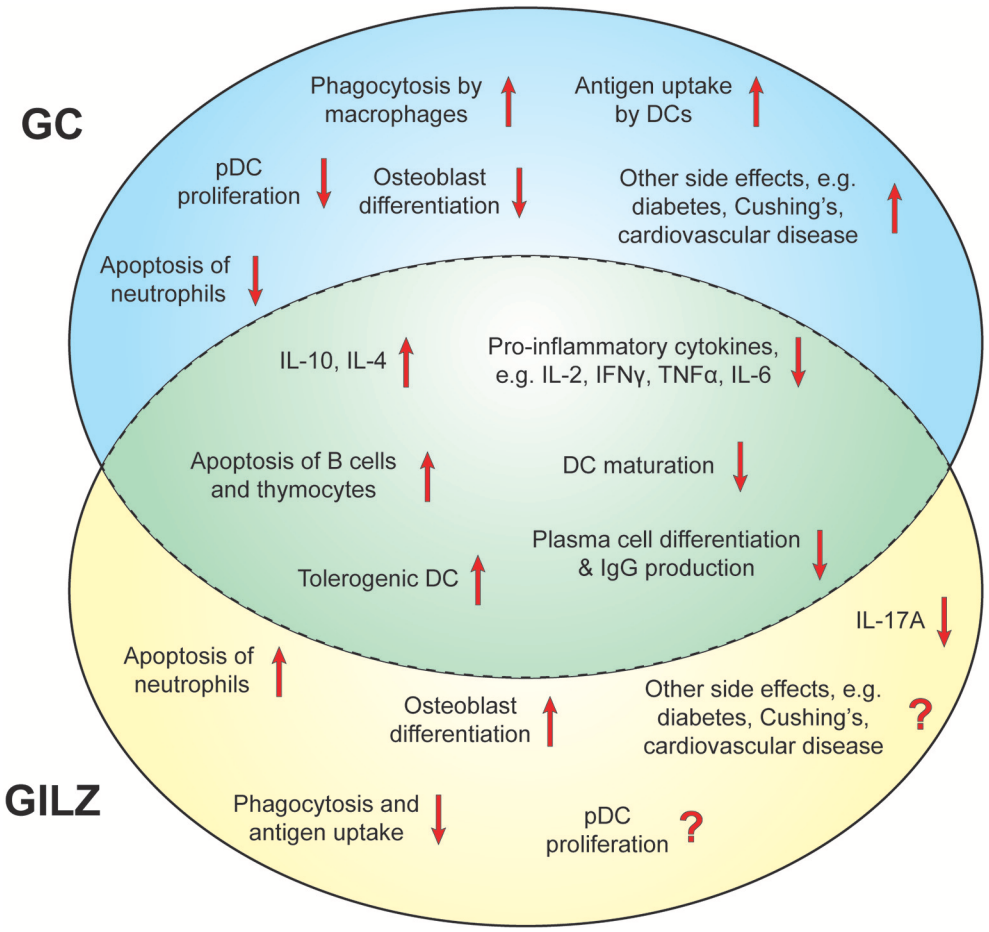

FIGURE 2 | Immunomodulatory effects of GC and GILZ. Overview of the effects of GILZ and GC that contribute to their suppressive capacities and potential side effects in SLE. Unique effects of GC are depicted in the blue circle, unique effects of GILZ in the yellow circle. Shared actions between GC and GILZ are shown in the middle green area.

suggest the possibility that GILZ deficiency contributes to SLE immunopathogenesis, and conversely that GILZ augmentation might be effective in SLE.

GILZ, also known as TSC22 domain family protein 3 (*TSC22D3*) is a 134aa protein in humans. It contains three main regions: an N-terminal domain with a tuberous sclerosis complex (TSC) domain, a leucine zipper, and a proline-rich C-terminal domain. The leucine zipper domain of GILZ largely mediates the homodimerization of GILZ, a requirement for many of its functions (101). The N-terminal and C-terminal domains of GILZ are regions where several protein-protein interactions occur, particularly with transcriptional and signaling molecules. These interactions are critical for the immunosuppressive effects of GILZ.

GILZ has been demonstrated to modulate immune cell activation and promote an anti-inflammatory phenotype (102, 103). This is mediated in part by inhibiting the nuclear translocation and DNA binding of NF-κB, largely through direct binding of the C-terminus of GILZ to the p65 subunit of NF-κB (101, 103). Additionally, GILZ binds to AP-1, a transcription factor consisting of c-Jun and c-Fos with a broad range of functions on immune cell activation (104), GILZ binds to AP-1 through its N-terminal domain and prevents AP-1 from binding to its target DNA (105).

GILZ also interferes with other cellular signaling molecules, such as extracellular signal-regulated kinases (ERK). The ERK pathway is involved in multiple regulatory processes and is key to many immune cell functions included cell differentiation, proliferation, survival, apoptosis, transcription and metabolism (106). GILZ suppresses the ERK pathway via direct binding to Ras via its TSC domain and, depending on the level of Ras activation, via formation of a trimeric complex with Ras and Raf (107, 108). This results in a decrease in activation of downstream targets of Ras and Raf, for example the ERK1/2 and protein kinase B (AKT/PKB) (107), and a subsequent decrease in cell proliferation.

The Effect of GILZ on Immune Events Involved in SLE Pathogenesis

As noted above, GILZ regulates pivotal transcription factors and cellular pathways involved in immune-inflammatory responses. Notably, many of these transcription factor interactions of GILZ,

mimic the transrepressive effects of GC. This raises the potential of GILZ as a replacement for GC. A comparison between the effects of GILZ and GC in animal models of inflammatory diseases has been summarized previously (39).

GILZ has a pro-apoptotic effect on thymocytes similar to GC, as demonstrated by GILZ overexpression systems in mouse models. GILZ overexpression led to a reduction of Bcl-xL expression and increased activation of caspase 3 and 8 (109). This results in a decrease in $CD4^+CD8^+$ thymocytes. However, in T cells, GILZ can exert an anti-apoptotic effect, inhibiting anti-CD3 antibody-induced cell apoptosis through binding to AP-1 (110). This leads to inhibition of FasL expression and inhibition of apoptotic pathways (105, 108).

Similar to GC, GILZ also promotes a Th2 over a Th1 phenotype in T cells. This was demonstrated in activated $CD4^+$ T cells from GILZ transgenic mice, which had increased expression of Th2 transcription factors GATA-3 and STAT6 but decreased production of the Th1 transcription factor T-bet (111). Additionally, GILZ induced a Th2 cytokine profile with increased IL-4, IL-5, IL-10, and IL-13 production and a reduction in IFN-γ production. Supporting the role of GILZ in reducing Th1 cytokine production, mouse GILZ-deficient T cells show an increase in IFN-γ production compared to wildtype T cells (112). GILZ also induces the production of Tregs and IL-10, further promoting a regulatory environment and increased production of IL-10 (113).

GILZ regulates cytokine production in T cells and other immune cells largely through inhibition of the transcriptional activity of AP-1, NF-κB, and NFAT transcriptional factors. For example, GILZ binding to NF-κB results in a reduction in IL-2 and IL-2R (103, 114) and regulation of T cell activation (103) and also regulates IL-2 production via binding to AP-1. In human T cells GILZ also inhibits IL-5 production via AP-1 binding, resulting in a negative correlation between expression of IL-5 and GILZ (115). These findings are particularly important for reducing inflammation in a chronic inflammatory disease like SLE, which is characterized by hyperactive T cell responses including T cell-dependent B cell activation.

Next to the classical Th1 and Th2 cells, GILZ also affects Th17 cells. We and others have shown that GILZ inhibits the differentiation of Th17 cells (116, 117). Mechanistically, this is thought to be mediated via direct binding of GILZ to the promoters of Th17 genes including Batf, Stat3, Irf4, and ROR-γt (117). Knockdown of GILZ increases expression of Th17 transcription factors (Rorc, Rbpj, and Batf), cytokines (IL-17A and IL-21) and also reduces Foxp3 expression (117). Furthermore, we have demonstrated, in studies of GILZ-deficient mice, that endogenous GILZ inhibits production of Th17-inducing cytokines IL-1β, IL-23, and IL-6 from bone marrow-derived dendritic cells, further limiting Th17 differentiation (116). Also, using the imiquimod-induced psoriasis model, we have found that GILZ deficiency increases IL-17A, IL-1β, IL-6, and IL-23 in skin lesions. However, these findings may contrast the results of Carceller et al. that demonstrate lesional expression IL-17F, IL-22, and IL-23 increases upon systemic exogenous GILZ overexpression in the same model (116, 118). In human psoriatic lesions, GILZ expression is decreased and correlates negatively with Th17-related pro-inflammatory cytokines IL-23, IL-17A, IL-22, and STAT3 demonstrated in human psoriatic lesions (116).

SLE is characterized by hyperactive B cells and a failure of B cell tolerance to self-antigens. GC have suppressive and cytotoxic effects on B cells (78, 119) and GILZ is able to mimic several of these effects, inhibiting cell proliferation, activation, differentiation, IgG production, and apoptosis (80, 81). We have also demonstrated a reduction in GILZ expression in B cells from both SLE patients and in a lupus prone mouse model (11). Our study also demonstrated that GILZ deficiency results in lupus-like autoimmunity in aged mice, manifesting as excessive B cell responses to T dependent stimulation and the upregulation of genes which promote germinal center B cell phenotype, lupus susceptibility genes and genes for B cell survival and proliferation (11). The consequences of GILZ deficiency *in vivo* in these experiments included spontaneous production of lupus-related autoantibodies including ANA, anti-dsDNA, and anti-Sm, as well as immune complex glomerulonephritis. Additionally, treatment of human B cells with GILZ protein suppressed their responsiveness to T dependent stimuli, providing more evidence that GILZ is a regulator of B cell activity and proof-of-principle that therapeutic supplementation of GILZ could negative regulate B cell activation in SLE.

Deletion of GILZ in mouse models has also been demonstrated to result in an increase in B cell numbers in bone marrow, blood, and lymph nodes (119). This is a corollary of the effect on B cell numbers of GC, which cause a decrease of B cells in several organs and circulation. Thus, GILZ is a key mediator in the regulation of B cell survival. This increase in B cell survival in GILZ-deficient mouse models correlates with an increased NF-κB activity and Bcl-2 expression (119). GILZ regulation of inflammatory immune responses is further demonstrated in a colitis mouse model where GILZ deficient mice had increased IFN-γ production by B cells, increased $CD4^+$ T cell activation and enhanced AP-1 activity (120). These mouse models demonstrate proof of principle for the potential therapeutic effect of GILZ in regulating B cell-dependent inflammatory diseases, wherein increased colitis in the setting of GILZ deficiency was reversible via GILZ protein administration (120).

The ability of GILZ to modulate the activation of several signal transduction pathways also affects the maturation of DCs. This is supported in GILZ overexpression models, where GILZ mimicked the inhibitory effects of GC on human DC maturation and activation. For example, GILZ caused a decrease in the expression of DC activation markers (CD80, CD86, and CD83), less IL-12 production and increased IL-10 production (121, 122). GILZ overexpression in DCs can also cause DCs to favor the induction of Tregs over T effector cells (123). Thus, it is suggested that GILZ supports an alternative pathway of activation and differentiation of DCs, leading to a more tolerogenic DC phenotype (122). This has also been demonstrated in mouse studies wherein GILZ affected splenic DC function by inhibiting macropinocytosis and inhibited antigen uptake by CD8a-positive mouse DCs, which had the highest level of GILZ of the splenic DC subsets (124).

Modulation of the NF-κB pathway by GILZ also reduces macrophage activation, as illustrated by the reduced expression of CD80, CD86, TLR 2, and chemokines CCL5 and CCL3 (102). TLR 4 stimulation of GILZ deficient bone marrow derived macrophages results in enhanced NF-κB and AP-1 activity (125). As well as cytokine and chemokine production, GILZ also decreases the phagocytic capacity of macrophages (126). Interestingly, this effect is opposite to the effect of GC, which promote phagocytosis by macrophages. Additionally, GILZ also regulates several neutrophil functions including their activation through inhibition of the MAPK pathway (127), their migration (via annexin A1) (128) and apoptosis (associated with caspases 3, 8, 9) (129, 130).

Interestingly, GILZ is not only regulated by GC, but can also be induced by IL-4, IL-10 and curcumin (102, 131–133). Furthermore, studies in macrophages, *in vitro*, *ex vivo*, and *in vivo*, have shown that TLR 1/2 and TLR 4 stimulation reduce GILZ mRNA and protein levels (126, 134). Similar findings were reported in T cells, where TCR triggering reduces GILZ expression (103). These data indicate a feedback loop where GILZ is higher in unactivated immune cells and decreases upon their activation.

THERAPEUTIC POTENTIAL AND DEVELOPMENT OF GILZ DELIVERY

In previous attempts to find safer GC replacement therapies, much research has focussed on selective glucocorticoid receptor agonists and modulators, termed SEGRAMs (135). These compounds were designed to address the hypothesis that GR transactivation was responsible for GC-induced adverse effects, and GR transrepression for anti-inflammatory effects, such that compounds targeting transrepression might be powerfully therapeutic without the side effects of GC. However, it is now known that transactivation effects of GC, such as the induction of GILZ and other GC-induced immune regulators such as DUSP1 (136), are required for the anti-inflammatory effects of GC *in vivo* (137), while adverse effects from GC such as osteoporosis are mediated by both transactivation and transrepression (138).

The potential of GILZ to be the target of a new therapeutic for SLE not only relies upon its immunosuppressive ability but also on a lack of detrimental metabolic effects. Evidence to date is encouraging, although more research is needed. GCs have an inhibitory effect on osteoblast formation, which accounts partially for the rapid bone loss seen in GC-treated patients (139). In contrast, GILZ may exert the opposite effect. Mesenchymal stem cells (MSC) can differentiate into osteoblasts or adipocytes; GILZ expression in MSC increases osteogenic differentiation and inhibits adipocyte formation (140, 141). Furthermore, osteogenic differentiation and development has been shown to be reduced by silencing GILZ (141). The underlying mechanism for this shift in differentiation includes GILZ binding to the tandem repeat of the CCAAT/enhancer binding protein (C/EBP) site in the promotor of peroxisome proliferator-activated receptor gamma-2 (PPARγ2). This decreases PPARγ2 expression, a regulator of adipocyte differentiation (140, 141). Thus, GILZ may play a role in enhancing or stabilizing bone density, rather than inhibiting osteoblast formation and inducing rapid bone loss as seen in GC treated patients. Furthermore, osteoblast-restricted GILZ overexpression resulted in a phenotype characterized by high bone mass, increased bone formation, and increased osteoblast numbers (142). Whereas, the effects of GILZ on osteoblast differentiation are opposite to the effects of GC, a study by Bruscoli et al. indicates that GILZ is required for the anti-myogenic effects of GC on skeletal muscle cells (143). Since both GILZ and GR expression are correlated with protein consumption, this may be mediated by increased protein catabolism that is associated with muscle atrophy (144). In relation to other metabolic adverse effects of GC, such as gluconeogenesis, skin thinning, cataracts and/or cardiovascular side effects, it is still unknown whether GILZ is protective or contributory. Therefore, further studies are essential to determine whether GILZ induces other metabolic adverse effects of GC in order to evaluate its value as a GC replacement therapy.

Studies to date investigating the clinical potential of GILZ-based therapies for autoimmune disease have largely used mouse disease models. Proof of principle studies have demonstrated that local upregulation of GILZ expression through the administration of adeno-associated virus vector system on the day of disease onset inhibits arthritis in the collagen-induced arthritis model (112). Additionally, transgenic mouse models, creating an overexpression of GILZ in T cells, were protective against Th1 mediated colitis (111). *In vitro*, a fusion protein of GILZ with a protein transduction domain (HHpH-GILZ), allowing entry of exogenously applied GILZ to the cell, was able to induce inhibition of Th17 activation and B cell activation (11, 116). Similarly, delivery of GILZ using a transactivator of transcription (TAT)-GILZ fusion protein was able to protect against dinitrobenzene sulfonic acid–induced colitis (145). However, it should be noted that systemic GILZ overexpression may not be beneficial for all disease states. For example, in the murine imiquimod-induced psoriasis model both GILZ deficiency (116) and GILZ transgenic overexpression led to worsening of skin inflammation (118); this is of interest given the clinical finding of glucocorticoid-withdrawal induced flares of psoriasis in humans. Thus, further studies are needed to address the therapeutic utility of GILZ in different disease settings and during established disease states.

Studies utilizing truncated regions of the GILZ protein have also demonstrated therapeutic promise. One study used a peptide targeting the C-terminus region of GILZ 115-137aa from the mouse GILZ sequence. This region binds to the p65 subunit of NF-κB, inhibiting NF-κB translocation to the nucleus and DNA binding (146). This peptide was demonstrated to have therapeutic potential in experimental autoimmune encephalitis (EAE), where it decreased T cell proliferation, decreased IL-12, IFN-γ, and IL-17 production and increased IL-10 production (146). Furthermore, the GILZ peptide decreased T-bet mRNA and increased GATA-3 mRNA levels creating a Th2 T cell phenotype. A single dose of GILZ peptide on day of disease induction was protective against the development of EAE in mice (146). Another study, utilizing a similar region of GILZ 98-134aa of the

human GILZ sequence, demonstrated the anti-inflammatory activity GILZ (147). Here GILZ administration similarly suppressed the nuclear translocation of NF-κB, inhibited cytokine production and inhibited the gliosis of Muller cells (147).

Although these studies utilizing the C-terminus of GILZ are promising for use of a GILZ-based therapy in autoimmune diseases, it should be noted that the N-terminal domain of GILZ is also important for inhibition of transcription factors and signaling pathways. Therefore, all or several regions of GILZ may be required for the potent regulation of multiple inflammatory immune responses, an essential requirement of a new SLE therapy. This implies that strategies targeting the endogenous expression of natural GILZ, or its degradation, may hold greater promise. A greater understanding of the mechanisms of regulation of GILZ and its gene targets is critical to advance this field. This knowledge is also important for a potential gene therapy approach to deliver GILZ, either via expressing peptides or the entire protein. An additional method which may hold promise as a future therapeutic mechanism could be to induce GILZ expression using small molecules. For example, two SEGRAM compounds under investigation, RU24858 and ORG 214007-0, induce GILZ (135). We consider that methods to induce GILZ expression that do not utilize the GR could avoid GR-dependent metabolic effects. Additionally, a GC replacement therapy which delivers or targets GILZ could alleviate the effects of GR down-regulation by chronic GC dosing. Thus, evaluation of multiple pathways is required to lead to the development of a therapy to therapeutically induce GILZ. As GILZ is also a *bona fide* transcription factor (117), studies cataloging in full the gene targets of GILZ are also required in order to understand the targets for GILZ mimics, both for comprehending the potential for potent immune modifying effects as well as potential adverse effects.

SUMMARY

SLE is a complex chronic autoimmune disease characterized by heterogeneous clinical features as a consequence of a failure of multiple immune checkpoints. This leads to hyperactive B and T cell responses, the production of autoantibodies and formation of immune complexes, activation of innate immunity, and consequently inflammation, organ damage, morbidity and mortality. Currently there is no cure for SLE and mainstay treatment with GC causes debilitating adverse effects. GILZ represents a novel target for the induction of a potent anti-inflammatory and immune suppressive response targeting multiple signaling pathways and immune cells. Proof of principle studies have demonstrated GILZ to have significant therapeutic effects in animal models of autoimmune disease. The immunosuppressive effects of GILZ are broadly similar to those of GC and to date, other than myogenic effects, there is no evidence of adverse metabolic effects of GILZ. Further research is required, to determine whether a GILZ based therapy would have metabolic effects, and into the molecular mechanisms for the induction and targeting of GILZ. The idea of a new therapy which enhances GILZ expression, and/or targets the same molecular pathways as GILZ, is a very attractive one with the potential to provide a critically-needed replacement for GC therapy in SLE.

AUTHOR CONTRIBUTIONS

JF and EM designed the review. JF and WD wrote the review. All authors critically revised the manuscript and approved it for publication.

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Toll-Like Receptor 2 Release by Macrophages: An Anti-inflammatory Program Induced by Glucocorticoids and Lipopolysaccharide

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Glucocorticoids (GCs) are widely prescribed therapeutics for the treatment of inflammatory diseases, and endogenous GCs play a key role in immune regulation. Toll-like receptors (TLRs) enable innate immune cells, such as macrophages, to recognize a wide variety of microbial ligands, thereby promoting inflammation. The interaction of GCs with macrophages in the immunosuppressive resolution phase upon prolonged TLR activation is widely unknown. Treatment of human alveolar macrophages (AMs) with the synthetic GC dexamethasone (Dex) did not alter the expression of TLRs −1, −4, and −6. In contrast, TLR2 was upregulated in a GC receptor-dependent manner, as shown by Western blot and qPCR. Furthermore, long-term lipopolysaccharide (LPS) exposure mimicking immunosuppression in the resolution phase of inflammation synergistically increased Dex-mediated TLR2 upregulation. Analyses of publicly available datasets suggested that TLR2 is induced during the resolution phase of inflammatory diseases, i.e., under conditions associated with high endogenous GC production. TLR2 induction did not enhance TLR2 signaling, as indicated by reduced cytokine production after treatment with TLR2 ligands in Dex- and/or LPS-primed AMs. Thus, we hypothesized that the upregulated membrane-bound TLR2 might serve as a precursor for soluble TLR2 (sTLR2), known to antagonize TLR2-dependent cell actions. Supernatants of LPS/Dex-primed macrophages contained sTLR2, as demonstrated by Western blot analysis. Activation of metalloproteinases resulted in enhanced sTLR2 shedding. Additionally, we detected full-length TLR2 and assumed that this might be due to the production of TLR2-containing extracellular vesicles (EVs). EVs from macrophage supernatants were isolated by sequential centrifugation. Both untreated and LPS/Dex-treated cells produced vesicles of various sizes and shapes, as shown by cryo-transmission electron microscopy. These vesicles were identified as the source of full-length TLR2 in macrophage supernatants by Western blot and mass spectrometry. Flow cytometric analysis indicated that TLR2-containing EVs were able to bind the TLR2 ligand Pam_3CSK_4. In addition, the presence of EVs reduced inflammatory responses

in Pam_3CSK_4-treated endothelial cells and HEK Dual reporter cells, demonstrating that TLR2-EVs can act as decoy receptors. In summary, our data show that sTLR2 and full-length TLR2 are released by macrophages under anti-inflammatory conditions, which may contribute to GC-induced immunosuppression.

Keywords: innate immunity, corticosteroid, pulmonary macrophage, exosome, microvesicle

INTRODUCTION

Glucocorticoids (GCs) represent the most effective anti-inflammatory drugs in the therapy of inflammatory lung diseases. Genes that are upregulated by GC treatment, such as dual-specificity phosphatase 1 (DUSP1), GC-induced leucine zipper (GILZ), and interleukin (IL)-10, are highly immunosuppressive and contribute to the overall effect of GC treatment (1–3).

Alveolar macrophages (AMs) are the tissue-resident macrophages in the lung alveolar space. They represent the first line of defense against pathogens in the lower airspace and recognize microbial ligands *via* pattern recognition receptors (4, 5). Toll-like receptors (TLRs) are the major pattern recognition receptors of the innate immune system that sense a wide range of "danger" signals or pathogen-associated molecular patterns (PAMPs) (6–8).

To date, 10 TLRs have been identified in humans. Surface-expressed TLRs (i.e., TLR1, −2, −4, −5, −6, and −10) recognize bacterial, fungal, and parasitic PAMPs, whereas endosomal TLRs (i.e., TLR3, −7/−8, and −9) sense nucleic acids of viral or bacterial origin. After recognition and binding of a specific PAMP, TLRs induce an intracellular signaling cascade that culminates in the activation of the activator protein (AP)-1, nuclear factor (NF)-κB, and interferon regulatory factors (IRFs). These signaling cascades result in the secretion of pro-inflammatory factors that ultimately protect the host from microbial infection (6, 9).

Although GCs usually dampen TLR signaling, GC-mediated induction of TLR2 has for example been shown in dendritic cells (10), THP-1 macrophages (11), and AMs (12). TLR2 recognizes a wide variety of pathogens, including bacteria, viruses, fungi, mycobacteria, and parasites. Unlike other TLRs, the formation of TLR2 heterodimers with other TLR family members (i.e., TLR1, TLR6, or TLR10) or non-TLR cellular molecules (e.g., CXCR4 or scavenger receptors) is a prerequisite for the initiation of cell activation (13).

Since TLR2 activity plays a prominent role in the pathogenesis of numerous acute and chronic inflammatory diseases, its activation has to be tightly regulated. In general, negative regulation of TLR signaling can be mediated by soluble factors, including soluble TLRs (sTLR) that act as decoy receptors and bind to PAMPs in the extracellular space, preceding their engagement with specific PRRs and reducing TLR signaling efficiency (14–16). sTLR2 is produced *via* proteolytic cleavage of the TLR2 trans-membrane protein, also referred to as ectodomain shedding, by disintegrin metalloproteinases (ADAMs) (17). Elevated sTLR2 plasma levels were observed in experimental models of human endotoxemia and sepsis patients and have therefore been suggested as a biomarker for infections (18, 19).

Sepsis represents a life-threatening systemic inflammation caused by bacterial infections. If sepsis patients survive the acute inflammatory response, compensatory mechanisms result in profound immunosuppression, often leading to lethal secondary infections. Several critical factors have been identified that contribute to the transition of the pro-inflammatory phase into the immunosuppressive phase, including endogenous GCs (1, 20, 21). In addition, prolonged exposure to bacterial components, such as lipopolysaccharide (LPS), skews monocytes and macrophages toward a hypo-responsive state termed LPS tolerance. LPS-tolerant cells are characterized by a decreased ability to produce pro-inflammatory mediators whereas their expression of mediators involved in immunosuppression and wound healing is elevated (20).

In the present study, we examined TLR2 expression in primary human AMs after GC administration and in chronic inflammation, as mimicked by prolonged LPS treatment.

Abbreviations: ACTB, beta actin; ADAM, a disintegrin and metalloproteinase; AFC, 7-amino-4-trifluoromethylcoumarin; AMs, alveolar macrophages; ANXA1, Annexin A1; AP-1, activator protein-1; APC, allophycocyanine; CCL, CC-chemokine ligand; COX2, cyclooxygenase-2; CXCL, C–X–C motif ligand; Dex, dexamethasone; DMEM, Dulbecco's modified Eagle medium; DUSP1, dual-specificity phosphatase-1; EVs, extracellular vesicles; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; flTLR, full length TLR; FPR2, formyl peptide receptor 2; GC, glucocorticoid; GILZ, glucocorticoid-induced leucine zipper; HEK, human embryonic kidney; HKSA, heat-killed *Staphylococcus aureus*; ICAM1, intercellular adhesion molecule 1; IL, interleukin; IL1RN, IL1 receptor antagonist; IFN, interferon; IRFs, interferon regulatory factors; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; NOS2, nitric oxide synthase 2; NTA, nanoparticle tracking analysis; MAPK, mitogen-activated protein kinase; PE, phycoerythrin; MARCO, macrophage receptor with collagenous structure; PMA, phorbol 12-myristate 13-acetate; Poly(I:C), polyinosinic:polycytidylic acid; PRR, pattern recognition receptor; RPMI, Roswell Park Memorial Institute; SELE, selectin E; STAT, signal transducer and activator of transcription; sTLR, soluble TLR; TCA, trichloroacetic acid; TEM, transmission electron microscopy; TNF, tumor necrosis factor; TLR, toll-like receptor; VCAM1, vascular cell adhesion molecule 1; VCAN, versican.

MATERIALS AND METHODS

Materials

RPMI1640 (#R0883), DMEM (#D6546), trypsin/EDTA (#T3924), fetal calf serum (FCS, #F7524), penicillin / streptomycin (#P433), kanamycin (#K0254), and glutamine (#G7513) were from Sigma-Aldrich. Endothelial cell growth media (#C-22010) including supplement mix (#C-39215) were from PromoCell. The anti-TLR2 antibody used for Western blot analysis was obtained from Abcam (EPNCIR133, #ab108998). The Phospho-p38 MAPK (Thr180/Tyr182, 3D7, #9215) and total

p38 MAPK (#9212, polyclonal) antibodies were from Cell Signaling. The anti-tubulin antibody (#T9026) was obtained from Sigma-Aldrich. Anti-rabbit IRDye 680- and anti-mouse IRDye 800-conjugated secondary antibodies were from LI-COR Biosciences (#926-68071, #926-32210). The anti-rabbit IRDye 800-conjugated secondary antibody was from Rockland (#612-132-120). APC-labeled anti-TLR2 and the respective isotype control were from ThermoFisher Scientific (TL2.1, # 17-9922-41; IgG2a kappa Isotype Control #17-4724-81). FITC anti-CD9 (HI9a, #BLD-312103), FITC anti-CD63 (H5C6, #BLD-353005), and the respective isotype control (MOPC-21, #BLD-400109) were purchased from Biozol. The Zombie Yellow™ Fixable Viability Kit (#423103) was from BioLegend. Ultrapure LPS from *Escherichia coli* K12 (#tlrl-peklps), Pam_3CSK_4 (#tlrl-pms), rhodamine-labeled Pam_3CSK_4 (#tlrl-rpms), Pam_2CSK_4 (#tlrl-pm2s), heat-killed *Staphylococcus aureus* (#tlrl-hksa), lipoteichoic acid (LTA, # tlrl-pslta), normocin (#ant-nr-1), and zeocin (#ant-zn-1) were obtained from Invivogen. Phorbol 12-myristate 13-acetate (PMA, # 524400) was from Cayman Chemical. Dexamethasone (#D8893) was obtained from Sigma-Aldrich. Dexamethasone stock solutions were either prepared in DMSO or ethanol (EtOH), and the appropriate vehicle control is indicated in the figure legends. Alternatively, water-soluble dexamethasone 21-phosphate disodium salt (Sigma-Adrich, #D1159) was dissolved in medium, and untreated cells served as a control (**Figures 6C,D**, **7**, and **8**). Primers and dual-labeled probes were from Eurofins MWG Operon. Taq polymerase (5 U/μL, #E00007), Taq buffer (#B0005), and the dNTP mix (#D0056) were from Genscript. Other chemicals were obtained from either Sigma-Aldrich or Carl Roth unless stated otherwise.

Cell Culture

Cell Lines

THP-1 (#TIB202) and L929 cells (#CRL-6364) were obtained from ATCC and grown in RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 2 mM glutamine. THP-1 were differentiated into macrophage-like cells by treatment with PMA (100 nM) for 48 h. HEK-Dual™ hTLR2 reporter cells (Invivogen, #hkd-htlr2ni) were grown in DMEM supplemented with 10% FCS, 2 mM glutamine, 50 U/mL penicillin G, 50 μg/mL streptomycin, 100 μg/mL normocin, and 100 μg/mL zeocin.

Human Alveolar Macrophages (AMs)

Human lung tissue was obtained from patients undergoing lung resection. The use of human material was reviewed and approved by the local ethics committee (State Medical Board of Registration, Saarland, Germany; permission no. 213/06). The informed consent of all participating subjects was obtained. AM isolation was performed according to a previously described method (4, 22, 23) with minor modifications. After visible bronchi were removed, the lung tissue was chopped and washed with PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The washing buffer was collected and centrifuged (15 min, 350 x g). Remaining erythrocytes were lysed by briefly resuspending the pellet in autoclaved water, followed by immediate washing with PBS and centrifugation. Cells were resuspended in AM medium (RPMI 1640 containing 5% FCS, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 2 mM glutamine). Unless stated otherwise, AMs were seeded at a density of 0.5–1 × 10^6 cells/well into a 12- or 6-well plate and incubated at 37°C for 2 h, washed with PBS, and cultured overnight before further use. AM preparations were 95% pure as judged by flow cytometric analysis of intracellular CD68 (4, 24).

Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs were isolated from umbilical cords provided by the Klinikum Saarbrücken (Saarbrücken, Germany; ethics committee permission no. 131/08). The informed consent of all donors was obtained. HUVEC isolation and culture was performed as described previously (25, 26). In brief, HUVECs were isolated by digestion of umbilical veins with 100 mg/L collagenase A (Roche, Mannheim, Germany). Cells were grown in endothelial growth medium with supplement mix, 100 U/mL penicillin G, 100 μg/mL streptomycin, 50 mg/mL kanamycin, and 10% FCS. For all experimental procedures, HUVECs were used in passage three. Cells were detached with trypsin/EDTA, seeded at a density of 1 × 10^5 cells per well in a 24-well plate and incubated overnight before further treatment. HUVECs were >95% pure, as assessed by flow cytometry using an antiserum against the von Willebrand factor (27).

TNF-α Bioassay

TNF-α concentrations in cell culture supernatants were quantified by bioassay as previously described (28). L929 cells were seeded into a 96-well plate (3 × 10^4 cells per well) and incubated overnight at 37°C, 5% CO_2. The medium was discarded, and 100 μL of actinomycin D solution (1 μg/mL in growth medium) was added. After incubation for 1 h at 37°C, AM supernatants (100 μL per well) were added. Dilution series of recombinant human TNF-α (100–2,500 pg/mL) were run alongside the samples to generate a standard curve. The plate was incubated for 24 h at 37°C, followed by incubation with MTT solution (0.5 mg/mL in medium) for 2 h. The supernatant was discarded, and cells were lysed in 100 μL DMSO. Absorbance measurements were carried out at 550 nm with 630 nm as the reference wavelength using a microplate reader (Tecan Sunrise).

RNA Isolation, Reverse Transcription, and Quantitative RT-PCR

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, #74134) or the High Pure RNA Isolation Kit (Roche, # 11828665001), and RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368813) according to the manufacturer's instructions. The cDNA was diluted with TE buffer (Applichem, #A0386) before use. The CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) was used for real-time RT-PCR. For *ACTB*, *CXCL10*, *IL10*, *TLR1, TLR2, TLR4, TLR6,* and *TNF*, one 25 μL reaction mix contained 2.5 U Taq polymerase, 500 nM sense and antisense primers, 60-100 nM probe, 200 μM dNTPs, 3-4 mM $MgCl_2$, 2.5 μL 10x Taq buffer, 3 μL Template, and molecular biology grade water (Applichem, #A7398). The reaction conditions were 95°C for 8 min followed by 40 cycles of 15 s at 95°C, 15 s at a reaction dependent temperature varying from 57 to 60°C, and 15 s at

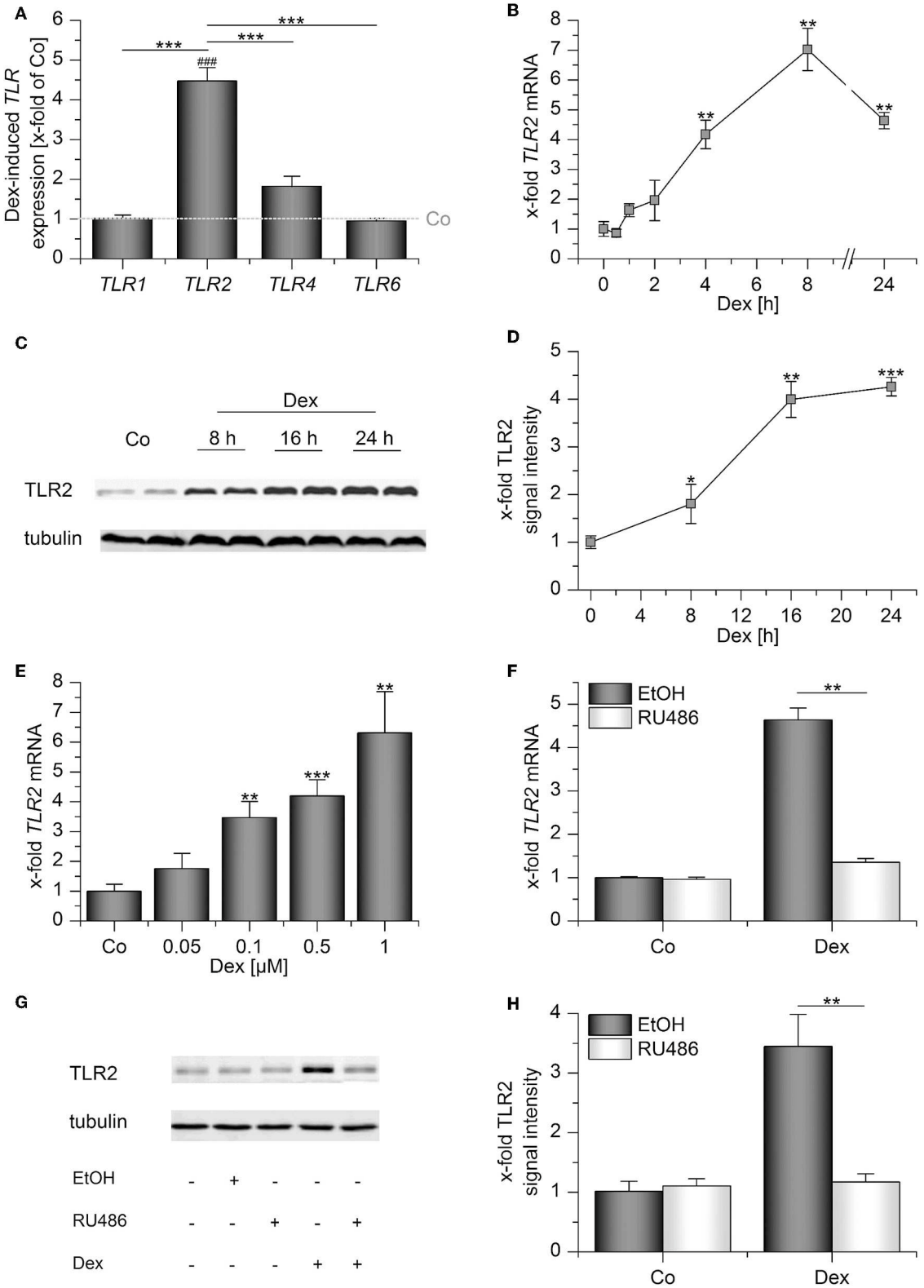

FIGURE 1 | Dexamethasone induces TLR2 in AMs. **(A–E)** AMs were incubated with solvent control (0.1% DMSO, Co) or dexamethasone (Dex, 1 μM) for up to 24 h **(A–D)** or at the indicated concentrations for 4 h **(E)**. **(E–G)** AMs were preincubated with the GR inhibitor RU486 (10 μM) or solvent control (0.1% EtOH) and treated

(Continued)

FIGURE 1 | with Dex (1 μM) for 24 h. Data from at least three independent experiments performed in duplicate with cells from different donors are presented as means ± SEM. TLR expression was measured by or qPCR **(A,B,E,F)** or Western blot **(C,D,G,H)**. **(A)** TLR expression upon Dex treatment was normalized to the TLR expression values for the respective vehicle-treated control (indicated by the dotted line). **(B–H)** TLR2 expression in solvent-treated cells were set as 1. **(C,G)** Representative blots. **(D,H)** Densitometric analysis. TLR2 signal intensities were quantified and normalized to the loading control tubulin. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#\#\#}p < 0.001$ vs. vehicle-treated cells. *p*-values were generated by ANOVA with Bonferroni's *post-hoc* test or Mann–Whitney *U*-test.

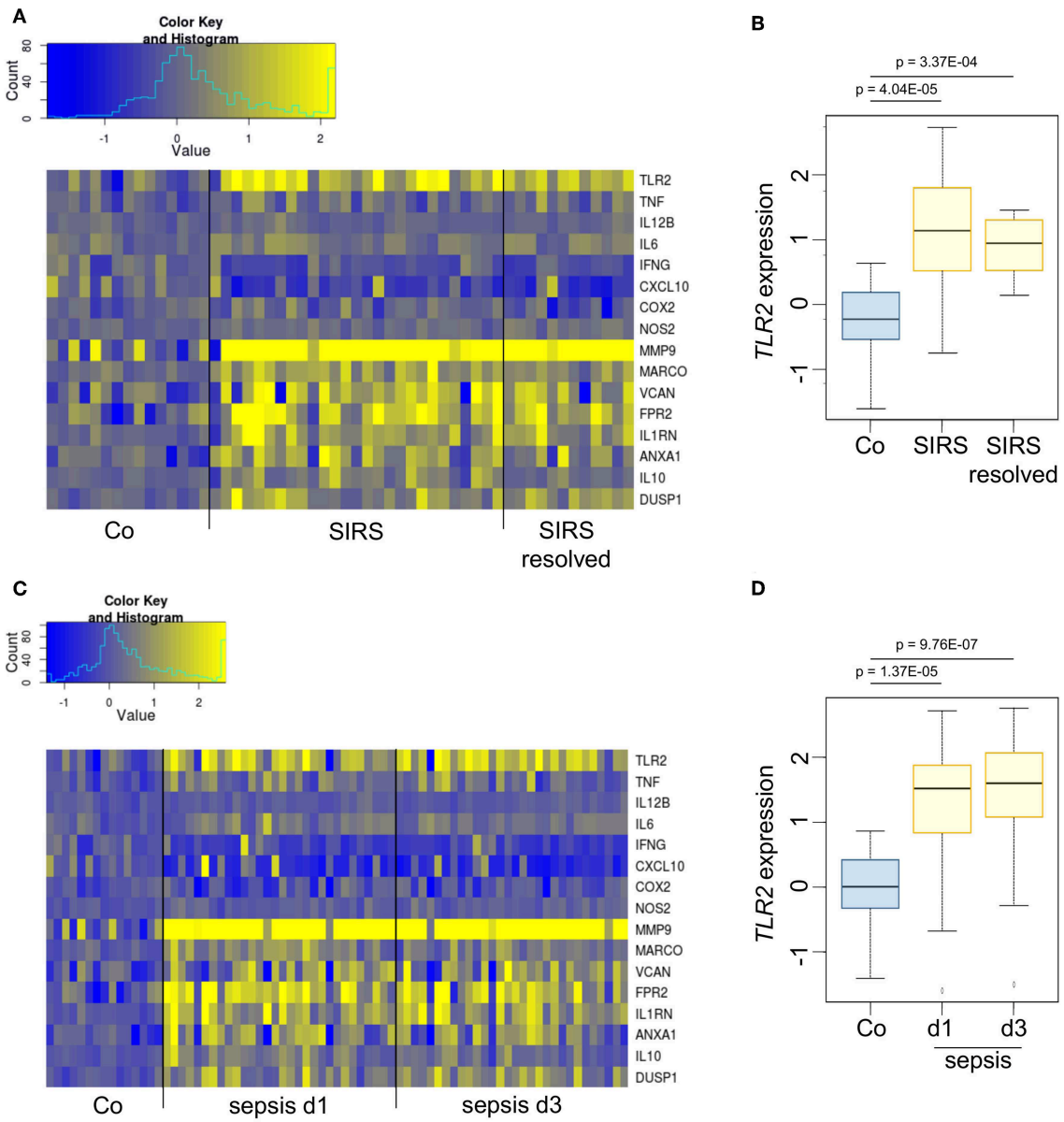

FIGURE 2 | TLR2 is overexpressed during the immunosuppressive phases of SIRS and sepsis. **(A,B)** Dataset GSE4607 was obtained from Gene Expression Omnibus (GEO) and normalized using log2-RMA. The dataset included transcriptional profiles of human whole blood samples of 15 healthy controls, 27 patients with non-infectious SIRS and 12 samples from patients with resolved non-infectious SIRS. Patients were classified as SIRS, or SIRS resolved (no longer meeting criteria for SIRS) on d3 after ICU admittance. **(C,D)** Dataset GSE8121 was retrieved from GEO and normalized using log2-RMA. The dataset included transcriptional profiles of human whole blood samples of 15 healthy controls and 30 patients with sepsis. Samples were obtained at d1 and d3 after admittance to the ICU. The statistical significance was determined by the Kolmogorov–Smirnov test.

72°C. For *ADAM10*, *ADAM17*, *CCL2*, *DUSP1*, *FPR2*, *ICAM*, *MMP9*, *SELE*, and *VCAM* detection, the 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis Biodyne, #08-25) was used according to the manufacturer's recommendations. Primer and probe sequences, as well as specific reaction conditions, are given in **Supplementary Table 1**. Standard curves were generated by using a dilution series of the PCR product cloned into pGEMTeasy (Promega, #A1360) (23, 28, 29). All samples

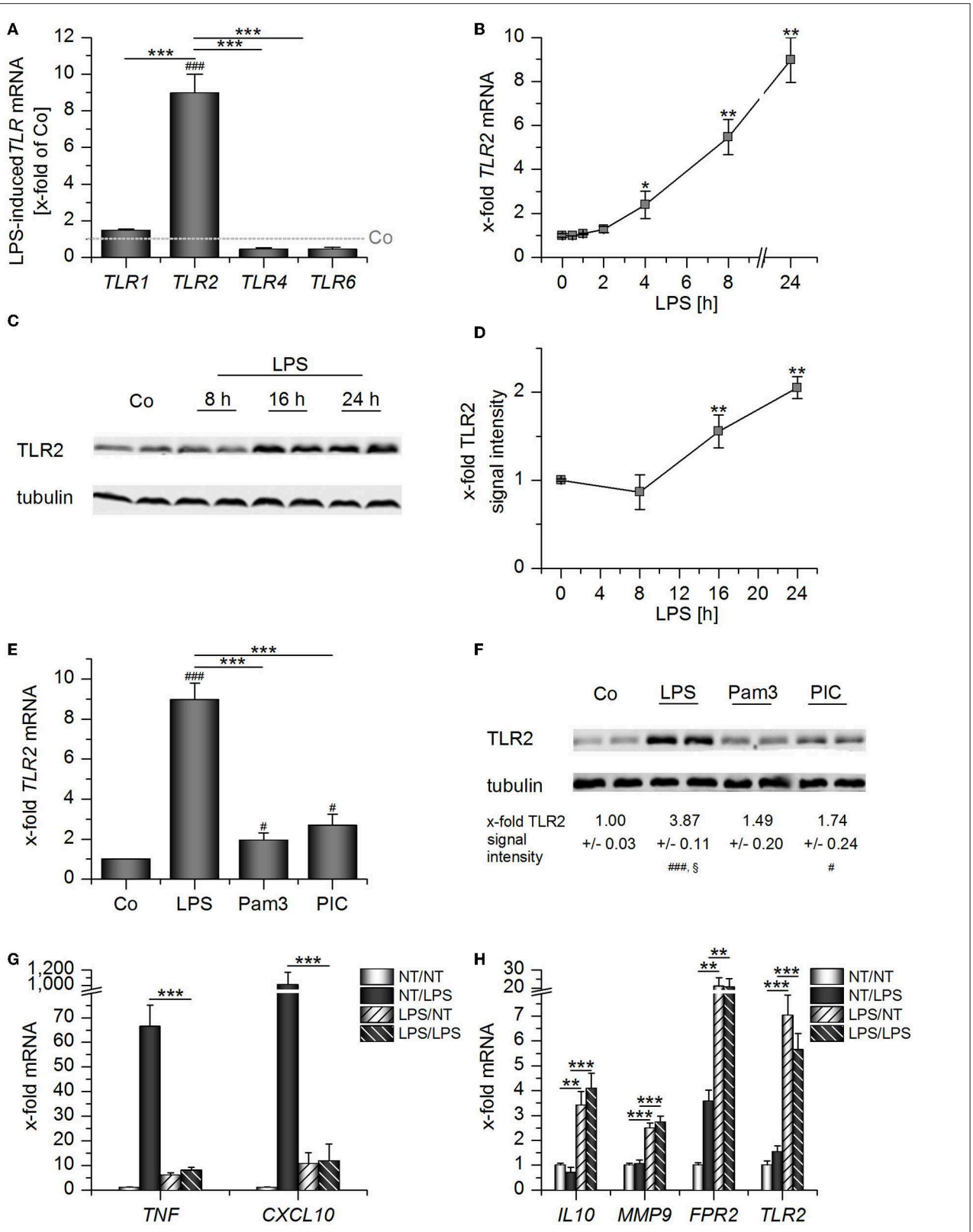

FIGURE 3 | Long-Term LPS exposure upregulates TLR2 in AMs. **(A–D)** AMs were incubated with LPS (100 ng/mL) for 24 h **(A)** or the indicated time points **(B–D)**. TLR expression was measured by qPCR **(A,B)** or Western blot **(C,D)**. **(A)** TLR expression was normalized to the TLR expression values for the respective

(Continued)

FIGURE 3 | vehicle-treated control which was set as 1 (indicated by the dotted line). **(E–F)** AMs were treated with LPS (100 ng/mL), Pam_3CSK_4 (Pam3, 100 ng/mL) or Poly(I:C) (PIC, 1 μg/mL) for 24 h. TLR2 expression was analyzed by qPCR **(E)** and Western blot **(F)**. **(G,H)** Long-Term LPS exposure results in LPS tolerance. AMs were pretreated with LPS (100 ng/mL) for 24 h and restimulated with LPS (1 μg/mL) for 2 h. *TNF*, *CXCL10*, *IL10*, *MMP9*, *FPR2*, and *TLR2* mRNA expression levels were determined by qPCR. NT/NT, not treated; NT/LPS, LPS stimulation without pretreatment; LPS/NT, LPS pretreatment only; LPS/LPS, LPS pretreatment followed by LPS stimulation. Data from at least three independent experiments performed in duplicate with cells from different donors are shown and are presented as x-fold of solvent-treated cells ± SEM. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. untreated cells, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as indicated, $^{\S}p < 0.05$ vs. Pam3-treated cells. *p*-values were generated with ANOVA and Bonferroni's *post-hoc* test.

and standards were analyzed in triplicate. All samples were normalized to the housekeeping gene *ACTB*.

Extracellular Vesicles (EVs)

Isolation

AMs or differentiated THP-1 cells were incubated for 3 d in FCS-free medium in the presence or absence of LPS (100 ng/mL) and/or dexamethasone (1 μM). EVs were purified from cell culture supernatants by sequential centrifugation as previously described (30). For THP-1-derived vesicles, 5×10^7 cells were used per preparation. After differentiation for 48 h, cells were washed with PBS, and FCS-free medium was added. Serum deprivation did not result in increased cell death, as indicated by caspase 3 assay and Zombie Yellow staining (**Supplementary Figure 1**).

Cell culture supernatants were collected and centrifuged at 300 × g for 10 min to remove remaining cells, followed by removal of dead cells and large cell debris by centrifugation at 2,000 × g for 10 min and 10,000 × g for 30 min. Supernatants were transferred into stable polycarbonate tubes (# 4416, Laborgeräte Beranek), and EVs were collected by ultracentrifugation at 100,000 x *g* for 90–120 min in an L70 ultracentrifuge with a 70Ti rotor (Beckman Coulter). EVs were washed with 25 mL sterile-filtered PBS and pelleted again by ultracentrifugation (100,000 × g, 90–120 min). The EV pellet was then resuspended in sterile-filtered PBS (AMs: 200–350 μL; THP-1: 200 μL) and stored at −80°C in protein LoBind microcentrifuge tubes (# Z666505, Eppendorf).

Nanoparticle Tracking Analysis (NTA)

For nanoparticle tracking analysis (NTA), EV suspensions were diluted 1:200 in sterile-filtered PBS. 300–500 μL of the dilution were injected into the sample chamber of a NanoSight LM10 (NanoSight Ltd). A video of 60 s was recorded and analyzed by the NTA software Nanosight NTA 2.3 to calculate vesicle size and concentration.

Protein Concentration

Total protein concentrations were determined with the Pierce BCA protein assay kit (ThermoFisher Scientific, #23225) using a GloMax® Discover Multimode Microplate Reader (Promega) according to the manufacturer's instructions.

Cryo-Transmission Electron Microscopy (TEM)

A 3 μL droplet of the aqueous EV dilution was placed onto a holey carbon covered TEM grid (Plano, type S147-4), plotted onto a thin liquid film for 2 s and plunged into a bath of liquid ethane at −165°C using a Gatan CP3 cryoplunger (Pleasanton). The frozen sample was transferred under liquid nitrogen to a Gatan cryo-TEM sample holder (model 914) and investigated at −173°C by low-dose bright-field imaging TEM (JEOL JEM-2100 LaB6). A Gatan Orius SC1000 CCD camera was used for image acquisition.

Proteomics

Thirty micrograms of EV protein were precipitated by trichloroacetic acid (TCA) precipitation with an end concentration of 20% TCA. Samples were washed thrice with acetone. After a final centrifugation of 15 min in a SeedVac Plus concentrator (Savant, Thermo Fisher, Waltham, USA), samples were resuspended in 2x Lämmli buffer (4% SDS, 20% glycerol, 120 mM Tris-HCl (pH 6.8), 0.02% bromophenol blue in Millipore water) and denatured at 95°C for 5 min. Proteins were separated on NuPAGE® 10% gels and prepared for mass spectrometry as described previously (31). Three protein bands per sample were cut out of the gel and incubated with porcine trypsin (Promega, #V5111) for in-gel digestion at 37°C overnight. Resulting peptides were extracted twice by shaking the gel pieces in aqueous extraction buffer (2.5% formic acid, 50% acetonitrile). Extracted peptides were concentrated via vacuum centrifugation and resuspended in 0.1% formic acid. Six microliters of each tryptic peptide extract were analyzed by online nanoflow LC-HR-MS/MS (Ultimate 3000 RSLC nano system equipped with an Ultimate3000 RS autosampler coupled to an LTQ Orbitrap Velos Pro, ThermoFisher Scientific) as described previously (31). Peptides were analyzed at a flow rate of 200 μL/min with buffer A (water and 0.1% formic acid) and B (90% acetonitrile and 0.1% formic acid) using the gradient given in **Supplementary Figure 2**. Fragmented peptides were identified using software Proteome Discoverer 1.4 (ThermoFisher Scientific) and database SwissProt 2015_01 (species human). For further data evaluation, software Scaffold4 (version 4.8.3) was used. In order to allow expression of x-fold values if a protein was absent in one of the treatments, log2 fold changes were calculated as log2[(mean of unique spectrum counts in $EV_{LPS+Dex}$) + 0.1) / (mean of unique spectrum counts in EV_{Co} + 0.1)]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (32) with the dataset identifier PXD013977 and 10.6019/PXD013977.

Flow Cytometry

For analysis of EV surface proteins by flow cytometry, vesicles were coupled to the surface of 4 μm aldehyde/sulfate latex beads (Invitrogen, #A37304). In detail, an amount of EVs resembling 10 μg protein or the same amount of the negative control BSA were allowed to bind to 10 μL latex beads for 15 min at room

temperature in a final volume of 100 μL in PBS. After adding 400 μL PBS, samples were incubated for 1 h at room temperature with gentle shaking. The reaction was stopped by adding 500 μL 200 mM glycine, followed by incubation for 30 min at room temperature. EV- or BSA-coupled beads were washed three times with 1% BSA in PBS, with centrifugation steps at 2,000 × g for 3 min in between. Samples were stained with fluorescently labeled antibodies directed against TLR2 or the EV markers CD9 and CD63 or the respective isotype controls on ice in the dark. Staining with rhodamine-labeled Pam_3CSK_4 was performed accordingly. Details are given in **Supplementary Table 2**. After 30 min, samples were washed twice with 1% BSA in PBS and analyzed on a BD LRS Fortessa (BD Biosciences) using BD FACSDiva 8.0. For graphical illustrations, BD FACSuite (version 1.0) software was used.

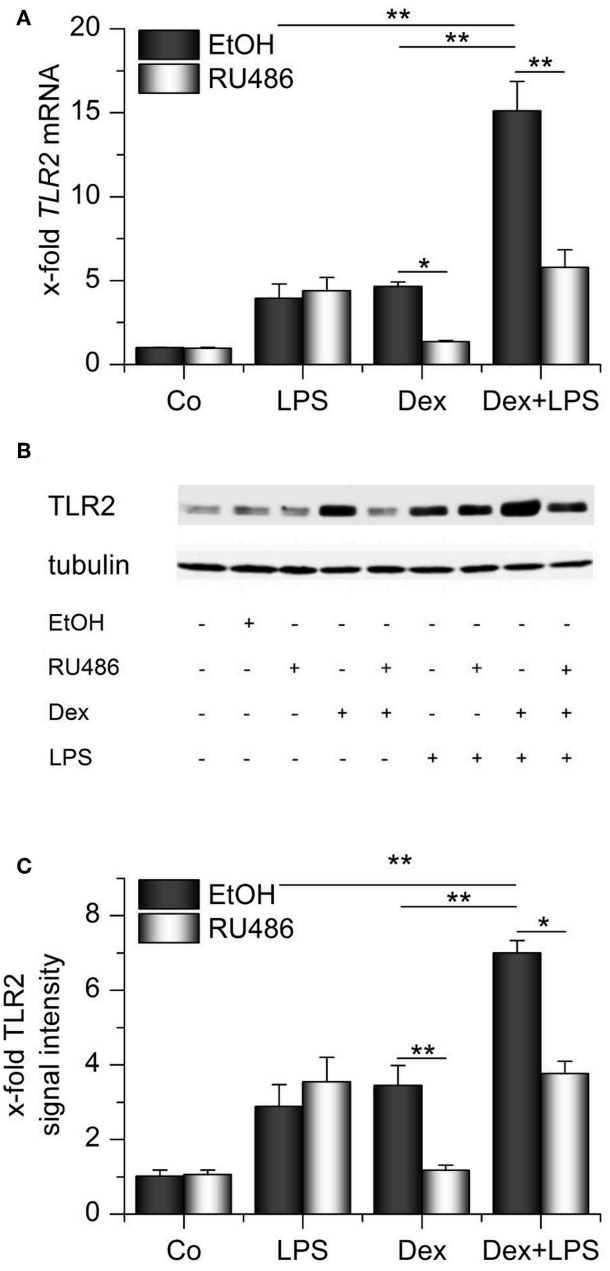

FIGURE 4 | Synergistic upregulation of TLR2 by dexamethasone and LPS. AMs were preincubated with the GR inhibitor RU486 (10 μM) or solvent control (0.1% EtOH) and treated with LPS (100 ng/mL), Dex (1 μM) or both for 24 h. TLR2 expression was measured by qPCR (A) or Western blot **(B,C)**. **(B)** Representative blot. **(C)** Densitometric analysis. TLR2 signal intensities were quantified, normalized to tubulin values, and expressed as x-fold of untreated cells. **(A,C)** Data from at least three independent experiments performed in duplicate with cells from different donors are presented as means + SEM. $^*p < 0.05$, $^{**}p < 0.01$. *p*-values were generated by ANOVA with Bonferroni's *post-hoc* test.

Western Blotting

For whole cell analysis, cells were lysed in lysis buffer (50 mM Tris-HCl, 1% (m/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.004% (m/v) bromphenol blue) supplemented with a protease inhibitor mix (cOmplete; Roche Diagnostics, #04693124001). Samples were sonicated, centrifuged at 10,000 × g for 10 min at 4°C, and stored at −80°C until further use (23, 28, 29, 33). Cell culture supernatants from AMs cultured in a 12 well plate (5×10^5 cells per well in 300 μL medium) were concentrated 10x by centrifugation at 15,000 × g for 8 min in Vivaspin® 500 tubes with 10 kDa cut off (Sartorius #VS0102). Concentrated supernatants (21 μL per lane), as well as isolated EVs (5×10^9 vesicles per lane), were supplemented with a 4x loading buffer (Carl Roth, Roti®-load 1, #K929.1). Before gel electrophoresis, all samples were denatured at 95°C for 5 min and subsequently kept on ice before gel loading.

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out using polyacrylamide gels (4% stacking gel, 12% resolving gel) and the Mini-PROTEAN® system (Bio-Rad). A prestained protein ladder was used to estimate the molecular mass (#26616, ThermoFisher Scientific). Samples were transferred onto an Immobilon FL-PVDF membrane (# IPFL00010, Millipore-Merck, Darmstadt, Germany) using a Mini Trans-Blot® Cell (Bio-Rad). The membrane was blocked for 1–4 h at room temperature in blocking buffer for near-infrared fluorescent Western blotting (#MB-070, Rockland) to saturate unspecific binding sites. Subsequently, the membrane was incubated with primary antibody dilutions (1:500–1:2,000 in Rockland blocking buffer) at 4°C, either overnight or for 48 h. After thorough washing with PBST (PBS + 0.1% Tween-20), the membrane was stained with IRDye680- or IRDye800-conjugated secondary antibodies (1:5,000–1:10,000) diluted in blocking buffer for 1.5-2 h at room temperature, washed again, and signals were detected and quantified using an Odyssey imager and software (LI-COR Biosciences). For densitometric analysis, signal intensities were normalized to the loading control tubulin except for pp38 which was normalized to values for total p38.

Viability Assays

Caspase 3-Like Assay

Cells were washed twice with ice-cold PBS. Seventy microliters ice-cold lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, 0.1% [v/v] Triton X-100) were added, and the samples

were stored at −80°C. After thawing on ice, the lysates were centrifuged (14,000 × g, 10 min, 4°C) and 10 µL of the supernatant were transferred to a black 96 well plate (TPP). 90 µL substrate solution (55 µM of fluorogenic substrate Ac-DEVD-AFC (Enzo, #ALX-260-032-M005) 50 mM HEPES, 0.1% [w/v] CHAPS, 1% [w/v] sucrose, 10 mM DTT, pH 7.5) were added, and the production of free 7-amino-4-trifluoro-methyl coumarin (AFC) at 37°C was determined by fluorescence measurement (excitation: 405 nm; emission: 495-505 nm) using a GloMax® Discover Multimode Microplate Reader (Promega).

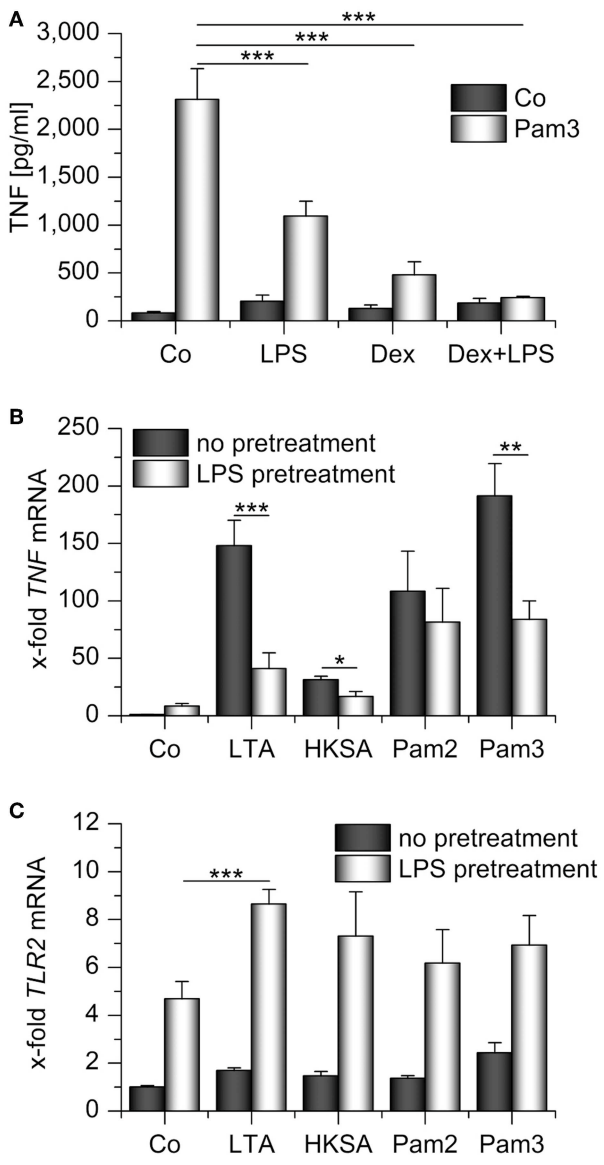

FIGURE 5 | Impaired response toward TLR2 ligands in LPS- and/or Dex-pretreated AMs. **(A)** AMs were preincubated with LPS (100 ng/mL), Dex (1 µM), or both for 24 h and treated with Pam_3CSK_4 (Pam3, 1 µg/mL, 4 h). TNF secretion was assessed by TNF bioassay. **(B,C)** Primary human AMs were incubated with LPS (100 ng/mL, 24 h) before restimulation with TLR2 ligands for 2 h. LTA: lipoteichoic acid (5 µg/mL), HKSA: heat-killed *S. aureus* (10^8 cells/mL), Pam2: Pam_2CSK_4 (1 µg/mL), Pam3: Pam_3CSK_4 (1 µg/mL). *TNF* **(B)** and *TLR2* **(C)** mRNA levels were determined by qPCR. Data from at least three independent experiments performed in duplicate with cells from different donors are presented as means + SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *p*-values were generated by ANOVA with Bonferroni's *post-hoc* test.

Zombie Yellow Staining

Cells were stained with the Zombie Yellow™ Fixable Viability Kit as recommended by the supplier. Samples were analyzed on a BD LRS Fortessa (BD Biosciences) using BD FACSDiva 8.0 software.

HEK-Dual hTLR2 Reporter Assay

HEK-Dual™ hTLR2 reporter cells express TLR2, an NF-κB/AP1-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene, and a secretable luciferase reporter gene (Lucia luciferase) placed under the control of the endogenous IL-8 promoter.

Cells were seeded into 96-well plates (5×10^5 cells/well) and immediately treated as indicated to monitor TLR2-dependent activation. After 24 h, supernatants were collected, and the activity of Lucia luciferase was determined using the QuantiLuc reagent (Invivogen, #rep-qlc1) according to the supplier's instructions. SEAP activity could not be used as a readout parameter in our setting because EVs interfered with the assay (data not shown).

Analysis of Publicly Available Datasets

Datasets were obtained from Gene Expression Omnibus (GEO) and normalized using log2-RMA. Dataset GSE4607 included transcriptional profiles human whole blood samples of 15 healthy controls, 27 patients with non-infectious SIRS, and 12 samples from patients with resolved non-infectious SIRS. Patients were classified as SIRS or SIRS resolved (no longer meeting criteria for SIRS) on d3 after ICU admittance. Dataset GSE8121 included transcriptional profiles of human whole blood samples of 15 healthy controls and 30 patients with sepsis. Samples were obtained at d1 and d3 after ICU admission. Statistical significances were determined by the Kolmogorov–Smirnov test. Detailed information about the patient cohort is given in the GEO database and the corresponding original publications (34, 35).

Statistics

All experiments were performed at least three times, and at least two replicates were analyzed for all experiments unless stated otherwise. Data distribution was determined by the Shapiro-Wilk test. For normally distributed data, means of two groups were compared with non-paired two-tailed Student's *t*-test or one sample *t*-test where applicable. For data that were not normally distributed, means of two groups were compared using the Mann-Whitney test. Means of more than two groups were compared by one-way ANOVA with Bonferroni's *post hoc* test (normal distribution) or Kruskal–Wallis ANOVA followed by Mann-Whitney test (no normal distribution). Statistical significance was set at $p < 0.05$, $p < 0.01$, or $p < 0.001$. Data analysis was performed using Origin software (OriginPro 2015G; OriginLab).

RESULTS

Upregulation of TLR2 by GCs

Human AMs express the surface TLRs −1, −2, −4, and −6 (4). Thus, we initially quantified the expression of these receptors after treatment with the GC dexamethasone (Dex) for 24 h by qPCR. Whereas Dex administration had no significant effect on *TLR1*, *−4*, and *−6* mRNA levels, *TLR2* was highly induced (**Figure 1A**). Further analysis showed that *TLR2* upregulation was already detectable 4 h after treatment (**Figure 1B**), and TLR2 protein production was maximal after 16 h, as shown by Western blot analysis (**Figures 1C,D**). Dex induced *TLR2* starting at a concentration of 100 nM (**Figure 1E**). Next, we evaluated whether Dex binding to the GR is necessary for TLR2 induction. To this end, we pretreated AMs with RU486, a specific GR antagonist, before Dex was added. RU486 completely abrogated Dex-mediated *TLR2* mRNA and TLR2 protein upregulation, indicating a GR-dependent mechanism (**Figures 1F–H**).

TLR2 Induction in SIRS and Sepsis

In vivo, endogenous GCs contribute to immunosuppression occurring at later stages of inflammatory processes (1, 20, 21). Analyses of publicly available datasets showed that *TLR2* mRNA was induced in whole blood samples from pediatric patients suffering from SIRS (**Figures 2A,B**) or sepsis (**Figures 2C,D**). In both groups, *TLR2* induction was paralleled by the upregulation of genes involved in the resolution of inflammation or wound healing (*MMP9*, *MARCO*, *VCAN*, *FPR2*, *IL1RN*, *ANXA1*, *IL10*, *DUSP1*), whereas the gene expression of pro-inflammatory factors (*TNF*, *IL12B*, *IL6*, *IFNG*, *CXCL10*, *COX2*, *NOS2*) was not elevated compared with healthy controls, suggesting the onset of anti-inflammatory feedback mechanisms.

TLR2 Induction by LPS in the Absence or Presence of GCs

Prolonged exposure to LPS, which often occurs in sepsis, can result in LPS tolerance in macrophages and monocytes, thereby contributing to immunosuppression (20). Thus, we wondered whether TLR2 levels might be altered by LPS stimulation. LPS treatment for 24 h potently induced *TLR2*, but not *TLR1*, *TLR4*, and *TLR6* mRNA expression in AMs (**Figure 3A**). *TLR2* mRNA and TLR2 protein upregulation were most evident at later time points after LPS addition (**Figures 3B–D**). Other

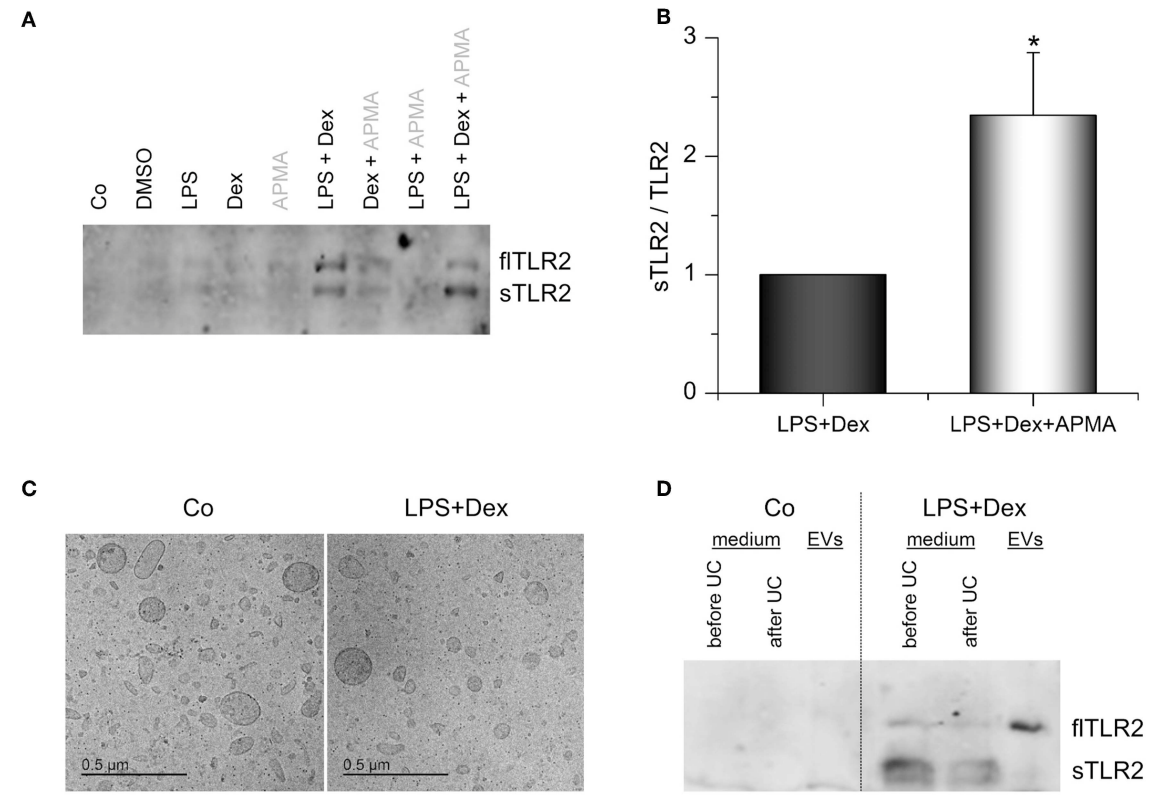

FIGURE 6 | TLR2 in AM supernatants. **(A,B)** AMs were incubated with solvent control (0.1% DMSO), LPS (100 ng/mL), Dex (1 μM), or LPS+Dex for 24 h. 4-aminophenylmercuric acetate (APMA, 10 μM) was added to the indicated samples 5 h before supernatants were harvested. Soluble TLR2 (sTLR2) and full-length TLR2 (flTLR2) were detected in the supernatants by Western blot. **(A)** representative blot. **(B)** Relative sTLR2/TLR2 signal intensities are presented as means + SEM ($n = 5$). *$p < 0.05$ (Student's *t*-test). **(C,D)** Cells were either left untreated (Co) or treated with LPS (100 ng/mL) + Dex (1 μM) for 3 days, and EVs were isolated by sequential centrifugation. **(C)** Representative cryo-TEM images of EVs from untreated (Co) and LPS+Dex-treated cells. **(D)** Representative Western blot analysis for TLR2 in AM supernatants before and after ultracentrifugation (UC) and in EVs is shown ($n = 5$).

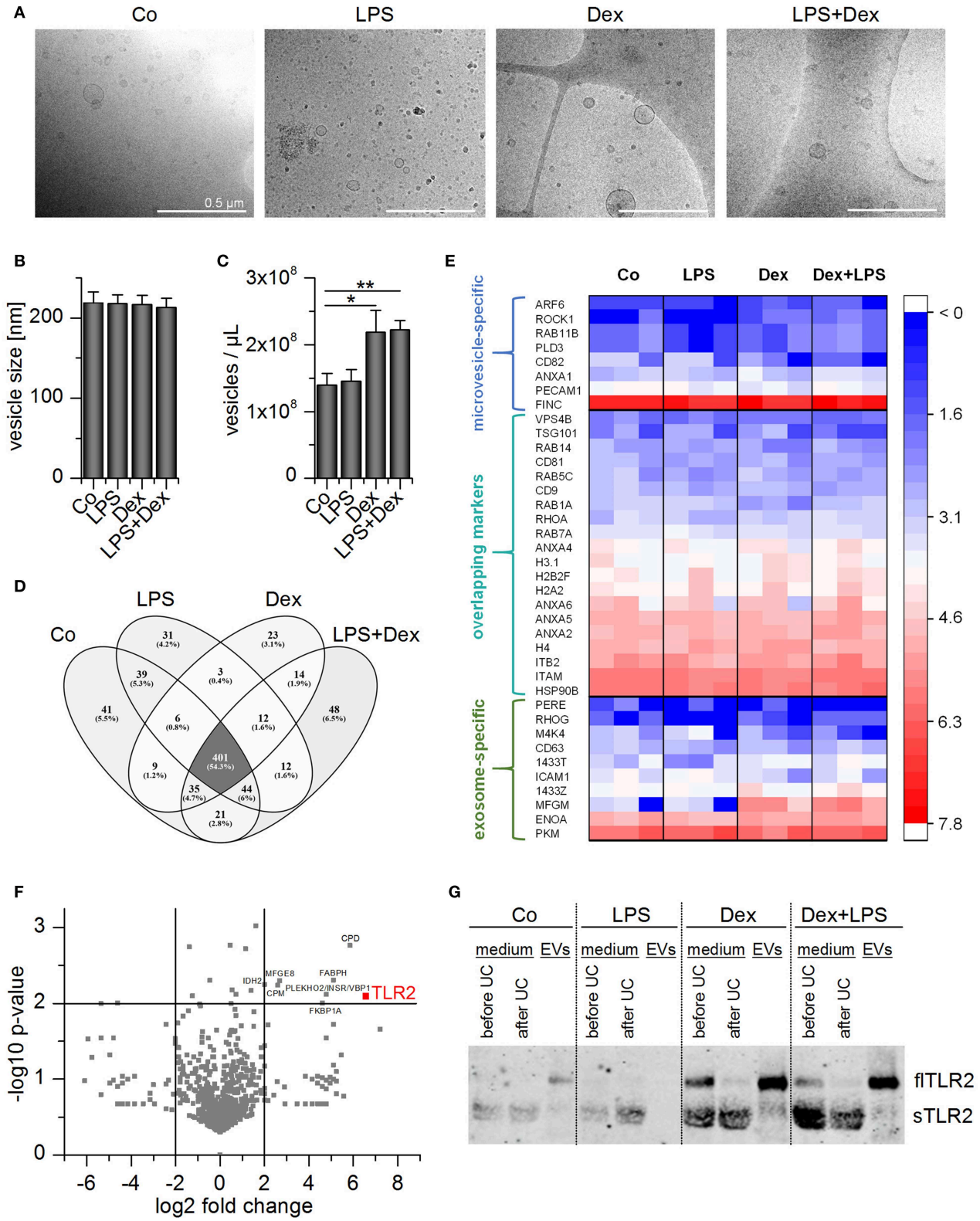

FIGURE 7 | Characterization of THP-1 vesicles. Cells were incubated with medium only (Co), LPS (100 ng/mL), Dex (1 µM), or LPS+Dex for 72 h and EVs were isolated by sequential centrifugation. **(A)** Vesicles were visualized by cryo-TEM. Scale bar = 500 nm. **(B,C)** Average EV size **(B)** and concentration **(C)** were

(Continued)

FIGURE 7 | determined by nanoparticle tracking analysis. Data are presented as means + SEM ($n = 7$). $^{*}p < 0.05$, $^{**}p < 0.01$. p-values were generated by Mann–Whitney U-test. **(D,E)** EVs originating from differentially treated THP-1 cells were subjected to proteomics analysis ($n = 3$). **(D)** Overlap of identified proteins among treatment groups. **(E)** TLR2 and EV marker distribution. Log2 values of unique spectrum counts are shown for all three independent preparations per treatment. **(F)** Volcano plot of p-value vs. fold change in expression level in $EV_{LPS+Dex}$ vs. EV_{Co}. Proteins that were upregulated at least 4-fold in $EV_{LPS+Dex}$ vs. EV_{Co} with a $p < 0.01$ are highlighted. **(G)** Representative Western blot result for TLR2 detection in THP-1 supernatant before and after ultracentrifugation (UC) and in EV fractions ($n = 3$).

TLR ligands, i.e., the TLR2 ligand Pam_3CSK_4 and the TLR3 ligand Poly(I:C), also induced TLR2, although to a lesser extent (**Figures 3E,F**). LPS-mediated TLR2 upregulation was accompanied by LPS tolerance, as indicated by the inability of LPS-primed AMs to produce the inflammatory cytokines *TNF* and *CXCL10* in response to repeated LPS stimulation (**Figure 3G**). As seen in whole blood samples from sepsis patients (**Figure 2**), *TLR2* induction correlated with the overexpression of genes associated with immunosuppression (*IL10*, *FPR2*) or wound healing (*MMP9*) (**Figure 3H**).

We next evaluated whether the presence of both GCs and LPS might elevate TLR2 expression even further. Indeed, we observed that both compounds cooperatively induced TLR2 mRNA and protein (**Figures 4A–C**). Binding of Dex to its receptor was required for the cooperative regulation of TLR2 because the GR antagonist RU486 blocked Dex-induced effects both in the absence or presence of LPS. LPS-mediated upregulation of TLR2 was not affected by RU486 administration (**Figures 4A–C**).

We hypothesized that TLR2 upregulation might rescue TLR2 signaling in otherwise immunocompromised AMs. Therefore, we treated AMs pretreated with LPS, Dex, or a combination of both with the TLR2 ligand Pam_3CSK_4 and measured TNF levels in AM supernatants. None of the pretreatment schemes sensitized AMs toward Pam_3CSK_4. Quite in contrast, TLR2 signaling was inhibited in each of the conditions tested (**Figure 5A**). Similar effects were observed when we used different TLR2 ligands, i.e., lipoteichoic acid (LTA) and heat-killed *Staphylococcus aureus* (HKSA), to stimulate LPS-tolerant AMs. The response to the TLR1/6 ligand Pam_2CSK_4 showed a comparable tendency, but the reaction to this ligand was heterogenous amongst cells from different donors (**Figure 5B**). Interestingly, LTA treatment even enhanced TLR2 induction in LPS-pretreated AMs (**Figure 5C**). The lack of responsiveness toward TLR2 ligands in AMs that highly expressed TLR2 suggested an entirely different function of TLR2 in this context.

TLR2 in Macrophage Supernatants

We hypothesized that the upregulated membrane-bound TLR2 might serve as a precursor for sTLR2, known to antagonize TLR2-dependent cell actions. Supernatants of LPS+Dex-primed AMs indeed contained the soluble 83 kDa form of TLR2, as indicated by Western blot analysis (**Figure 6A**). As previously shown by Langjahr et al. (17), activation of metalloproteinases by 4-aminophenylmercuric acetate (APMA) resulted in enhanced sTLR2 shedding (**Figure 6B**). sTLR2 is produced via proteolytic cleavage of the TLR2 trans-membrane protein by ADAM10 and ADAM17 (17). These ADAMs were also expressed by alveolar macrophages, and *ADAM17* was even induced when LPS and Dex were present (**Supplementary Figure 3**). Thus, an involvement of ADAM17 in sTLR2 shedding is suggested.

Surprisingly, we also detected full-length TLR2 (flTLR2, ∼ 102 kDa) and assumed that this might be due to the production of TLR2-containing extracellular vesicles (EVs). Therefore, EVs from macrophage supernatants were isolated by sequential centrifugation. Both untreated and LPS+Dex-treated cells produced vesicles of various sizes (50–300 nm) and mostly round in shape, as shown by cryo-TEM (**Figure 6C**). These vesicles were identified as the source of full-length TLR2 in macrophage supernatants, as indicated by Western blot analysis (**Figure 6D**).

Vesicle Characterization

For vesicle characterization and functional analysis, we used differentiated THP-1 cells as an easily accessible EV source. THP-1-derived EVs were similar to AM-derived EVs regarding size and shape (**Figure 7A**). Nanoparticle tracking analysis (NTA) was used to determine the EV size and concentration. Treatment schemes did not influence the vesicle size (∼220 nm), but the number of vesicles slightly increased with Dex- or LPS+Dex-treatment (**Figures 7B,C**). This was not due to increased apoptosis, as determined by caspase-3 activity (**Supplementary Figure 1**). Vesicle numbers correlated with protein concentrations of the vesicle preparations (**Supplementary Figure 4**).

EV preparations were analyzed by high-resolution tandem mass spectrometry (MS/MS) to determine whether the treatment scheme had an impact on vesicle composition. A total of 709 proteins was detected in each of the independent experiments, and 401 proteins occurred in all four EV types (**Figures 7D,E**). The preparations did not show differences in vesicle marker abundance, and both exosome- and microvesicle-specific markers (36, 37) were detected (**Figure 7E**). Several proteins were enriched in EVs from LPS+Dex-treated cells ($EV_{LPS+Dex}$), including TLR2 (**Figure 7F**). As seen in AM-derived EVs, TLR2 was most abundant in $EV_{LPS+Dex}$ preparations when compared with other treatment schemes (**Figure 7G**).

THP-1-derived EVs were further analyzed by flow cytometry. To this end, vesicles were coupled to aldehyde/sulfate latex beads. The presence of vesicle markers, tetraspanins CD9 and CD63 (36, 38), indicated that the vesicles were attached to the beads (**Figures 8A,B**). TLR2 staining confirmed that TLR2 was present in $EV_{LPS+Dex}$ samples, but not in preparations from vehicle-treated cells (EV_{Co}) (**Figures 8C,E**). In addition, staining of bead/EV complexes with fluorochrome-labeled Pam_3CSK_4 showed that $EV_{LPS+Dex}$ were able to bind the TLR2 ligand, whereas EVs_{Co} were not (**Figures 8D,F**).

To examine the functional implications of TLR2-EV production, we treated primary human umbilical vein endothelial cells (HUVECs) with a mix of Pam_3CSK_4 and either EV_{Co} or $EV_{LPS+Dex}$. Subsequently, the expression of

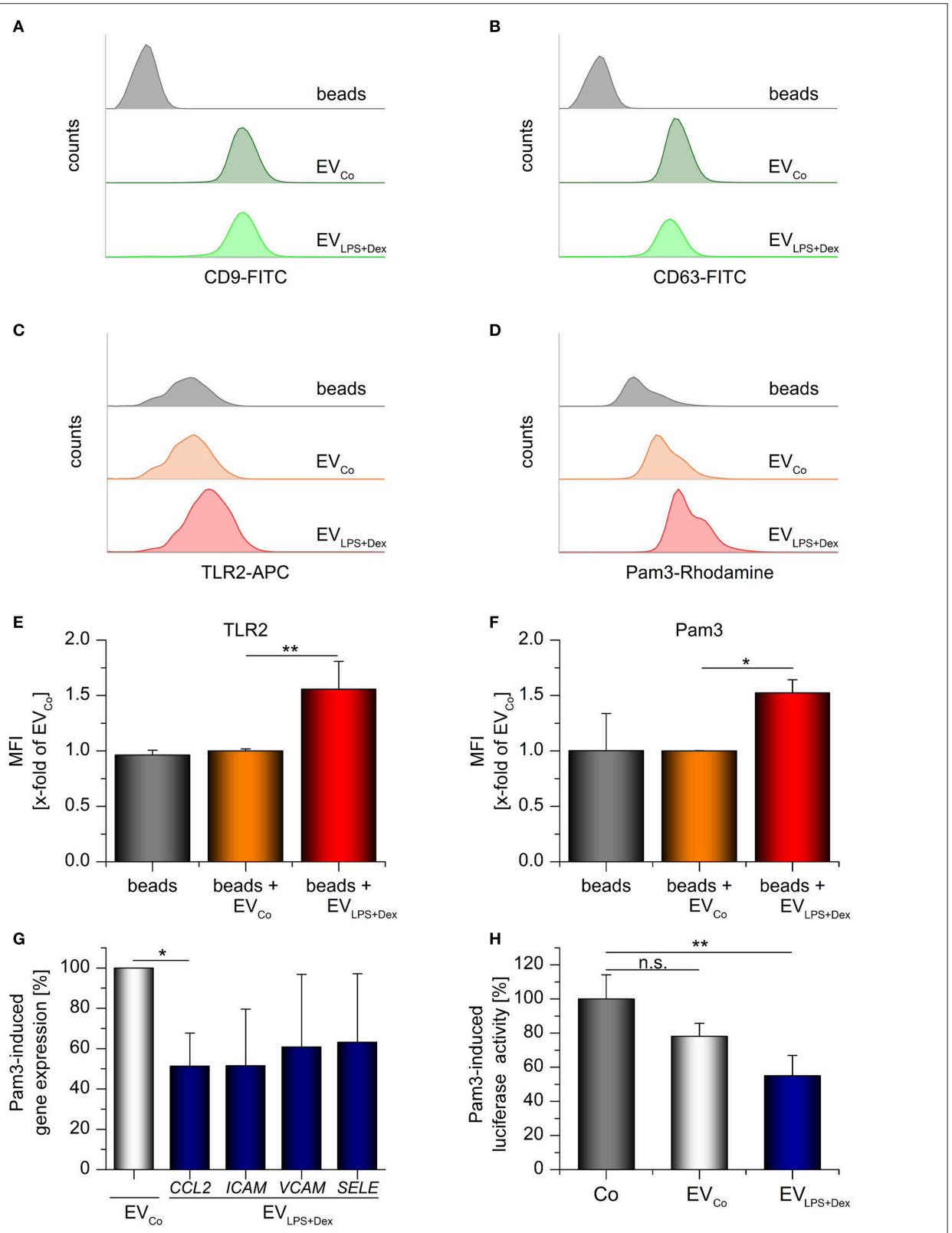

FIGURE 8 | TLR2-containing vesicles act as decoy receptors. Differentiated THP-1 cells were incubated with medium only (Co) or LPS (100 ng/mL) + Dex (1 μM) for 72 h and EVs (EV_{Co} and $EV_{LPS+Dex}$, respectively) were isolated by sequential centrifugation. **(A–F)** Bead-bound EVs from untreated or LPS+Dex-treated

(Continued)

FIGURE 8 | THP-1 cells were analyzed by flow cytometry. Unloaded latex beads served as controls. Histograms show bead counts vs. log fluorescence intensity. **(A,B)** EV loading was confirmed by staining for vesicle markers CD9 **(A)** and CD63 **(B)**. Representative histograms are shown ($n = 3$). **(C–F)** TLR2 staining and binding of rhodamine-labeled Pam_3CSK_4 (Pam3). **(C,D)** Representative histograms. **(E,F)** Mean fluorescence intensities were expressed as x-fold of EV_{Co} values + SEM ($n = 3$, duplicates). **(G)** Pam3-induced gene expression in HUVECs was measured by qRT-PCR. Pam3 was preincubated with the specified vesicles for 30 min at 37°C (2×10^{10} EVs/μg Pam3), and HUVECs were treated with the Pam3/EV mix (2×10^{10} EVs and 1 μg Pam3/mL) for 3 h. Data from 3 independent THP-1 vesicle preparations and HUVEC donors are presented as a percentage of EV_{Co}-treated cells + SEM. H: Pam3-induced *CXCL8* promoter-dependent luciferase activity was quantified in hTLR2 HEK-Dual cells. Cells were either treated with Pam3 only (1 ng/mL, Co) or co-treated with Pam3 (1 ng/mL) and the specified vesicles (5×10^9 EVs/ml) for 24 h. The Pam3/vesicle mix was preincubated for 30 min at 37°C before it was added to the cells. Data are expressed as percentage of Co-values + SEM ($n = 4$, duplicates). $^*p < 0.05$, $^{**}p < 0.01$, n.s.: not significant. *P*-values were generated by ANOVA with Bonferroni's *post-hoc* test or Student's *t*-test.

the chemokine *CCL2* and the adhesion molecules *ICAM*, *VCAM*, and *SELE* were measured. Pam_3CSK_4-induced *CCL2* expression was decreased in HUVECs treated with $ECV_{LPS+Dex}$ (**Figure 8G**). The expression of the three adhesion molecules tended to be reduced, although not significantly so due to high inter-individual differences between donors. Additionally, TLR2-responsive HEK reporter cells expressing a luciferase reporter gene under the control of the *CXCL8* promoter were used to study the influence of EVs on Pam_3CSK_4-induced inflammatory responses. We found indeed that TLR2-containing vesicles derived from LPS+Dex-treated cells were able to inhibit Pam_3CSK_4-induced luciferase production (**Figure 8H**). In summary, these data suggest that TLR2-EVs can exert decoy functions.

DISCUSSION

AMs are one of the first lines of defense against the invasion of airborne pathogens. The stimulation of TLRs triggers the production of proinflammatory cytokines, which in turn activate the hypothalamic-pituitary axis to induce the synthesis and secretion of anti-inflammatory GCs by the adrenal cortex, thereby limiting inflammation (39). Therapy of pulmonary diseases, such as asthma and chronic obstructive pulmonary disease, with inhaled GCs mimics the effects of endogenous GCs, resulting in decreased production of pro-inflammatory mediators by AMs (12).

Paradoxically, glucocorticoids have also been suggested to enhance inflammation and innate immune responses, particularly by upregulating TLR2 (40). An increase in TLR2 expression after GC administration was observed in many cell types, including epithelial cells (41–43), keratinocytes (44, 45), dendritic cells (10), and macrophages (11, 12, 45). Several studies showed that TLR ligands or inflammatory cytokines cooperate with GCs to induce TLR2 (12, 41–45). In line with our findings, Ji et al. (12) reported that coadministration of the GC budesonide and LPS resulted in elevated *TLR2* mRNA levels in human AMs, whereas *TLR4* was not affected.

Different mechanisms were suggested to underly the cooperative induction of TLR2 by GCs and pro-inflammatory stimuli. For example, *Haemophilus influenzae*-mediated TLR2 upregulation was enhanced by GCs *via* negative cross-talk with the mitogen-activated protein kinase (MAPK) p38 (41). Likewise, GC-mediated TLR2 induction was reported to depend on p38 inhibition *via* the GC-inducible phosphatase DUSP1 in keratinocytes and epithelial cells (44, 46). An entirely different mechanism was suggested to drive TLR2 expression in TNF-α/GC-treated A549 cells, requiring the collective recruitment of NF-κB, signal transducer and activator of transcription (STAT) transcription factors, and the GR to the *TLR2* promoter (42). In our hands, Dex-mediated TLR2 induction was GR-dependent and accompanied by DUSP1 induction (**Supplementary Figure 5A**). High DUSP1 expression levels correlated with the repression of p38 phosphorylation (**Supplementary Figures 5B,C**), suggesting that Dex-induced p38 inhibition may indeed play a role in TLR2 upregulation. However, direct binding of GC/GR complex to the *TLR2* promoter might also contribute to the overall effect.

Although GC-induced TLR2 upregulation has been suggested to enhance inflammation, a link between TLR2 induction and enhanced TLR2 responsiveness in immune cells has not been shown so far. In contrast, TLR2 upregulation has been reported to be paralleled by immunosuppression (10, 12), which was confirmed by our study. The lack of cytokine release upon TLR2 stimulation of GC-treated cells has been explained by the downstream blockade of the TLR2 receptor signaling and lack of TLR2 heterodimerization partners (10, 12, 40, 45).

In addition to GC treatment, we showed that long-term LPS exposure results in elevated TLR2 levels. Similar to GC-mediated effects, chronic exposure to LPS represses pro-inflammatory macrophage responses to recurring stimulation with LPS or other TLR ligands. TLR signaling is inhibited on many levels upon constant LPS stimulation, including downregulation of TLR adapter molecules and upregulation of anti-inflammatory factors (3, 20). In accordance, increased TLR2 expression did not lead to improved TLR2-mediated inflammatory responses in LPS-primed AMs in our study. Thus, we speculated that TLR2 induction by LPS and/or Dex might have anti-inflammatory effects, e.g., by sTLR2 release.

Secretion of sTLR2 balances responses to both viral and bacterial infections by binding a wide range of PAMPs and DAMPs, thereby inhibiting the activation of cellular TLR2 (16). We observed that sTLR2 was indeed produced by AMs, in particular after LPS+Dex-treatment. sTLR2 was enriched after activation of MPs, indicating that ectodomain shedding led to sTLR2 production (17).

In addition, we detected an unexpected protein that resembled full-length TLR2. In a previous study, Langjahr et al. (17) also observed a full-size TLR2 glycoprotein in human macrophage supernatant and hypothesized that it might correspond to the full-length protein associated with membrane vesicles. This

hypothesis is supported by our results showing that flTLR2 is present in isolated extracellular vesicles (EVs). Of note, quantification of sTLR2 by ELISA, as used to analyze plasma samples from LPS-exposed volunteers or septic patients (18, 19), would also detect vesicular flTLR2. Thus, it presently remains elusive whether sTLR2, flTLR2, or both are present in the circulation in response to LPS or sepsis. Elevated sTLR2 plasma levels have been suggested as a biomarker for infections (18, 19). Therefore, it might be interesting to investigate whether TLR2-EVs might serve as diagnostic markers to assess disease progression, as currently discussed for various types of EVs (47).

Under physiological and pathological conditions, almost all cell types release cell-derived phospholipid-based bilayer membrane vesicles equipped with functional surface and membrane proteins and encapsulating various cargoes, including proteins, cytokines, lipids, and nucleic acids (48, 49). They are categorized as exosomes, microvesicles (MVs), and apoptotic bodies based on their size, pathway of formation, and membrane composition (49). Exosomes, which are 30–200 nm in size, derive from the late endosome. In contrast, MVs are between 100 and 1,000 nm in diameter and are formed through outward budding of the plasma membrane. Apoptotic bodies derived from apoptotic cells are very heterogeneous in size and morphology, and are, therefore, different from the other two EV subtypes (36, 50). Since exosomes and microvesicles display a similar appearance and composition as well as an overlapping size distribution, it is difficult to define their origin once isolated (36). Thus, we made no further distinction between these vesicle types in this work.

Flow cytometric analysis confirmed the presence of TLR2 in EV preparations derived from LPS+Dex-treated AMs and indicated an intact ligand binding ability. The overall inhibitory function of these vesicles suggests that they may act as a decoy, as previously shown for sTLR2 (17, 51). This decoy activity may involve competition for not only the microbial ligand but also the heterodimerization partners (51). Further studies are required to elucidate the anti-inflammatory potential of TLR2-containing EVs. These investigations might comprise more complex *in vitro* (52) or *in vivo* models (53, 54).

In summary, we showed for the first time that sTLR2 and full-length TLR2 are released by macrophages under anti-inflammatory conditions. Our data suggest that vesicle-bound flTLR2 has decoy functions, which may contribute to immunosuppression induced by GCs and chronic infections.

DATA AVAILABILITY

The mass spectrometry proteomics datasets generated for this study have been been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD013977 and 10.6019/PXD013977. In addition, the publicly available datasets analyzed in this study were obtained from Gene Expression Omnibus (GEO), accession numbers GSE4607 and GSE8121.

ETHICS STATEMENT

Human lung tissue was obtained from patients undergoing lung resection. The use of human material was reviewed and approved by the local ethics committee (State Medical Board of Registration, Saarland, Germany; permission no. 213/06). The informed consent of all participating subjects was obtained.

AUTHOR CONTRIBUTIONS

JH, AD, RL, CD, AB, CF-T, MK, AK, and AKK designed, performed, and analyzed the experiments. JH wrote the paper. All authors contributed to drafting the manuscript. GF and HH provided materials and discussed the data. 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.2019.01634/full#supplementary-material

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REVIEW
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Glucocorticoids—All-Rounders Tackling the Versatile Players of the Immune System

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Glucocorticoids regulate fundamental processes of the human body and control cellular functions such as cell metabolism, growth, differentiation, and apoptosis. Moreover, endogenous glucocorticoids link the endocrine and immune system and ensure the correct function of inflammatory events during tissue repair, regeneration, and pathogen elimination via genomic and rapid non-genomic pathways. Due to their strong immunosuppressive, anti-inflammatory and anti-allergic effects on immune cells, tissues and organs, glucocorticoids significantly improve the quality of life of many patients suffering from diseases caused by a dysregulated immune system. Despite the multitude and seriousness of glucocorticoid-related adverse events including diabetes mellitus, osteoporosis and infections, these agents remain indispensable, representing the most powerful, and cost-effective drugs in the treatment of a wide range of rheumatic diseases. These include rheumatoid arthritis, vasculitis, and connective tissue diseases, as well as many other pathological conditions of the immune system. Depending on the therapeutically affected cell type, glucocorticoid actions strongly vary among different diseases. While immune responses always represent complex reactions involving different cells and cellular processes, specific immune cell populations with key responsibilities driving the pathological mechanisms can be identified for certain autoimmune diseases. In this review, we will focus on the mechanisms of action of glucocorticoids on various leukocyte populations, exemplarily portraying different autoimmune diseases as heterogeneous targets of glucocorticoid actions: (i) Abnormalities in the innate immune response play a crucial role in the initiation and perpetuation of giant cell arteritis (GCA). (ii) Specific types of CD4+ T helper (Th) lymphocytes, namely Th1 and Th17 cells, represent important players in the establishment and course of rheumatoid arthritis (RA), whereas (iii) B cells have emerged as central players in systemic lupus erythematosus (SLE). (iv) Allergic reactions are mainly triggered by several different cytokines released by activated Th2 lymphocytes. Using these examples, we aim to illustrate the versatile modulating effects of glucocorticoids on the immune system. In contrast, in the treatment of lymphoproliferative disorders the pro-apoptotic action of glucocorticoids prevails, but their mechanisms differ depending on the type of cancer. Therefore, we will also give a brief insight into the current knowledge of the mode of glucocorticoid action in oncological treatment focusing on leukemia.

Keywords: glucocorticoids, immune system, inflammation, giant cell arteritis, rheumatoid arthritis, systemic lupus erythematosus, allergic diseases, leukemia

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INTRODUCTION

Hormones enable intercellular communication as well as the exchange of information between different organ systems throughout the human body. They are involved in a variety of processes such as growth, development, and metabolism. The synthesis and secretion of hormones is subject to stringent regulations, comprising positive, and negative feedback loops as crucial mechanisms. Steroids are lipophilic hormones that are subdivided into mineralocorticoids produced in the zona glomerulosa of the adrenal cortex, glucocorticoids produced in the zona fasciculata as well as sex hormones produced in the zona reticularis and to a great extent in the gonads. Since it has been demonstrated that natural glucocorticoids also have some mineralocorticoid effects, the classification into these groups is not completely accurate. The term "glucocorticoids" is more suitable when talking about synthetic glucocorticoids (e.g., prednisolone or dexamethasone), because these drugs are more restricted to glucocorticoid effects only (1).

The initial step of steroid hormone biosynthesis is the conversion of cholesterol to the precursor pregnenolone in the mitochondria. Steroid hormone biosynthesis is mainly realized by enzymes of the cytochrome P450 family (2). Sex hormones affect growth, development and reproductive cycles whereas mineralocorticoids regulate sodium and water balance and glucocorticoids influence energy and metabolic processes as well as immune and stress responses.

Between 5 and 30 mg of the active endogenous (physiological) glucocorticoid cortisol is produced per day, regulated by the hypothalamic–pituitary–adrenal (HPA) axis. Glucocorticoids bind to glucocorticoid receptors that are present in cells throughout the body, including cells in the hypothalamus and pituitary gland, which are part of the negative feedback loop controlling the glucocorticoid production. Furthermore, the hormone concentration varies in a circadian manner peaking at 9 a.m. in the morning and reaching the lowest plasma concentration at midnight.

The dehydrogenation of cortisol to its inactive form cortisone is promoted by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1 in the liver. The same enzyme also exhibits reductase activity promoting the reverse reaction. The type 2 11β-HSD is only able to convert the active into the inactive form due to its sole dehydrogenase activity. Depending on the balance and activity of both enzymes, the intracellular glucocorticoid concentration and thus the tissue sensitivity for glucocorticoids varies (3). In addition to that, glucocorticoids have been demonstrated to possess immunomodulating effects which depend on concentration and time of administration: While an immunostimulatory effect is observed at lower concentrations (below serum level), higher concentrations (therapeutic range) lead to an immunosuppression (4). Due to their strong immunosuppressive, anti-inflammatory and anti-allergic effects, synthetic glucocorticoids have been established as important drugs in the treatment of diseases driven by immune and inflammatory dysregulation.

Glucocorticoid Signaling

Glucocorticoids are lipophilic substances with a low molecular weight that can easily pass cellular membranes and bind to the glucocorticoid receptor in the cytosol. The cytosolic glucocorticoid receptor is ubiquitously expressed by nucleated cells and resides in the cytoplasm as a multi-protein complex. Proteins and co-factors stabilize the receptor and support a specific conformation leading to a high binding affinity for its ligands (5–9). Two main receptor isoforms are described, the α glucocorticoid receptor, which is activated by glucocorticoids, and the β isoform with a deformed ligand-binding domain that cannot bind ligands (10–13). Further receptor isoforms which differ in their transcriptional activity as a result of alternative splicing and/or post-translational modifications, have been extensively described elsewhere (12–14).

The hormone-receptor complex is translocated into the nucleus as a homodimer and binds to palindromic DNA-binding sites in the promoter region of different target genes, so called glucocorticoid response elements. This genomic mechanism of glucocorticoid action is known as transactivation, which describes the binding to positive glucocorticoid response elements leading to the activation of the transcription of anti-inflammatory but also regulatory proteins. These include for example IL-10, Annexin 1, and IκB as well as enzymes of gluconeogenesis such as tyrosine aminotransferase, serine dehydrogenase, or phosphoenol pyruvate carboxykinase. In contrast, the term transrepression refers to an impairment of the expression of immunoregulatory and proinflammatory proteins caused by (i) competition for nuclear co-activators between the hormone-receptor-complex and transcription factors; (ii) direct or indirect interaction with transcription factors like NF-κB and AP-1. Similarly, glucocorticoids diminish gene expression by a mechanism referred to as cis-repression, which involves binding to negative glucocorticoid response elements. Genomic mechanisms of glucocorticoid action result in "delayed effects," meaning that the protein level does not change directly after glucocorticoid administration. The duration of the delay depends on different factors, including transport within the bloodstream, onset of activation/translocation of the hormone-receptor complex and the transcriptional and translational processes themselves. Nevertheless, the description of rapid improvements which are observed within a few minutes—especially after intravenous or intraarticular injection of high glucocorticoid doses—demonstrates the existence of non-genomic effects. These are triggered by (i) proteins released from the multi-protein complex after the binding of glucocorticoids to the cytosolic receptor, (ii) interactions with membrane-bound receptors, and (iii) nonspecific effects resulting from the interaction of glucocorticoids with cellular membranes (15, 16).

More pronounced glucocorticoid effects are observed with increasing glucocorticoid dosages, as receptor saturation is achieved (17). Unfortunately, rising dosages and duration of administration simultaneously increase the risk of adverse events. While the long-term use of dosages $\leq$5 mg prednisone equivalent per day is generally associated with a low risk of adverse effects, the application of dosages >10 mg/day increases

the frequency of the latter (18). These adverse effects are thought to depend on the mechanism of glucocorticoid action: Repression of cytokines such as IL-1, IL-2, IL-6, TNF-α, IFN-γ, and prostaglandins mediates the positive anti-inflammatory effects, while transactivation is thought to be responsible for the majority of adverse effects (19–21). However, this classification is not absolute. In contrast, it has been demonstrated that transactivation also contributes to the anti-inflammatory effects, e.g., by the upregulation of genes like GILZ and DUSP1. In addition, in a mouse model the prevention of receptor dimerization and thereby inhibition of DNA-binding impaired the anti-inflammatory capacity of glucocorticoid action (22–26).

Glucocorticoids and Inflammation

These findings clearly show that our knowledge concerning the mechanisms of glucocorticoid action—including the desirable anti-inflammatory and the undesirable adverse effects—is yet insufficient. Nevertheless, these drugs still represent an indispensable component of the treatment of most inflammatory diseases because of their efficient and cost-effective characteristics. However, the considerable toll taken by adverse events must not be neglected and the development of an equally effective alternative with a more favorable side-effect profile would be most desirable. The extent and importance of glucocorticoid toxicity has been reviewed elsewhere (27, 28) and will not be discussed in detail in this article.

The immune system consists of two major components: The innate immune response represents our first line of defense and includes physical and chemical barriers such as the skin and tears. In addition, non-specialized cells recognize foreign invaders by components like bacterial lipopolysaccharide and destroy them by phagocytosis or release of toxic substances. The adaptive immune response—our second line of defense—includes B and T lymphocytes. While the former are responsible for antibody production, the latter can differentiate into distinct subpopulations that participate in B cell maturation or possess cytotoxic potential (29–31). The two lines of defense are linked by cytokines and cell-cell interaction, which is crucial for the initiation of the adaptive response. The most notable attribute of the adaptive immune response is memory, enabling an immediate and very specific pathogen defense following previous exposure. The protective actions of the immune system are accompanied by pain, swelling, itching, redness and heat, typical signs of an inflammation. At the same time, these symptoms represent a significant burden in autoimmune diseases. Normally, the immune response is strictly regulated to discriminate self from non-self—a mechanism known as tolerance (29, 30). It is realized by positive and negative selection of lymphocytes in the bone marrow or thymus. In more detail (for T cells), T cells that cannot bind MHC class 1 or class 2 complexes undergo apoptosis due to the lack of survival signals. The subsequent negative selection determines if T cells bind self-peptides presented by epithelial cells of the thymus. Naive T cells that have passed both, the positive and the negative selection are qualified to migrate into secondary lymphoid organs (29). Autoimmune diseases originate from a dysregulation of the immune response, while the particular cause of the disease is often unknown. Some factors, including genetic predisposition,

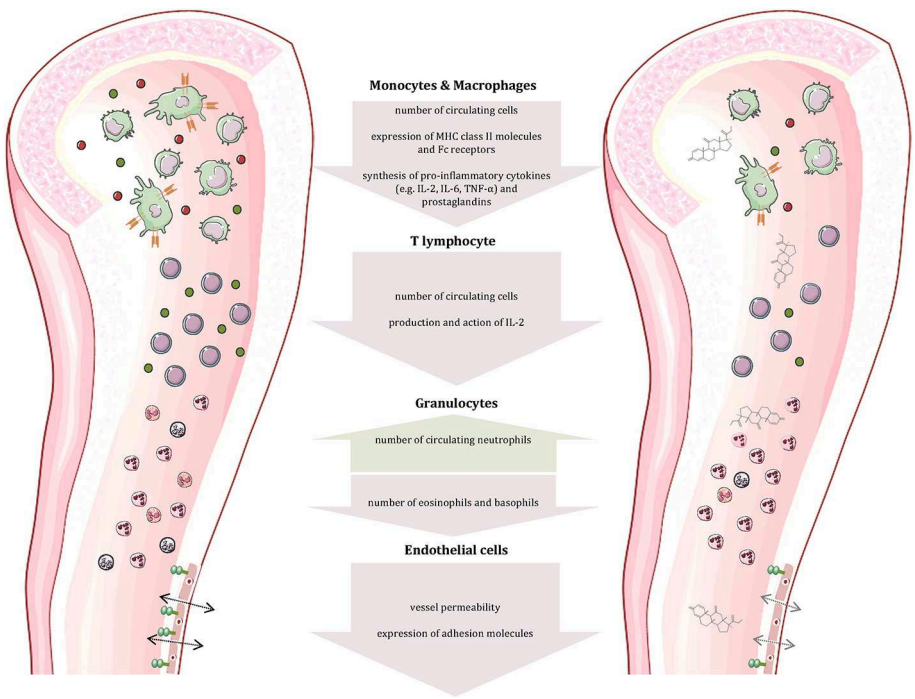

FIGURE 1 | Effects of glucocorticoids on immune and other cells. Glucocorticoids affect the number and function of immune cells (cells and compartments adapted from Servier Medical Art, 2007; Les Laboratoires Servier, München, Germany).

sex, and environment have been identified to promote the establishment of autoimmune diseases. Due to their strong anti-inflammatory and immunosuppressive effects on almost all immune cells (summarized in **Figure 1**), glucocorticoids are indispensable in the treatment of autoimmune diseases. In general, glucocorticoids inhibit leukocyte traffic and thereby the access of leukocytes to the site of inflammation. Furthermore, glucocorticoids interfere with immune cell function and suppress the production and actions of humoral factors involved in the inflammatory process.

Since the establishment and the course of autoimmune diseases are driven by different cell populations, glucocorticoid application targets diverse leukocyte populations and thus the mechanism of glucocorticoid action varies. Recently, Franco et al. have investigated the transcriptional effects of glucocorticoids on nine primary human cell types. They found 9,457 genes to be differentially expressed in response to glucocorticoids, whereas only 25 of them (0.3%) involved all cell types examined, demonstrating that the transcriptional response of each cell type is quite distinct (32).

The next chapters will illustrate the versatile modulating effects of glucocorticoids on the immune system on the basis of exemplary diseases involving the respective leukocyte population. Glucocorticoid regimens used in daily practice according to current guidelines are presented in **Table 1** for the selected diseases.

ABNORMALITIES IN INNATE IMMUNE RESPONSE PLAY A CRUCIAL ROLE IN THE INITIATION AND THE PERPETUATION OF GIANT CELL ARTERITIS

Giant cell arteritis (GCA) is defined as a granulomatous large-vessel vasculitis, which primarily involves medium- and large-caliber branches of the aorta (34, 58, 59). Both the innate and the adaptive immune responses are involved in the pathogenesis of this disease which can be divided into different phases (59). The initiation of inflammation is followed by its amplification and the constitution of feed forward loops leading to arterial remodeling and ultimately vascular damage. Recently, the current knowledge on the pathophysiology of GCA has been discussed in detail in two reviews (59, 60). Al-Mousawi et al. describe this disease as being mainly mediated by T cells (60). The first step, however, is the abnormal maturation of vascular dendritic cells (DC) in the adventitia of the affected vessels. An unknown trigger, perhaps microorganisms or viral agents, drives this initial step (59). Predisposing factors include a certain genetic background, female sex, and alterations of the immune and arterial systems related to aging (59). The activated DC recruit and activate CD4+ naïve T cells in the arterial wall where they polarize into T helper (Th) 1 cells, Th17 and regulatory T (Treg) cells (59, 60). The secreted products of these cells, namely and most importantly interferon-γ, interleukin (IL)-2, and IL-17, facilitate both the recruitment and activation of neutrophils, macrophages and vascular smooth muscle cells, and the formation and activation of multinucleated giant cells (**Figure 2**). These giant cells are also capable of secreting cytokines and growth factors. Of note, Th17 cells also secrete other cytokines such as IL-21, IL-22, IL-8, and IL-26. Macrophages produce IL-6 and IL-1β within the adventitia. The latter cytokines are thought to mainly drive the systemic manifestations of GCA such as fatigue, fever, and weight loss. The fact that the levels of these cytokines largely determine glucocorticoid requirements underlines the importance of the innate immune response in the pathogenesis of GCA (61). Macrophages also produce matrix metalloproteinases (MMP) such as MMP-9, a type IV collagenase. Watanabe et al. have very recently identified this enzyme in vasculitic lesions of GCA and have shown MMP-9 to control the access of monocytes and T cells to the vascular wall. MMP-9–producing monocytes facilitate migration of T cells through the collagen IV-containing basement membrane. The enzymatic activity of MMP-9 is required for invasion of vasculitogenic T cells and monocytes, formation of neoangiogenic networks, and neointimal growth (62). As a consequence, the elastic lamina and growth factors are destroyed, which propagates intimal hyperplasia. Of note, macrophages also produce reactive oxygen species which contribute to the damage of smooth muscle cells in the media (60). Ultimately, the injured arterial cells respond to the damaging immunological events mentioned above by initiating dysfunctional repair processes. This vascular remodeling leads to inflammatory wall thickening, decreased luminal diameter, and ischemic manifestations of GCA with potential organ damage (34).

Glucocorticoids represent a most effective therapy and, therefore, remain—despite the recently shown favorable effects of the IL-6 receptor inhibitor Tocilizumab (63)—the primary treatment in GCA (34). These drugs have been the mainstay of treatment since the 1950s. Their genomic and non-genomic effects contribute to the successful treatment of this disease. We have recently summarized details regarding glucocorticoids in the management of polymyalgia rheumatica and GCA (64). In brief, glucocorticoids induce important anti-inflammatory and immunosuppressive effects on both primary and secondary immune cells involved in the pathophysiology as described above. Glucocorticoids inhibit some of their crucial functions with key mechanisms being the suppression of the production of pro-inflammatory cytokines, and the prevention and inhibition of activation of T cells and monocytes/macrophages.

Innate immune cells that are predominantly responsible for the features of systemic inflammation present in GCA are most susceptible to glucocorticoid treatment (65, 66). By inhibiting the NFκB pathway by direct or indirect interaction with this transcription factor as described in the introduction, glucocorticoids efficiently suppress the production of central cytokines (**Figure 2**) (67). In this context, Linden and Brattsand demonstrated that GM-CSF showed the highest susceptibility to glucocorticoid treatment compared to IL-1β and IL-6 (68). These findings conform to the beneficial effects of IL-6 blockade in GCA therapy (63). Of note, higher glucocorticoid sensitivity has been attributed to monocytes compared to more differentiated macrophages (68). Consequently, it can be inferred that glucocorticoids are most potent in inhibiting freshly attracted monocytes in states of acute inflammation. In addition, glucocorticoids affect the recruitment of cells of the

TABLE 1 | Glucocorticoid regimens in selected diseases.

Disease	Induction	Tapering	Maintenance	Relapse
INFLAMMATORY RHEUMATIC DISEASES				
Giant cell arteritis	- Immediate treatment with 40–60 mg/day* for induction of remission in active GCA** (33)	- Tapering is recommended when the disease is under control to achieve a target dose of 15–20 mg/day* within 2 to 3 months - After 1 year target dose should be ≤5 mg/day* (33)	- If long-term therapy is required a dose of 5 mg/day* or less should be used - GC therapy should ideally be tapered to zero as early as clinically feasibly (18)	- Increase to pre-relapse dose or by up to 5–10 mg/day* - Taper within 4–8 weeks to pre-relapse dose - Repeat induction therapy for ischemic complications (34)
Rheumatoid arthritis	- When initiating/changing csDMARDs short-term GC therapy should be considered (35)	- GC tapering should start as soon as clinically feasible (35)	- If long-term therapy is required a dose of 5 mg/day* or less should be used - GC therapy should ideally be tapered to zero as early as clinically feasibly (18)	- Usually doses between 10 and 20 mg/day* are sufficient to treat flares in this disease
Systemic lupus erythematosus	- Therapy depends on disease manifestations and severity (36) - In acute, organ-threatening disease high-dose intravenous pulse therapy (usually 250–1,000 mg/day* for 3 days) is often used (36)	- GC should be tapered or at least minimized as rapidly as clinically feasible	- Long-term aim is to minimize daily dose to ≤7.5 mg/day* or to discontinue GC therapy (36)	- The characteristic of flare therapy depends on disease, as has been similarly stated for the induction therapy
ATOPY				
Atopic dermatitis	- Stepwise approach: adjust treatment based on disease severity assessed by SCORAD (37) → Mild disease: class II topical glucocorticoids (e.g., flumethasone 0.02%) (38) → Moderate disease: class II/III topical glucocorticoids (e.g., mometasone 0.1%) (39) → Severe disease: short-term oral glucocorticoids may be considered in adults (38)			
Allergic rhinitis	- Moderate to severe rhinitis: nasal glucocorticoids, e.g., fluticasone, mometasone, beclametasone (40, 41) - Oral glucocorticoids should only be used in severe persisting disease (40, 41) - stepped-care approach according to disease severity (42)			
Asthma	- Most patients initially receive low dose ICS (e.g., 200–400 μg/d budesonide) (43) - Frequent troublesome symptoms justify medium (400–800 μg/d) to high dose ICS (>800 μg/d) (44) - Low dose oral corticosteroids (≤7.5 mg/day *) should only be considered in adults with severe asthma or poor symptom control (45)	- ICS should not be stopped completely, cessation is associated with a higher risk of exacerbations (46) - In stable disease ICS doses can be reduced by 25–50% every 3 months (47)	- ICS are recommended as controller treatment in all asthma patients either as-needed or daily depending on disease severity (43) - Dose adjustment according to a stepwise approach*** ranging from 200–400 to >800 μg/d budesonide or comparable doses of other formulations in adults, reduced doses are used in the treatment of children <12 years (48)	- *Worsening symptoms:* adjustment of the treatment (increase reliever/controller use, step up to higher dose) according to a written asthma action plan*** - Severe exacerbation: → adults: 40–50 mg/d prednisolone → Children: 1–2 mg/kg/d, max. 40 mg/d prednisolone to be continued for 5–7 days (49, 50)
Anaphylactic shock	- Glucocorticoids are used to prevent protracted anaphylactic symptoms, while their efficacy in the acute phase is limited due to slow onset of action (51, 52) - 250–1,000 mg i.v. prednisolone (weight-adjusted dosing in children) (53)			
LEUKEMIA				
Chronic lymphoblastic leukemia****	- Patients with diagnosed limited-stage Hodgkin's lymphoma (HL) and a positive interim positron-emission tomography after two cycles of ABVD (adriamycin, bleomycin, vinblastine, and dacarbazine) should be treated with two cycles of bleomycin/etoposide/doxorubicin/cyclophosphamide/vincristine/procarbazine/prednisone in escalated dose before ISRT - Patients with refractory or relapsed HL dexamethasone can be given in combination with high-dose cytarabine/cisplatin (DHAP) before high-dose chemotherapy followed by autologous stem cell therapy - Patients diagnosed for nodular lymphocyte predominant Hodgkin lymphoma benefit from the combination of rituximab/cyclophosphamide/doxorubicin/vincristine/prednisone (R-CHOP) - CLL patients with transformation into a diffuse large B-cell lymphoma benefit from therapies used in DLBCL such as rituximab plus CHOP (cyclophosphamide, vincristine, doxorubicin, and dexamethasone) (54–56)			

(Continued)

TABLE 1 | Continued

Disease	Induction	Tapering	Maintenance	Relapse
Chronic myeloid leukemia	N/A			
Acute myeloid leukemia	N/A			
Acute lymphoblastic leukemia	- Glucocorticoids are given as a so-called pre-phase therapy (usually prednisone 20–60 mg/day or dexamethasone 6–16 mg/day, both *i.v.* or *p.o.*) alone, or in combination with another drug (e.g., vincristine, cyclophosphamide), but often given together with allopurinol and hydration for ~5–7 days. The response to pre-phase therapy defines the chemosensitivity of the disease, and is included in some studies for risk assessment, since good responders to prednisone may have a better outcome. - Regimens of induction therapy are centered on vincristine, glucocorticoids, and anthracycline (daunorubicin, doxorubicin, rubidazone, idarubicin), with or without cyclophosphamide or cytarabine. Dexamethasone is often preferred to prednisone, since it penetrates the blood–brain barrier and also acts on resting leukemic blast cells (LBCs). - In adult ALL glucocorticoids are often used in the hyper-CVAD (cyclophosphamide, vincristine, doxorubicin, dexamethasone) protocol, preferentially used in the United States, but also in other parts of the world - Maintenance therapy usually consists of daily 6-mercaptopurine and weekly methotrexate. In some treatment regimens, repeated cycles of vincristine, dexamethasone or other drugs in monthly or longer intervals are given (57)			

*Doses are given as prednisone-equivalent. **In patients with GCA suffering from acute visual loss or amaurosis fugax, the use of very high GC dosages, namely 0.25–1 g i.v. methylprednisolone daily for up to 3 days should be considered. ***Details are provided by the Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention, 2019. Available from: www.ginasthma.org N/A: glucocorticoids are not used as standard therapy in these diseases. ****The transformation into a diffuse large B-cell lymphoma (DLBCL) or Hodgkin's lymphoma occurs in 2%–15% of CLL patients during the course of their disease.

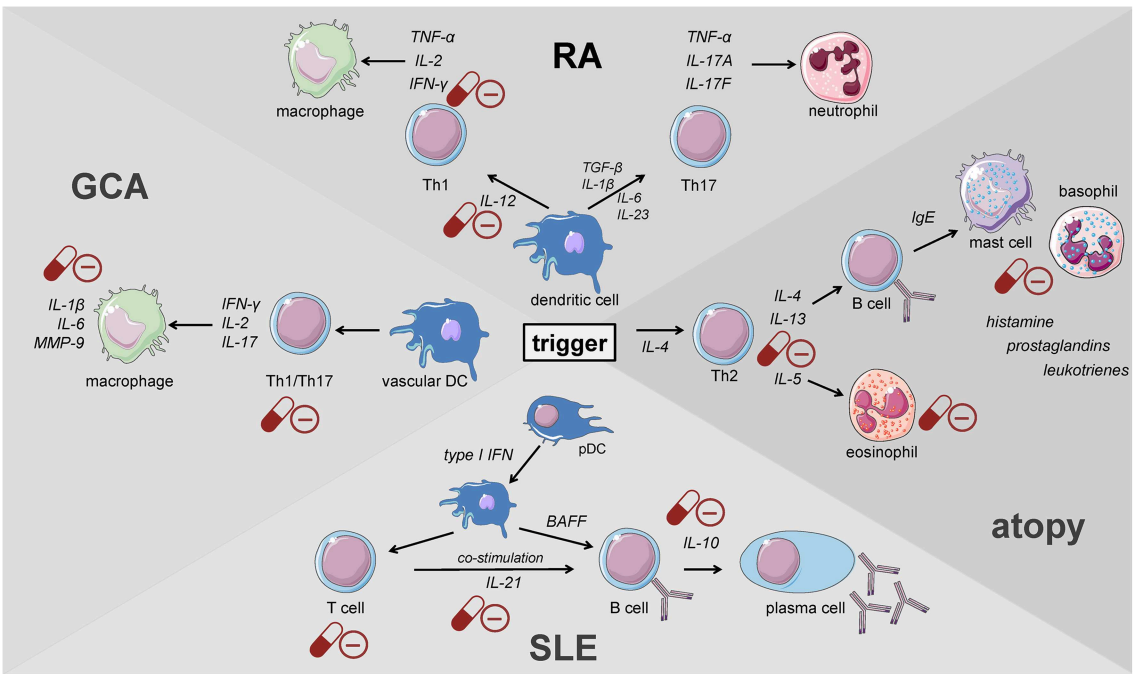

FIGURE 2 | Key players of the immune system driving the pathogenesis of immune-mediated diseases. GCA, giant cell arteritis; DC, dendritic cell; pDC, plasmacytoid dendritic cell; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus (cells adapted from Servier Medical Art, 2007; Les Laboratoires Servier, München, Germany).

mononuclear phagocytic system by suppressing the expression of adhesion molecules on the surface of the endothelium (69). With respect to monocyte function, Blotta et al. demonstrated that the incubation of monocytes with dexamethasone led to a decreased IL-12 production *in vitro* (**Figure 3**) (70). In line with this, they presented a limited capacity to induce Th1 differentiation.

Deng et al., however, have shown that glucocorticoids suppress the production of Th17-promoting cytokines (IL-1β, IL-6, and IL-23) (**Figure 3**), but IFN-γ-producing Th1 responses persist in treated patients (71). Also, patients presenting prominent expression of IL-17A in temporal artery biopsies demonstrated favorable responses to glucocorticoid treatment (72). Therefore, it was assumed that the IL-6-IL-17 cluster is highly responsive to glucocorticoid therapy, whereas the IL-12-IFN-γ cluster is resistant to glucocorticoid-mediated immunosuppression (73). Nevertheless, there are reports of a reduction in Th1 response

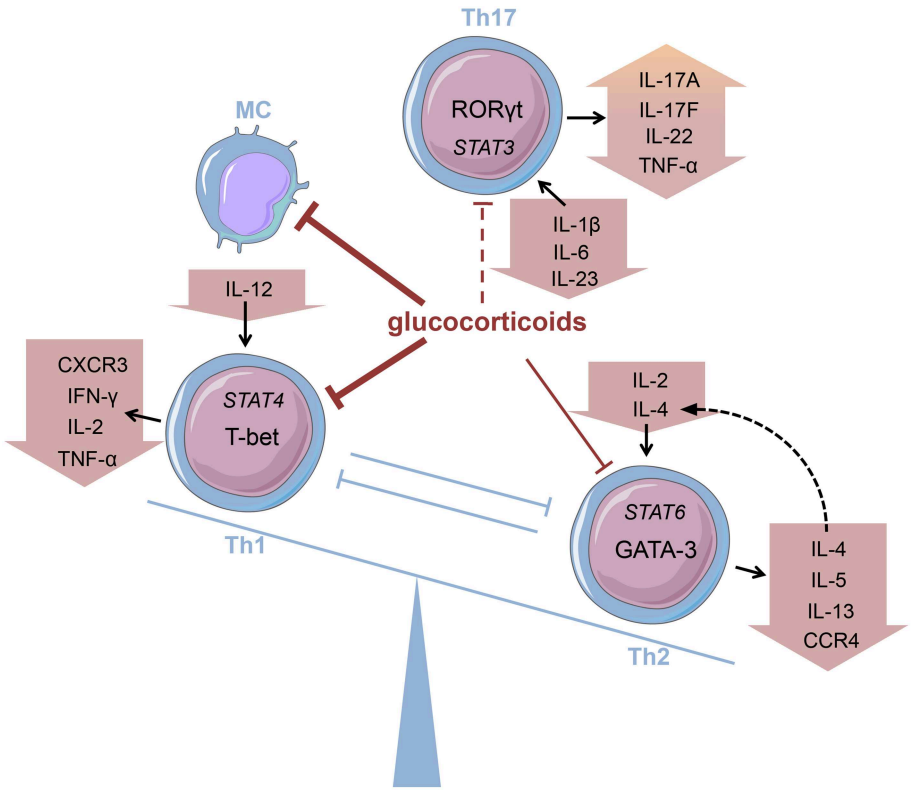

FIGURE 3 | Glucocorticoids modifying the Th balance Glucocorticoids affect the predominance of different T helper (Th) cell subsets, e.g., by influencing cytokine production. MC, monocyte (cells adapted from Servier Medical Art, 2007; Les Laboratoires Servier, München, Germany).

after glucocorticoid treatment in patients with Takayasu's arteritis—a condition closely linked to GCA (74). Moreover, further studies revealed a decrease in both Th1 and Th17 cells, and a reduction of IFN-γ in GCA patients after glucocorticoid treatment (75, 76). Reviewing the pathogenesis of GCA, Samson et al. thus concluded that the conflicting results regarding glucocorticoid response result from prevalent plasticity between Th1 and Th17 cells influenced by the surrounding cytokine milieu (77).

At higher glucocorticoid dosages, for instance in form of pulse therapy in complicated GCA and in case of established visual loss, rapid non-genomic effects as already described in the introduction contribute to their therapeutic efficacy [reviewed in (64)].

AUTOIMMUNE DISEASES DRIVEN BY IRREGULARITIES IN THE ADAPTIVE IMMUNE SYSTEM

Th1 and Th17 Cells Represent Important Players in the Establishment and Course of Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disease that affects up to 1% of the population worldwide with a higher prevalence in women than in men. RA patients suffer from pain, immobility, and fatigue leading to decreased quality of life (78). The pathogenesis of RA is characterized by chronic inflammation mainly localized in the synovial joints leading to the destruction of articular cartilage and the establishment of bone erosions. Joint inflammation is accompanied by the infiltration of the synovium with immune cells such as T cells, B cells, macrophages, and dendritic cells and the proliferation of fibroblast-like synoviocytes of the synovial sub-lining layer which finally contribute to the joint destruction (79).

Glucocorticoids play a very important role in the treatment of RA, rapidly suppressing inflammatory activity especially at disease onset and during flares (15, 16, 80–82). Although glucocorticoids satisfactorily suppress inflammation and reduce symptoms such as pain and morning stiffness, data regarding their ability to manage cartilage degradation and bone erosions remain controversial (83, 84). Only limited success with regard to remission rates using glucocorticoids has been reported, e.g., in early treatment of undifferentiated arthritis (85) but also the SAVE trial (remission-rate: 17%) (86) and the STIVEA trial (remission-rate: 20%) (87). However, glucocorticoids still efficiently limit inflammation. Although the exact mechanism of RA pathogenesis remains unclear, it has become evident that Th cell subsets play an important role in the course of the disease. CD4+ T cells, especially Th1 and Th17 cells, play a major role in RA (88). RA patients present an enrichment of effector memory CD4+CD45RO+ T cells in the affected joints (89) and a massive

expansion CD4+ T cell clones in synovial tissue of early disease, which suggests a local antigen-induced proliferation (90). In this context, it should be noted that blocking co-stimulation targeting CD80/CD86-CD28 interaction significantly improved the signs and symptoms of RA illustrating the importance of T cells in the pathogenesis (91). Moreover, genetic association of certain HLA-DRB1 alleles with increased susceptibility to RA further supports the central role of Th cells in RA (92).

When the Th1/Th2 paradigm dominated the understanding of the pathogenesis of autoimmune diseases, RA was defined as a Th1-driven disease because CD4+ T cells identified to be enriched in synovial fluids from RA patients were prone to secrete IFN-γ but not IL-4 (93, 94). These findings were further supported by the enrichment of the IFN-γ-induced chemokines CXCL9 and CXCL10 and the chemokine receptor CXCR3 binding both of the latter in RA synovium (95–98). Th1 cells classically activate macrophages and increase their capacity to produce pro-inflammatory cytokines present in RA synovium such as TNF (**Figure 2**) (99). Moreover, IL-12, IL-18, and IFN-γ, the drivers of Th1 differentiation have been also identified in synovial tissues of RA patients (100, 101), although the levels of Th1-mediated IFN-γ were relatively low compared with those of TNF-α, IL-1, or IL-6 derived from synovial fibroblasts (102, 103).

The discovery of Th17 cells (100, 101) and the delineation of the IL-17 family members (104) as well as the shift from Th17 cells to Th1 cells (i.e., "non-classic Th1 cells") being more pathogenic than Th17 cells *per se* shed new light on the contribution of inflammatory Th subsets to the initiation of RA (**Figure 2**) (105–108). Th17 cells are highly unstable and easily shift to Th1 cells but can also transdifferentiate back as demonstrated for Th1 cells in the gut (109–112). At the onset and in the early phase of the pathogenesis of RA, Th17 cells shift to Th1 cells, whereas methotrexate (MTX) reduced the ratio of Th17 cells but not Th1 cells (113). Finally, these finding demonstrate that Th17 and ex-Th17 or "non-classic Th1 cells" cells play important roles in the early phase of RA and for the treatment using a combination of MTX and glucocorticoids according to the EULAR recommendations for the management of rheumatoid arthritis (35). While MTX reduces the ratio of Th17 cells, which are—depending on the immunopathological setting—resistant to glucocorticoid mediated suppression in terms of survival and the production of IL-17A and IL-17F but not IL-22 (114), glucocorticoids induce Th1 cell apoptosis via induction of BIM (114, 115). Moreover, glucocorticoids decrease IFN-γ production by T cells from patients with rheumatoid arthritis *ex vivo* and *in vitro* mechanistically via their suppressive action on the IL-12-induced STAT4 phosphorylation and by direct protein-protein interaction with the transcription factor T-BET—described as transrepression in the introduction (**Figure 3**) (116–119). Inhibition of Th1 activity by glucocorticoids may reduce overall inflammation in RA patients while the glucocorticoid resistant joint destruction can be assumed to be Th17 mediated. Mechanistically, glucocorticoid resistant joint destruction may be maintained by the glucocorticoid-mediated promotion of intrinsic Th17 differentiation (120), and the induction of bone resorption via synovial IL-17 (121). IL-17 also contributes to neutrophil recruitment (122) and an increase in neutrophil survival, a hallmark of RA synovial fluid promoting joint damage (**Figure 2**) (122–124).

B Cells Have Emerged as Central Players in Systemic Lupus Erythematosus

Components of the innate and the adaptive immune system play an important role in the pathogenesis of systemic lupus erythematosus (SLE). Clinical manifestations of this autoimmune disease are diverse, affecting a wide spectrum of organs and tissues. The pathogenesis of the disease is not yet fully understood, but beside environmental factors a genetic susceptibility to SLE has been described including a variety of nucleotide polymorphisms [reviewed elsewhere (125)]. Plasmacytoid dendritic cells (pDC) produce type I interferon in response to viral infections. A large number of SLE patients possess an ongoing production of type I interferons and subsequently an increased expression of type I interferon regulated genes, termed IFN-signature, which correlates with autoantibodies and disease activity (126–128). This type I interferon synthesis is induced by immune complexes containing nucleic acid via Toll-like receptor (TLR) ligation. In addition to their antiviral features, type I interferons contribute to the activation of the adaptive immune system, e.g., by activation of autoreactive T and B cells (**Figure 2**) (129, 130). T cell signaling alterations and hyperactive B cells, producing and presenting autoantibodies against nuclear complexes to T cells, constitute the main drivers of SLE. The important role of B cells has been demonstrated in a murine model lacking this lymphocyte population (131). In addition to that, the same group showed that B cells also play an antibody-independent role in murine lupus in their function as antigen presenting and cytokine secreting cells (132).

Alterations in B cell maturation and differentiation affect several B cell subsets, targeting different checkpoints of B cell development. In SLE patients the frequency of antibody producing plasma cells in the peripheral blood is increased and correlates with autoantibody production and disease activity (133). It has been demonstrated that amongst others the overexpression of BAFF/BLyS (B-cell activating factor/B-lymphocyte stimulator), type I interferon and Blimp-1 (B lymphocyte-induced maturation protein-1) is responsible for these alterations in SLE patients (134–136).

Although B cells have emerged as central players in SLE, B cell depletion failed repeatedly as a therapeutic strategy in clinical trials. For example, the EXPLORER study demonstrated that rituximab, a CD20 antibody, did not show any statistically significant efficacy in achieving treatment response compared to placebo. Moreover, a recent reanalysis confirmed these findings, reevaluating the data with the help of newly available disease activity scores (137, 138).

There is only one therapeutic antibody approved by the FDA and the EMA for SLE therapy, namely belimumab, which neutralizes BAFF/BLyS and thereby decreases the number of newly formed B cells (139, 140).

The management of SLE strongly depends on the course of the disease. Glucocorticoids represent highly effective agents in order to immediately control the inflammatory process in SLE. Systemic glucocorticoids are required as initiation therapy in severe SLE, whereas maintenance immunosuppressive therapy is added in order to enable steroid tapering. Nevertheless, especially in acute, organ-threatening disease high-dose intravenous pulse therapy (usually 250–1,000 mg prednisone equivalent per day for 3 days) is often used to reduce disease activity (36). Interestingly, Guiducci et al. demonstrated that oral glucocorticoids (5–20 mg per day) modulate multiple gene expression pathways but the IFN pathway (including 36 type-I-IFN-inducible transcripts) is not affected in SLE patients. In contrast, the IFN signature was normalized after intravenous pulse therapy, which correlates with a reduction in pDC. The IFN-α production was reduced after a combined inhibition of TLR7 and 9 in purified pDC indicating that continuous triggering of TLR7 and 9 in these cells by immune complexes containing nucleic acid in SLE patients counteracts the activity of glucocorticoids on the IFN pathway. Thus, TLR7, and 9 inhibitors could be effective as glucocorticoid-sparing drugs (128).

However, the mechanism of glucocorticoid action in SLE patients is largely unknown. A study in MRL/MpSlac-lpr mice with systemic autoimmune symptoms similar to human SLE analyzed prednisone action on plasma cell differentiation with regard to the impact of regulatory factors, including IL-21, Blimp-1, and Bcl-6 (B cell lymphoma-6—essential for germinal center development). The percentages of plasma cells and plasma cell precursors as well as activated T cells were decreased after 13 weeks of prednisone treatment (**Figure 2**). In addition, serum IL-21 and the expression of splenic Blimp-1 and Bcl-6 were reduced, which may be correlated with the restriction of B lymphocyte differentiation into plasma cells in these mice (141).

Haneda et al. went further in order to analyze which step of B cell differentiation is affected by glucocorticoids. They differentiated human B cells by sequential addition of cytokines and other agents in a three-step culture system to obtain activated B cells [CD19(hi)CD38(lo)IgD(-)], plasmablasts [CD19(hi)CD38(hi)IgD(-)], and plasma cells [CD19(lo/-)CD38(hi)IgD(-)]. They added low and high concentrations of prednisolone at the beginning of each differentiation step and found a significant inhibition of B cell proliferation and differentiation in the last step, whereas IgG production was decreased in step 2 and 3 only at high glucocorticoid concentrations (100 ng/ml) (142). Interestingly, the number of circulating B cells was less affected by glucocorticoids compared to T cells which showed a rapid depletion in the circulation. In contrast, plasma cells and naive B cells are markedly decreased in the peripheral blood of SLE patients upon immunosuppressive therapy (143)—indicating that the inhibition of T cell help might contribute to the immediate glucocorticoid responses in SLE (144). Using transcriptome data to generate a pathway-level map of glucocorticoid effects across immune cell types, Franco et al. identified that glucocorticoid treatment (i) up-regulated the expression of *PRDM1*, which encodes BLIMP-1 involved in terminal differentiation and reduced proliferation of B cells and *IL10*, (ii) functionally impaired BCR signaling by suppressing *CR2* and *CD19* which encode the two components of the B cell co-receptor complex that serve as an enhancer of BCR-mediated signaling and (iii) selectively impaired TLR signaling by downregulation of *TLR1*, *TLR6*, and *TLR7* (32).

However, responses to glucocorticoids differ from patient to patient suffering from SLE. This may, at least in part, be related to the glucocorticoid receptor α whose alteration has been demonstrated in several autoimmune diseases (145–150). In SLE patients, the receptor expression is reduced compared to healthy controls. In addition, treatment with glucocorticoids further reduces the receptor mRNA and protein expression and it has been demonstrated that the receptor expression is negatively associated with SLE disease activity. Thus, the determination of receptor expression may be of importance with regard to insensitivity to glucocorticoids or determination of therapeutically effective dosages (151).

The glucocorticoid-induced leucine zipper (GILZ), an anti-inflammatory protein whose expression is upregulated by endogenous and exogenous glucocorticoids, has been in the focus of an *in vitro* study in human B cells. In general, GILZ mRNA and protein expression in peripheral blood mononuclear cells obtained from patients with SLE were downregulated compared to controls and correlated negatively with different markers of disease activity. An analysis of human B cell subsets revealed that intracellular GILZ was significantly decreased in circulating HLA-DR^{lo} plasmablasts [precursors of HLA-DR^{hi} cells which indicate active disease (152)] in patients with SLE. Treatment with prednisolone restored the GILZ expression to the level of control donors, a process described as transactivation—the activation of the transcription of anti-inflammatory proteins—in the introduction. Furthermore, an impaired induction of GILZ in SLE patients under glucocorticoid treatment was associated with an increased disease activity (153).

In the past decade, several additional factors including p-glycoprotein and the macrophage migration inhibitory factor (MIF) have been identified in the context of glucocorticoid resistance in SLE [reviewed in (154)]. P-glycoprotein (P-gp), a product of the multidrug resistance gene MDR-1, mediates the excretion of numerous drugs including antibiotics and cytotoxins but also glucocorticoids (155). P-gp is widely expressed in a variety of tissues, including peripheral blood T and B lymphocytes (156). However, P-gp expression is increased in these cells in SLE patients and is correlated with disease activity (157). Thus, elevated levels of P-gp lead to poor disease control by systemic glucocorticoid therapy and are associated with glucocorticoid resistance (157, 158). Beside P-gp, the inflammatory cytokine macrophage MIF actively reduces glucocorticoid action, participates in multiple stages of the inflammatory response and is widely associated with autoimmune disorders such as RA and SLE (159). MIF is also known as a naturally occurring counter-regulator of glucocorticoid action, correlates with disease activity in SLE and mediates the development of glucocorticoid resistance in SLE (159–162).

Although glucocorticoids are highly effective in the treatment of SLE, these drugs bear the risk of severe adverse effects,

especially when given over a longer period of time and/or at higher dosages. A study analyzing the relationship between glucocorticoids and damage accrual in SLE demonstrated that medium to high mean daily prednisone doses and higher cumulative doses were associated with an increased occurrence of adverse effects. Eighteen patients developed new damage attributable to glucocorticoid treatment including cataracts, osteoporotic fractures, avascular necrosis, and diabetes mellitus (163). New drug developments or improved formulations for SLE therapy are promoted with the objective of reducing the glucocorticoid dosage and thereby attenuating adverse effects. The therapeutic effect of a liposome-based steroidal methylprednisolone nano-drug has been evaluated in a murine model of SLE compared to the free agent. The study revealed that the steroidal nano-drug formulation is significantly more effective in suppressing anti-dsDNA antibody levels, proliferation of lymphoid tissue and renal damage, and in prolonging survival compared to free methylprednisolone given at the same dosage (164). The advantage of nano-liposomes is that they passively reach the inflamed site due to the enhanced permeability of the inflamed tissue vasculature, ensuring a reduced level in non-inflamed tissues (165, 166). Due to these advantages, liposomal glucocorticoids are also of great interest in the treatment of other inflammatory diseases (167–170).

Th2 Lymphocytes Constitute Major Contributors to the Pathogenesis of Allergic Diseases

Contrary to Th1 cells CD4+ T helper cells type 2 (Th2) are mainly involved in eosinophil activity as well as IgE production caused by an immunoglobulin class-switch in B cells (171). Th2 cells are characterized by the expression of GATA-3 and the secretion of Th2 cytokines, namely interleukin (IL)-4, IL-5, and IL-13 (172). Their development is promoted by a milieu abundant in IL-2 and IL-4 that activate STAT6 signaling and thereby promote Th2 differentiation (**Figure 3**). Thus, the key role of IL-4 consists in both mediating Th2 cell function and maintaining Th2 predominance by autocrine secretion.

Physiologically, Th2 cells exert their main function in the control of helminth infections. This mechanism of defense, referred to as the "type 2 response," involves players of both the innate and adaptive immune system. Besides the activation and proliferation of Th2 cells and the secretion of their characteristic cytokines, this cascade comprises eosinophil and basophil granulocytes, mast cells as well as IgE secreted by plasma cells (173). Considered to possess anti-inflammatory characteristics, the type 2 response is thought to have evolved as a mechanism of parasite control that simultaneously confines collateral damage and promotes tissue repair (174). In this regard, the antibody isotype IgE fulfills an important function in responding to metazoan infections. Cross-linking of IgE bound to high-affinity receptors (FcεRI) on mast cells and basophils triggers the release of mediators that facilitate healing without activating complement. However, rising hygienic standards have reduced the necessity of antihelminthic defense mechanisms, thereby depriving Th2 cells of their original target pathogens. In this context, the role of a dysregulated type 2 response in the pathogenesis of immune-mediated diseases has attracted increasing attention.

With a lifetime prevalence of about 40%, allergic diseases represent the most common immune disorder in western countries, affecting both children and adults (175). The German Health Interview and Examination Survey for Children and Adolescents (KiGGS) revealed a prevalence of 22.6% among children and adolescents with three main diagnoses in descending order: atopic dermatitis (AD), allergic rhinoconjunctivitis (AR), and asthma (176). The pathogenesis of atopic disorders is defined by a predominant type 2 response involving all major players described above (177–180). The allergic cascade is set into motion by IL-4 and thymic stromal lymphopoietin (TSLP) secreted from basophils (181). This step promotes Th2 differentiation followed by the secretion of IL-4 and IL-13 from activated T lymphocytes (**Figure 2**). Subsequently, these cytokines cause B cells to undergo a class-switch to IgE producing plasma cells. Upon allergen exposure cross-linking of these antibodies bound to mast cells results in a release of histamine, prostaglandins, and leukotrienes that enhance paracellular permeability (**Figure 2**). As a result, dendritic cells (DCs) infiltrate the affected tissue and maintain T cell stimulation in their role as antigen-presenting cells (APCs). Activated Th2 cells produce type 2 cytokines that sustain the mechanisms underlying allergic reactions. While IL-4 mainly induces the class-switch toward IgE production, IL-13 additionally causes mucus production and airway hyperresponsiveness (182, 183). On the other hand, IL-5 supports eosinophil survival and function (**Figure 2**) (184, 185). The substantial role of the type 2 response in the pathogenesis of allergic diseases has also been highlighted by the examination of samples from patients suffering from AD (186), AR (187), and asthma (188–191) demonstrating the preponderance of Th2 cells and cytokines in the affected tissues.

Glucocorticoids, administered both topically and systemically, represent indispensable agents in the treatment of atopic disorders (192). Generally, these drugs are capable of reducing the number of immune cells present at the site of allergic reactions (**Figure 2**) (193, 194). On examining the effect of glucocorticoids on Th2 cells in greater detail, a contradiction becomes evident. Although these agents are successfully administered to atopic patients, glucocorticoids have been described to promote Th2 cell predominance (**Figure 3**)—a well-described driver of allergic diseases (195–201). In order to solve this apparent conflict, the mechanism of action of glucocorticoids in Th2-driven disorders needs to be reviewed more closely.

Firstly, one has to distinguish between short-term and long-term drug effects. Temporary application of supraphysiological glucocorticoid doses results in an inhibition of Th2 cytokine production (**Figures 2**, **3**) (202–205). This effect is mainly mediated by glucocorticoid action on transcription factors as described in the introduction (206–208). For instance, binding of the GR to the IL-5 gene promoter region results in the repression of the cytokine by interfering with GATA-3 signaling (209). Moreover, this process seems to involve

histone deacetylation. Similarly, inhibition of GATA-3, the key transcription factor of Th2 differentiation, plays an important role (210). Maneechotesuwan et al. showed that ligand-activated GR and GATA-3 compete for importin-alpha interaction enabling nuclear localization (211). Application of inhaled fluticasone propionate (FP) prevented nuclear transport of GATA-3 by means of this mechanism in asthmatic patients. The authors also demonstrated that the induction of MAPK phosphatase-1 (MPK-1) by FP results in the inhibition of p38 MAPK function, thus preventing GATA-3 phosphorylation. Also, dexamethasone treatment decreased GATA-3 expression in an asthmatic mouse model by inhibiting Notch1 signaling (212). In contrast, chronic exposure to glucocorticoids may cause a shift toward Th2 predominance. On the one hand, this thesis is underlined by multiple studies analyzing the role of stress in atopic diseases. Periods of stress are marked by elevated levels of endogenous cortisol that promote Th2 predominance and thereby susceptibility to allergy (213). The impact of psychological stress on the course of disease in asthmatic patients has been reviewed by Miyasaka et al. (214). On the other hand, Ramirez revealed that prior glucocorticoid exposure provokes type 2 cytokine production in T cells (201).

Secondly, the effect of glucocorticoid administration on Th2 cells in atopic patients appears to differ from the Th2 enhancement generally caused by these drugs. Hydrocortisone significantly reduced the presence of IgE, histamine, and type 2 cytokines in serum and skin samples from AD patients (215). Correspondingly, AR patients presented a decrease in eosinophils, IgE, IL-4, and IL-5 in their nasal fluid after topical and oral glucocorticoid application (**Figure 2**) (187, 216–218). Lastly, the suppression of the type 2 response by glucocorticoid treatment was also observed in bronchial tissue and bronchoalveolar lavage fluid from asthmatics (219–221).

Finally, the beneficial glucocorticoid actions in allergic diseases are not only caused by their impact on Th2 lymphocytes. On the contrary, several players of the type 2 immune response are equally affected by glucocorticoid treatment. Namely, mast cell maturation and activation, FcεRI expression as well as mediator production and release are inhibited by glucocorticoid exposure (222–227). Furthermore, glucocorticoids impede histamine release from basophils and induce eosinophil apoptosis (228, 229). Recruitment and function of APCs as well as the class-switch to IgE in B cells are also restrained (230–232). Additionally, Klaßen et al. demonstrated the importance of non-hematopoietic cells in mediating glucocorticoid effects in a mouse model of allergic asthma (233). In the end, it has to be mentioned that recent findings emphasize the involvement of other CD4+ T helper cell subsets in the pathogenesis of allergic diseases. Increasing importance has been ascribed to Th17, Th9, Th22, and Th25 cells in this context (234). Similarly, the impact of regulatory T cells (Treg) must not be neglected. Several studies describe defective Treg activity as a major contributor to the development and maintenance of atopy (235–242). In this regard, glucocorticoids greatly contribute to the restoration of Treg function, thereby controlling the dysregulated type 2 immune response (243–246).

MECHANISMS OF GLUCOCORTICOIDS IN THE TREATMENT OF MALIGNANCIES WITH A FOCUS ON LEUKEMIA

The last chapter will give a brief insight into the mechanisms of glucocorticoid action in cancer therapies. Interestingly, the effects of glucocorticoids on different cancer subtypes and thereby the underlying mechanisms vary, even regarding opposite effects. This may be related to the subtype of cancer itself including its location, the affected cell type, the microenvironment and emerging comorbidities. Also, the glucocorticoid dose ranging from low to high daily dosages, and the level of glucocorticoid receptor expression and activity play an important role. In addition to that, the co-existence of other receptors of the steroid receptor family, namely the androgen and the estrogen receptors, can affect glucocorticoid action, especially in breast or prostate cancer, since there are also differences in receptor positive and receptor negative cancer subtypes. Another beneficial effect on different subtypes of cancer should not be neglected: Glucocorticoids are used as co-therapy during chemotherapy or radiotherapy in order to reduce side effects. They have been shown to improve mood, increase appetite and thereby lessen weight loss, reduce fatigue, diminish ureteric obstruction, prevent vomiting, and alleviate pain (247–250).

In the following, we will concentrate on hematopoietic malignancies which form a particular subset of cancerous conditions that were first discovered as such in the Nineteenth century when Rudolf Virchow coined the term "leukemia," meaning "white blood." Glucocorticoids play a crucial role in the treatment of these malignancies, among others as part of the CHOP regimen to treat non-Hodgkin lymphoma as well as in myeloma therapy. Nevertheless, due to the considerable differences of glucocorticoid effects on diverse cancer types, we will focus on one subtype here, namely leukemia. The epidemiology of the disease is summarized in **Table 2** according to the German and Austrian cancer register (www.gekid.de; www.statistik.at).

Four different types of leukemia are described: chronic lymphoblastic leukemia (CLL), chronic myeloid leukemia (CML), acute myeloid leukemia (AML), and acute lymphoblastic leukemia (ALL). CLL is the most common leukemic disease in western industrialized countries, where the disease constitutes 95% of the overall cases in older individuals (50 years and older) (251). The main reason for the therapeutic use of glucocorticoids in leukemia is their pro-apoptotic action.

Chronic Lymphoblastic Leukemia (CLL)

The inhibition of B cell apoptosis and the dysregulation of proliferation and differentiation are the main causes of CLL. They lead to an accumulation of mature CD5-positive, CD10-negative, CD20 weakly positive, and CD23-positive B cells within blood, bone marrow and solid lymphoid organs (252–254). Therefore, the B cell itself, the B cell receptor and the subsequent signaling pathways are novel targets of therapies using e.g., monoclonal antibodies like rituximab or small molecules such as the kinase inhibitor ibrutinib [reviewed in (253)].

TABLE 2 | Epidemiology of leukemia.

Registry	Cancer type	Female		Male		Total
		Rate	Number of cases	Rate	Number of cases	Number of cases
Germany	Cancer (total)	336.7	223.019	436.5	259.013	482.032
	Leukemia	8.1	5.550	13.2	7.489	13.039
	Leukemia (mortality)	4.0	3.575	6.4	4.168	7.743
Austria	Cancer (total)	421.8	19.393	581.4	21.342	40.735
	Leukemia	9.0	420	16.5	586	1.006
	Leukemia (mortality)	7.3	354	11.9	386	740

Rate and absolute numbers of cases of total cancer (without other tumors of the skin) and leukemic (including mortality) in Germany and Austria in the year 2014. Rate is given per 100,000 individuals per year (new disease) according to the German and Austrian cancer registry (www.gekid.de; www.statistik.at).

In healthy subjects, the administration of glucocorticoids affects different subsets of the peripheral blood leukocytes, resulting in a transient lymphocytopenia (255). This has been demonstrated to be mostly caused by a glucocorticoid induced redistribution of lymphocytes from the blood into the tissue, affecting mainly T cells and B cells to a lesser extent (252, 256). In CLL patients however, the administration of glucocorticoids leads to an increase in blood lymphocytes accompanied by a rapid reduction in spleen and lymph node sizes. Following the therapy, the number of lymphocytes decreased even beneath pretreatment levels (257, 258). Unfortunately, the underlying mechanism is still unknown and glucocorticoids are consequently not commonly used to treat CLL. Nevertheless, these drugs are currently of interest to complement treatment with monoclonal antibodies or small molecules. In 2016, Manzoni et al. analyzed the *in vitro* effects of the combination of ibrutinib and dexamethasone on the proliferation and metabolic stress markers in lymphocytes obtained from patients suffering from CLL. They demonstrated an enhanced inhibition of cell cycle progression, an increase in apoptosis and a decrease in DNA damage in lymphoid B cells by a combination of dexamethasone and ibrutinib compared to the tyrosine kinase inhibitor alone (259).

Chronic Myeloid Leukemia (CML)

Tyrosine kinase inhibitors also show remarkable success in controlling CML, a disease of myeloid progenitor cells. This is due to the knowledge of the underlying molecular pathogenesis of this disease which arises mainly from a translocation $t_{(9,22)}$ (q34;q11), resulting in transcripts and fusion proteins with unusual tyrosine kinase activity (260). Thus, tyrosine kinase inhibitors, e.g., imatinib and dasatinib, are used as standard therapy with a high rate of remission (261). Consequently, the use of glucocorticoids has become dispensable. Unfortunately, this kind of molecular-targeted therapy is exceptional since the molecular target is unknown in all other types of leukemia.

Acute Myeloid Leukemia (AML)

The heterogeneous character of AML impedes such targeted therapies. Therefore, the treatment largely relies on the use of aggressive chemotherapy (262). AML is characterized by an infiltration of the bone marrow, blood, and tissues by hematopoietic progenitor cells which lose their ability to differentiate physiologically due to heterogeneous clonal disorders. The extent of the genetic variability of AML patients has been the focus of different studies aiming at customized therapeutic approaches (263). In contrast to more recent findings, it has been demonstrated in 2006 that short-term treatment with high-dose methylprednisolone resulted in an induction of differentiation and apoptosis of leukemic cells in children with AML. Furthermore, the addition of this high-dose glucocorticoid therapy to chemotherapy led to increased remission rates and improved patient outcome (264). However, high rates of glucocorticoid resistance in AML patients have been reported in the last years, so that glucocorticoids are not suitable as standard therapy (265).

Acute Lymphoblastic Leukemia (ALL)

In contrast, leukemic cells in ALL are much more sensitive to glucocorticoids. Therefore, the administration of high-dose glucocorticoids (i.e., dexamethasone and prednisolone) represents the standard induction therapy in ALL (266). The specific genotypes of ALL are diverse, including aberrant expression of proto-oncogenes, chromosomal translocations resulting in fusion genes and hyperdiploidy involving more than 50 chromosomes [reviewed in (267)]. These genetic alterations contribute to changes in cellular function, such as a dysregulation of differentiation, proliferation, and programmed cell death of hematopoietic stem cells (254, 267, 268).

The glucocorticoid-induced cell death in leukemia is mediated by the glucocorticoid receptor via transrepression and transactivation (please see Introduction). It has been demonstrated that the repression of anti-apoptotic BCL2 and the activation of the antagonizing pro-apoptotic BIM induce cell death in ALL (269, 270). Other genes and even microRNAs have been described to be regulated by glucocorticoids and thereby mediate apoptosis (271–273). In addition, cell death is also triggered by calcium release from the endoplasmic reticulum into the cytosol and by an enhanced expression of thioredoxin-interacting protein (TXNIP) which induces cell death by increasing reactive oxygen species and/or blocking glucose transport (270).

Finally, the underlying mechanisms which mediate glucocorticoid-induced cell death in leukemia are

diverse and not yet well-understood. The existence and the development of glucocorticoid resistance after long-term therapy aggravate treatment strategies or reverse the achieved remission. This applies to both the treatment of cancer and the treatment of inflammatory autoimmune diseases.

CONCLUDING REMARKS

After highlighting the effects of glucocorticoids in different immune cells in the context of a variety of immunopathologies, we have to conclude that the understanding of the mode of glucocorticoid action in the scope of immune responses and glucocorticoid resistance is still incomplete. Although glucocorticoids have ranked among the most potent immunosuppressive drugs in daily clinical care for more than 70 years, knowledge on their mechanisms of action on cellular and sub-cellular levels in an immune cell type-specific manner and in the context of the respective immunopathology remains scarce. Further research into this topic will enhance our comprehension of the capacity spectrum of glucocorticoid action and the establishment of glucocorticoid resistance, also providing guidance for personalized therapy.

AUTHOR CONTRIBUTIONS

TG, LE, and CS designed the concept. CS and LE prepared the figures. All authors wrote the manuscript and read and approved the final version of the manuscript.

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REVIEW
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Fighting the Fire: Mechanisms of Inflammatory Gene Regulation by the Glucocorticoid Receptor

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For many decades, glucocorticoids have been widely used as the gold standard treatment for inflammatory conditions. Unfortunately, their clinical use is limited by severe adverse effects such as insulin resistance, cardiometabolic diseases, muscle and skin atrophies, osteoporosis, and depression. Glucocorticoids exert their effects by binding to the Glucocorticoid Receptor (GR), a ligand-activated transcription factor which both positively, and negatively regulates gene expression. Extensive research during the past several years has uncovered novel mechanisms by which the GR activates and represses its target genes. Genome-wide studies and mouse models have provided valuable insight into the molecular mechanisms of inflammatory gene regulation by GR. This review focusses on newly identified target genes and GR co-regulators that are important for its anti-inflammatory effects in innate immune cells, as well as mutations within the GR itself that shed light on its transcriptional activity. This research progress will hopefully serve as the basis for the development of safer immune suppressants with reduced side effect profiles.

Keywords: glucocorticoid receptor, inflammation, macrophages, mouse models, gene regulation

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INTRODUCTION

Glucocorticoids as Immunomodulators

Glucocorticoids (GCs) are steroid hormones secreted in a diurnal and stress responsive manner, under the control of the hypothalamic-pituitary-adrenal (HPA) axis (1).GCs regulate numerous essential physiological and developmental processes, ranging from lung maturation to glucose metabolism and immune responses. This is clearly demonstrated in mice with abrogated GC signaling, which die perinatally due to pulmonary atelectasis (2). The effect on lung maturation is not merely limited to mice: in clinical practice, pre-term neonates are given GCs to accelerate pulmonary development (3). In adult mammals, endogenous GCs play important homeostatic roles. For instance, GCs increase glucose production through glycogenolysis and gluconeogenesis in the liver upon fasting, and as part of daily rhythmic energy mobilization (4, 5).

Pharmacologically, GCs are widely used to treat acute and chronic inflammatory diseases, such as asthma, allergies, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis etc., due to their potent anti-inflammatory actions. In addition, GCs are commonly prescribed to prevent graft-vs.-host immune responses after organ transplantation and for certain cancer types, such as lymphoma (6, 7). Currently, it is estimated that 1–3% of the adult Western population are receiving

GCs, demonstrating their broad applications (8). GCs have been used for over 70 years as anti-inflammatory drugs, despite their adverse effects on systemic metabolism, which were noted soon after their first clinical use (9). Long term exposure to GCs induces adipocyte hypertrophy, glucose intolerance and insulin resistance, hypertension, muscle and skin atrophy, osteoporosis, glaucoma, impaired wound healing and psychological effects such as mood changes, insomnia, and depression (4, 10). Long term GC exposure due to increased secretion from endocrine tumors or chronic exogenous administration, often causes a pathological condition known as Cushing's syndrome (11). Cushing's manifests as debilitating muscle wasting, fat accumulation, and susceptibility to infection and can be fatal if left untreated.

Separating beneficial therapeutic properties from detrimental side effects based on a molecular understanding of GC action is a long-term goal of biomedical research. Furthermore, the glucocorticoid receptor (GR) has been key to understanding the basic molecular concepts of GC action. There have been several paradigm shifts of the molecular understanding of GC/GR mechanisms since cloning of the receptor more than 30 years ago (12). The generation of GR mutants that interfere with specific functions of the receptor, the introduction of several mutants into preclinical models and the characterization of genome wide profiles all revolutionized our view of GC action. In this review, we summarize recent insights into the anti-inflammatory effects of GR, focusing on mechanisms of macrophage gene regulation, GR co-regulators, novel GR target genes, and mouse models of inflammation. We also summarize the current understanding of immune modulatory mechanism in the innate immune system based on mouse mutants. These might explain why, despite much progress, developing novel immune modulators that match the efficacy of GCs but avoid the adverse effects remains a major challenge for the field.

The Glucocorticoid Receptor

The endogenous GC, cortisol in humans and corticosterone in rodents, binds to the GR, encoded by the *NR3C1* gene. GR belongs to the nuclear receptor superfamily of ligand activated transcription factors. It consists of three major domains, the central DNA binding domain (DBD), the N-terminal transactivation domain (NTD), and the C-terminal ligand binding domain (LBD) [(12); **Figure 1**].

The *NR3C1* gene encodes several isoforms that are generated by alternative splicing and alternative initiation of translation (10, 13). The full-length isoform GRα-A is the focus of this review. GRβ, a second splice variant, and other GR isoforms, are known to modify GC sensitivity, but are discussed in detail elsewhere (14).

In the absence of ligand, GR resides in the cytoplasm, bound to heat shock proteins 70 and 90 (Hsp70 and Hsp90) together with other chaperones and immunophilins (15). Upon binding of GCs, GR translocates to the nucleus where it binds to DNA sequences. In this way, GR is recruited to target gene enhancers and promoters where it can both activate and repress transcription (16, 17). Canonical binding sites for the GR are called glucocorticoid response elements (GREs) and are composed of two 6bp palindromes (half sites) separated by a 3bp spacer, with the consensus AGAACAnnnTGTTCT. However, GR binding sites (GBS) in the genome vary to a certain degree of motif mismatch, expanding the number of possible target sequences. Furthermore, the context of neighboring transcription factor binding sites and the ensuing crosstalk is relevant for the regulation of inflammatory genes by the GR. The beauty of using GR as a model transcription factor is that its ability to regulate genes can be easily controlled *in vitro* and *in vivo* by the absence or presence of the GC ligand.

Chromatin Residence Time and Multimerization of the Glucocorticoid Receptor

GR, along with other transcription factors, was assumed to bind DNA in a relatively static manner, "sitting down" for long periods of time to regulate gene expression. However, visualization of the dynamics of fluorescent-tagged GR in living cells led to the insight that occupancy of dimeric GR molecules at GREs is rather in the order of seconds and less (18). Only a small portion of available molecules are specifically bound to chromatin at a given time, suggesting that transcription factors and co-factors have a transient rather than stable interaction at genomic response elements (19).

GR acts as a monomer (20), dimer (21, 22), and even tetramer (23–25) depending on the subcellular localization, presence of ligand, GREs, or artificial response elements such as the MMTV array. Interestingly, DNA binding was proposed to trigger allosteric regulation of GR, followed by a change in its oligomeric state (24). Ligand bound GR is mainly nuclear and dimeric. Interestingly, upon DNA binding, the structural LBD rearrangement promotes the formation of higher order oligomers, predominantly tetramers, through unstudied LBD surfaces (25). The physiological relevance and implications of a tetrameric GR, however, are still open for debate and further investigation.

In general, chromatin binding and gene regulation by GR appear to be much more dynamic than previously thought, and the residence time of GR on chromatin may have differential effects. The LBD seems to regulate the number of GR molecules bound at a specific genomic region, which may also affect the transcription of target genes.

Glucocorticoid Receptor Co-regulators

All nuclear receptors (NRs), including GR, require a host of co-activators and co-repressors to ultimately control the transcriptional apparatus.

Steroid receptor coactivator-1 (SRC-1, also known as nuclear receptor co-activator 1, NCOA1) was one of the first identified (26), followed by glucocorticoid receptor interacting protein (GRIP1, SRC-2, and NCOA2) (27). Originally found to be a co-activator of the progesterone receptor (PR), SRC-1, and GRIP1 were shown to directly interact with GR and other steroid receptors. This direct co-activator interaction with GR depends on the evolutionarily conserved LXXLL motif, or NR-box, and without this motif, GR loses transcriptional activity (28). SRC-1

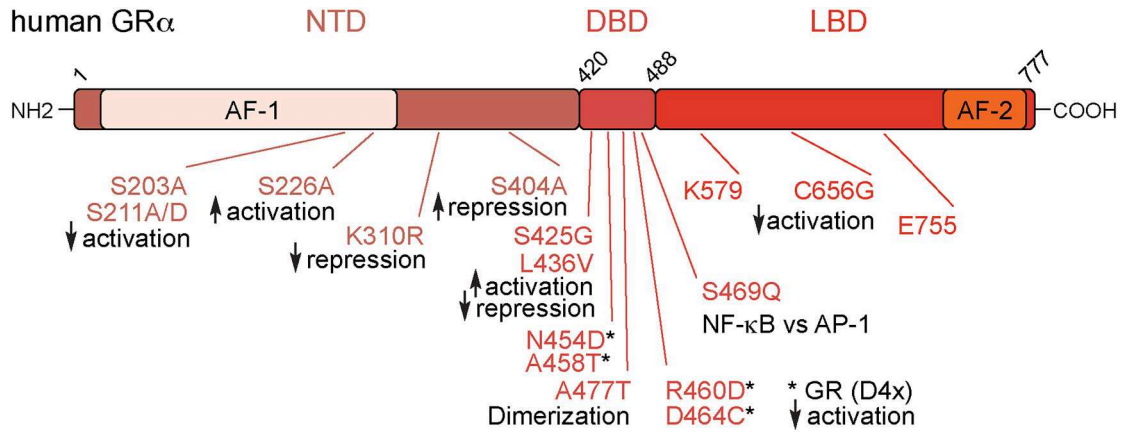

FIGURE 1 | Overview of the glucocorticoid receptor protein. The Glucocorticoid Receptor (GR) is organized into three main domains: the N-terminal Transactivation Domain (NTD), the DNA-Binding Domain (DBD), and the Ligand Binding Domain (LBD). In addition, there are the transactivation domains 1 and 2 (AF-1 and AF-2). These mutations numbered above are relevant for GR's immunomodulatory effects. Numbers are amino acids of the human protein.

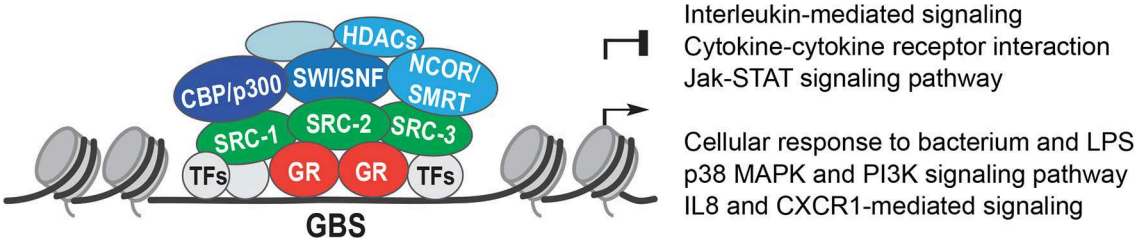

FIGURE 2 | Glucocorticoid receptor co-regulators. The Glucocorticoid Receptor (GR) binds to Glucocorticoid Receptor Binding Sites (GBS) in open chromatin. GR interacts with other transcription factors (TFs) and recruits co-activators or co-repressors, such as: the Steroid Receptor co-activators 1, 2, and 3 (SRC-1, SRC-2, and SRC-3); the histone acetyl transferases CREB binding protein (CBP) and p300; the Nuclear Receptor co-repressors NCOR1 and NCOR2 (NCOR, SMRT), which recruit histone deacetylases 1 and 3 (HDACs); and the SWItch/Sucrose-Non Fermentable (SWI/SNF) chromatin remodeling complex.

directly activates genes with its histone acetyltransferase (HAT) domain that decondenses chromatin [(29); **Figure 2**].

The strength of GR's interaction with SRC-1 and GRIP1 might determine the steroid responsiveness of cancer cells, suggesting that the loss of GC-induced apoptosis or growth arrest is due to, at least in part, co-activator recruitment (30). However, GR seems to preferably interact with GRIP1 over SRC-1, while the opposite is true for PR, which confers selectivity of GR activation and PR activation on chromatin modifications (31).

Importantly, the co-activator GRIP1 can also act as a co-repressor. Depending on the individual GR target gene, GRIP1 functions as either an activator or repressor by using its co-repressor domain. For example, GRIP1 was described to act as a co-repressor at the *osteocalcin* promoter (32). Moreover, the functionality of GRIP1 is modulated by post-translational modifications. CDK9 mediated phosphorylation of GRIP1 was shown to increase GR dependent activation, but had no effect on repression (33).

SRC-3 (NCOA3), another member of the SRC family, was originally identified through interaction with the estrogen receptor (ER) (34). Similar to SRC-1 and GRIP1, SRC-3 is recruited in a locus-specific manner (35).

In the mid-1990s, the discovery of two nuclear receptor co-repressors (NCOR)—NCOR1 (36), and NCOR2 (otherwise known as SMRT, silencing mediator co-repressor) drove further research into the field of NR co-regulators (37). The NCOR family interacts with nuclear receptors via the coRNR-box, consisting of the consensus sequence LXX I/H I XXX I/L, which contacts the AF-2 domain of NRs (38, 39). This is analogous to the LXXLL sequence in co-activators and occupies a similar location on the receptors.

While the NCOAs display intrinsic HAT activity, the co-repressors NCOR/SMRT were described to interact with the histone deacetylase HDAC3 (40). Both NCOR1 and SMRT were able to recruit HDAC3 to condense chromatin as part of their repressive mechanism (41).

SUMOylation of mouse GR at K310 was shown to be essential for repression, and in point mutant mice, neither NCOR1, SMRT nor the associated HDAC3 complex were recruited (42, 43). GCs down-regulate expression of GR itself, through a negative feedback loop. This occurs by recruitment of a GR-NCOR1-HDAC3 complex to an nGRE in exon 6 of the *NR3C1* gene (44). GC-mediated suppression of natural killer cells activity however, was described to be mediated by HDAC1 and SMRT specifically

(45). The differential control of GR action by recruitment of alternative co-activators and co-repressors, in tissue or signal specific contexts, is still an open area of investigation. Different GR ligands selectively recruit alternate co-factors (46), suggesting that ligand induced conformational changes might have discrete effects on GR target genes, adding another level of complexity to GR mediated gene regulation.

Two major proteins that are recruited by co-activators are CBP (CREB binding protein) and p300. Both CBP and p300 are histone acetyl transferases (HATs), and induce chromatin relaxation (47) (**Figure 2**). SRC-1 was shown to recruit p300 into a complex with nuclear receptors to activate transcription (48). Part of GR's repressive action might involve competition for CBP and p300, as GR repression of an AP-1 (Activator Protein 1) reporter was abolished by overexpression of CBP and p300 (49). Moreover, enhanced engraftment of hematopoietic stem cells in response to GCs was described to be controlled by SRC-1 and p300 recruitment to the *CXCR4* gene, with acetylation of histones H4K5 and H4K16 upregulating *CXCR4* (50).

GR and the tumor suppressor protein 53 (p53) were shown to interact in a ligand dependent manner via Hd2m (a transcription factor), which enhanced the GC-induced degradation of both GR and p53 (51). In fact, the interaction between GR and p53 is important for the repression of NF-κB (nuclear factor-κB) responsive genes. Without p53, GR did not repress inflammation in a mouse model of endotoxic shock (52).

Finally, GR interacts with components of the SWI/SNF complex (SWItch/Sucrose-Non Fermentable). These highly evolutionarily conserved ATP-dependent chromatin remodelers use energy from ATP hydrolysis to alter nucleosome positioning. GR was shown to directly interact with the Baf250, Baf57, and Baf60a subunits of SWI/SNF complexes, further demonstrating the ability of GR to modify the chromatin architecture [(53–56); **Figure 2**].

In summary, GR recruits co-activators such as SRC family members, which in turn assemble a transcriptional complex containing histone modifying enzymes and chromatin remodelers to control the transcriptional machinery and RNA Pol II activity. These interactions are crucial for its anti-inflammatory actions and might present novel therapeutic targets in the future.

Mechanistic Insights Into Immunomodulation From GR Point Mutations *in vitro*

Introducing point mutations into the *NR3C1* gene significantly contributed to our understanding of the molecular mechanisms of GR action. Here, we briefly address the insights gained from specific residues that revealed certain GR functions essential to suppress inflammation in cultured cells.

Besides promoter/enhancer occupancy, post-translational modifications of GR play a major role for transcriptional control. Three key phosphorylation sites were identified in the human GR: S203, S211, and S226 (57–59). All of them are located in the AF-1 domain, which is crucial for protein-protein interactions with TATA-box binding protein and others (60). By using phospho-deficient (S211A) or phospho-mimetic (S211D) mutations, it was shown that phosphorylation of GR at S211 increases association with the MED14 subunit of the mediator complex, a key bridge to the transcriptional machinery (59). In confirmation, the S211A mutant displays reduced expression of the GR targets *GILZ* and *IRF8*. S226A mutation however, had the opposite effect. The phosphorylation-deficient mutant S226A showed increased expression of *GILZ* and *IRF8*, suggesting an inhibitory role (59). In addition, S404, a site for GSK3β phosphorylation, regulates GR transcriptional activity. Mutation to S404A rewired the GR-regulated transcriptome, interestingly increasing its repressive capacity (61). Moreover, the SUMOylation-deficient murine GR K310R was shown to affect repression and the recruitment of co-regulators [(42, 43); **Figure 1**].

The AF-2 domain, located within the LBD (62), has additional sites modulating GR function. The mutation C656G within the AF-2 domain of the rat GR (C638 in human) reduced the ligand concentration required for activation of the *PEPCK* promoter (63). Mutations within the "charge-clamp"—that is the co-activator interaction site of K579 and E755—resulted in loss of transcriptional activation, but had no effect on repression (64).

Applying a random mutagenesis approach in yeast, Yamamoto and colleagues showed that multiple mutations within the zinc finger of the DBD impede GR binding to GREs *in vitro*, demonstrating the importance of this particular domain (65). Further mutagenesis studies in the 1990s identified a multitude of important amino acids involved in activation and repression. For example, the mutations S425G and L436V in the DBD could double the activation in a reporter assay, but almost completely abolished repression by GR (66).

Mutations in the dimer interface are also central for the understanding of GR biology. The GR^{dim} (human A458T), corresponding to rat A477T (67), and GR^{mon} (mouse A465T/I634A) (68) mutations disrupt the dimer interface. Further mutation of A458T outside the D-loop to the double N454D/A458T further increased the capacity of GR to repress a reporter *in vitro* (66). Generation of the GR(D4X), a quadruple mutant GR with the residues N454D, A458T, R460D, and D464C in the dimerization region of mouse GR provided deeper insight into the monomer/dimer action of GR. The GR (D4X) had equivalent repressive activity to wild type, while activation capacity as measured in reporter assays was near zero. This mutant demonstrated that opposition of TNF-α involved both activation of IKKB and repression, since mutant GR was unable to induce IKKB, but repressed the production of TNF-α (69). There is significant work on the GR^{dim} mutation *in vivo*, covered in the next section. Early *in vitro* work however, showed that the A477T mutation induced loss of the dimer interface and reduced DNA residence time, making target gene regulation by A477T rather difficult to interpret (70). Both wild type GR and GR^{mon} bound GRE half sites, but A447T was incapable of binding classic, full length GREs, which are occupied by receptor dimers [(67); **Figure 1**].

Another mutation in the second zinc finger of the DBD in rat GR R488Q (R469 in the human GR) was designed to discriminate between interactions with NF-κB and AP-1. Overexpressing GR R488Q in activated CV-1 cells under inflammatory conditions failed to suppress NF-κB reporter

activity, whereas AP-1 inhibition was preserved (71). Additional GR mutations with less impact on inflammation are reviewed in more detail elsewhere (72).

Taken together, these GR point mutants show the importance and complexity of GR interactions with transcription factors and chromatin modifiers. In fact, several discrete mutations within the GR AF-1, AF-2 domains and the dimer interface alter its activity in a gene-specific manner, indicating that different parts of the receptor are dispensable for certain gene regulatory events, but essential for others (32). Differentially interfering with GR function therefore affects multiple physiological processes, and distinct types of inflammatory responses.

Lessons Learned From Genome-Wide Studies

Chromatin as a key determinant of GR function has been highlighted in multiple genome-wide ChIP-sequencing studies since the early 2010s. For instance, GR gene regulation is determined by the chromatin architecture of the responsive cell. GR does not act as its own pioneer factor, but rather cell-type-specific gene regulation is dependent on pre-existing available binding sites, determined by chromatin accessibility (73). The pro-inflammatory transcription factor AP-1 governs a large subset of GR regulatory sites, making areas of DNA accessible to GR (74). As GR is largely dependent on pre-existing open chromatin for binding, it cemented the possibility that stimuli which are known for chromatin remodeling, for example inflammation, alters GR binding. Indeed, treatment with TNF-α amends the transcriptional response to GCs, as well as chromatin occupancy of GR, and surprisingly GR activation also transformed the occupancy of NF-κB (75). Recent data showed that GR could indeed act as a pioneer factor for other transcription factors, such as FOXA1, but only at a minority of genomic sites, and thus far this effect has not been demonstrated in immune cells (76).

When assessing GR activity in a more relevant cell-type, macrophages treated with LPS, GR, p65 (part of the NF-κB complex), and c-Jun (one of the members of the AP-1 dimer) binding overlapped significantly (see below). However, the directionality of the gene regulatory response did not correlate well with the type of interaction. That is, contrary to established models, GR binding to NF-κB loci did not only result in repression of target genes, but either repression or activation depending on the particular locus. The inverse is also true, that GR binding to canonical GREs did not only result in up-regulation of transcription at the assigned gene. Rather than the presence or absence of GR as the determining factor, the recruitment of different chromatin modifiers, such as GRIP1, were the prime measure of whether the particular gene would be activated or repressed (77).

Moreover, GR effects can be dependent on the timing of the inflammatory signal. Pre-treatment of macrophages with GCs before LPS stimulation resulted in differential gene regulation compared to treatment with GCs after LPS stimulation. In addition, a large part of GR's anti-inflammatory action can be accounted for by the induction of negative regulators of inflammation such as Mkp1, GILZ, and A20, see below (78). GR^{dim} macrophages treated with LPS and Dex also showed that the dimerization impaired GR preferentially occupied GR-half sites (16), a phenomenon also observed in cells overexpressing GR A477T (67).

Importantly, all these studies showed that GR not only binds to GREs, but occupies motifs near lineage determining factors, such as PU.1 in macrophages. Again this underscores the idea that GR requires open, pre-programmed chromatin for finding its genomic target sites (16, 74, 77–79). The chromatin landscape is cell-specific and depends on pioneer factors, cell lineage transcription factors and epigenetic marks that all predetermine GR binding. Only a minority of GR peaks are found in inaccessible chromatin and trigger chromatin remodeling upon hormone treatment (16, 73, 79–82). These findings strongly suggest that other DNA-binding proteins prime the chromatin landscape prior to GR arrival. The collaborative binding of lineage-determining transcription factors results in nucleosome remodeling, which generates open regions of chromatin. This provides access to signal-dependent transcription factors to bind open regions and modulate gene transcription in a cell-specific manner (83). In the context of macrophages, PU.1 and C/EBP are essential for the development of the myeloid lineage and have been shown to establish the monocyte-specific enhancer landscape (83, 84). PU.1 deletion results in loss of macrophages, neutrophils and B cells (85, 86). Importantly, PU.1 and C/EBP transcription factors often co-localize with GR in macrophages (16).

This new methodology has given deeper insights into the mechanisms by with GR regulates gene expression, identifying chromatin remodeling, and cooperation with other transcription factors, as a key determinants of GR activity. Importantly, GR's reliance on other factors to define its binding sites underscores the necessity of studying GC responses in a tissue-specific manner, rather than extrapolating effects from one cell-type to another.

Molecular Mechanisms of Immunomodulation by the Glucocorticoid Receptor

Non-genomic Actions of GR

Some therapeutic GC effects, such as bronchodilation, resolution of airway irritation or suppression of inflammation, occur almost too rapidly to result from transcription, raising the possibility of non-genomic GR actions (87, 88). These could be GR-unspecific interactions with cellular membranes, functions of membrane-bound GR or specific interactions with cytosolic GR, thereby altering posttranslational modifications like phosphorylation, or other mechanisms (89).

Membrane-bound GR was described in human monocytes and B cells (90, 91), and non-genomic functions have been found in macrophages (92), lung epithelial cells (93), and T-cells (94).

Downstream of inflammatory MAPK signaling, mitogen- and stress-activated protein kinase-1 (MSK1) is an essential kinase for NF-κB p65 S275 phosphorylation (95).Interestingly, GC-mediated repression of NF-κB targets involves loss of MSK1

kinase recruitment at inflammatory promoters and nuclear export of MSK1 via cytosolic GR (96). Putatively, GR can also crosstalk with AKT, GSK-3β, and mTOR signaling (93).

These non-genomic effects might be very interesting for the development of novel therapeutics, and will benefit from future studies, for example with novel cell lines or mouse models to dissect these complex interactions.

Genomic Actions of GR

Lipopolysaccharide (LPS) is a molecular component of the cell wall of Gram-negative bacteria commonly used to study inflammation (97, 98). On macrophages, LPS binds to Toll-Like Receptor 4 (TLR4) and activates a signaling cascade that results in NF-κB and AP-1 nuclear translocation. Together with other inflammatory transcription factors, these two protein complexes then activate pro-inflammatory gene expression (99, 100). TLR4 activates AP-1 via the MAPK signaling pathway and NF-κB via degradation of the cytosolic IKK complex that frees the NF-κB transcription factor (**Figure 3**).

GR can antagonize or synergize with pro-inflammatory signaling, depending on the context of promoters or enhancers. For antagonism of pro-inflammatory signaling, several mechanisms are proposed. These include the direct interference with MAPK or JNK signaling (101, 102), leading to repressive actions at the gene regulatory level. Conversely, repression of GR-target genes might be explained by tethering to other transcription factors or trans-repression, negative GREs (nGREs, with a different sequence), composite GREs, non-canonical novel GREs, DNA as a modulator of GR, and consensus classical GREs.

Most frequently, GR tethering to AP-1 or NF-κB via protein-protein interactions (trans-repression), instead of direct DNA binding, was suggested to underlie its repression of inflammatory responses (103, 104). In other words, GR has been shown to represses genes via protein-protein interactions with AP-1 (105), NF-κB (106), STAT3 (107), and other DNA-bound transcription factors (**Figure 3**). Interestingly, STAT3 tethering to GR resulted in synergistic gene regulation, and increased target gene expression in AtT-20 cells. On the other hand, GR tethering to DNA-bound STAT3 resulted in transcriptional repression (107).

Negative GREs (nGREs) were originally described as GREs motifs in the promoters of repressed target genes. nGREs can be found in very different cell types and genes involved in various processes, for example: HPA axis (*POMC* and *CRH*) (108, 109), lactation (*PRL3*) (110, 111), bone homeostasis (*osteocalcin*) (112), skin structure (*keratins*) (113), and inflammation (*IL-1β*) (114).

However, the definition of nGREs has not yet reached consensus in the literature, and subsequently, GBS with non-classical consensus sequences, near repressed targets, are also named nGREs. One study described a variation of nGREs, termed "inverted repeat (IR) nGRE." IR nGRE is a complex GBS with the following consensus motif: CTCC(n)$_{0-2}$GGAGA, which differs from the classical GRE (AGAACAnnnTGTTCT) or nGRE (115). These elements however, have not been identified by ChIP-seq, questioning how relevant they are to GR responses.

Similar to nGREs, composite elements, such as degenerate GREs overlapping with other transcription factor consensus motifs, may also affect the transcription of inflammatory targets. For example, a 25-base pair composite element (plfG element) in the promoter of the *proliferin* gene, is regulated by GR and AP-1 (116, 117). Furthermore, the GR DNA-binding domain (DBD) can bind a newly identified motif inside NF-κB consensus sequences. Crystal structures of the GR DBD demonstrated direct binding of GR to the AATTT nucleotides within the NF-κB motif from the promoter regions of *CCL2*, *IL-8*, *PLAU*, *RELB*, and *ICAM1*. This cryptic GR-binding site overlapping the NF-κB response element was named κBRE and was highly conserved between species (118).

An important aspect is the concept of DNA being an allosteric modulator of the GR. Here, the precise nucleotide sequence in a GBS is proposed to function as a shaping ligand that specifies GR's transcriptional activity. X-ray crystallography of GR DBD dimers bound to different GBSs showed that conformation of the lever arm in the DBD appeared to be influenced by the DNA sequence (24, 119). Furthermore, the addition of a single GR-binding site was sufficient to convert a gene, which was normally not regulated by GR, into a target gene, such as *IL-1β* and *IL1R2* in U2OS cells (120). The presence of classical GREs in GR-bound enhancers near both activated and repressed genes in murine bone marrow-derived macrophages (BMDM) stimulated with LPS and Dexamethasone (Dex) challenge these models. These findings suggest that first, direct GR:GRE binding is relevant for repression of inflammatory genes. Secondly, that the classical models described above are not sufficient for prediction of GR mediated activation or repression. Therefore, the presence of a different combination of cofactors in activated vs. repressed sites could explain or contribute to the up- or down-regulation of GR target genes (77, 118, 121, 122).

Taken together, how GR activates one set of target genes while repressing another is still an open question, and the molecular mechanisms specifying the repression of inflammatory genes remain unknown. Repression by GR is a complex process which likely involves different determinant factors. One factor is GR itself (phosphorylation, post-translational modifications and ligand-specific conformations), another factor is the DNA sequence, the cell type-specific chromatin landscape and the cooperation with co-regulators and other transcription factors. All of these, together with potentially unknown factors, ultimately determine which target genes are up- or down-regulated.

Mechanistic Insights Into Immunomodulation From GR Point Mutations *in vivo*

As described above, one particular class of point mutations, which interfere with GR dimerization, caught considerable attention. In tissue culture experiments expressing these GR^{dim} mutants (human GR A458T, mouse GR A465T, and rat A477T), the concept was developed that abrogation of dimerization could be beneficial to limit side effects of anti-inflammatory treatments. Therefore, pharmaceutical companies directed their research to develop dissociated ligands favoring GR monomer

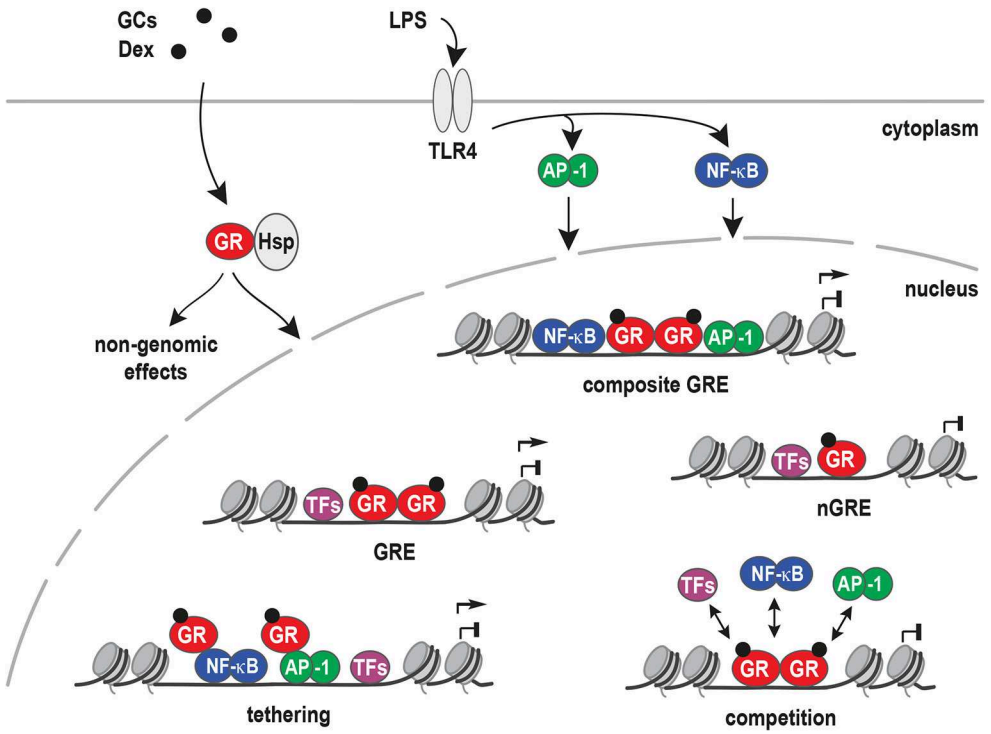

FIGURE 3 | Models for inflammatory gene regulation by the glucocorticoid receptor. Upon ligand binding (GCs), the glucocorticoid receptor (GR) is released from heat shock proteins (Hsp) and translocates to the nucleus. Inflammation can be activated by lipopolysaccharide (LPS) binding to Toll-like receptor 4 (TLR4). TLR4 signaling results in the activation of NF-κB, AP-1, and other inflammatory transcription factors that bind and regulate pro-inflammatory target genes. Different mechanisms have been proposed for GR's potent anti-inflammatory actions, i.e., binding to Glucocorticoid Response Elements (GREs), to composite GREs together with other transcription factors, to negative GREs (nGRE), by tethering to DNA-bound transcription factors, by competing with other factors for DNA binding sites or by non-genomic actions.

dependent favorable effects and reducing unwanted GR dimer action (123, 124).

Various selective GR agonists (SEGRAs), such as RU24858, RU24782, and non-steroidal ligands (LDG552, ZK216348, Compound A), were examined for desired anti-inflammatory effects with the hope that there would be minimal metabolic actions (124, 125). Only a few of these compounds, however, showed promise in preclinical trials (126). Their limited success arose from the generalized and oversimplified view that the GR monomer mediates trans-repression (anti-inflammatory) and the GR dimer regulates only unwanted effects (127). The disappointing conclusion of these programs for SEGRAs and non-steroidal ligands and their translation to the clinic called for new perspectives in the context of pathophysiology (10, 16, 104, 127–129). With knowledge gained from the GR^{dim} mouse and others, the development of selective monomerizing GRagonists or modulators (SEMOGRAMs) and selective dimerizing GRagonists or modulators (SEDIGRAMs) has begun to make progress (130). To find SEDIGRAMs, a screening identified Cortivazol and AZD2906 as compounds that increase GR dimerization and enhance the transactivation capacity. Both chemicals, however, still have GR monomer activity, indicating that these are not yet the ideal SEDIGRAMS (129). Efforts are still ongoing to identify perfect GR modulators separating dimer from monomer.

In 1998, the GR A465T mutation was introduced into mice (131, 132). Intriguingly, mice born with this mutation survived in certain backgrounds (131), and simple inflammatory models, such as phorbol ester induced skin irritation, responded to GC treatment in these animals. This indicated that GR monomer and thus transrepression by tethering might be sufficient to reduce inflammation. However, for most other inflammatory models, GCs failed to have an effect in these GR^{dim} mice (**Figure 4A**).

For instance, during LPS, CLP (cecal ligation and puncture), and TNF-α induced shock, GR^{dim} mice were highly susceptible to inflammation and cytokine production, impaired thermoregulation and metabolic alterations (133–135). Furthermore, macrophages from GR^{dim} mice were unable to efficiently repress cytokines in response to LPS (135). Moreover, GR^{dim} mice treated with exogenous GCs showed impairment of anti-inflammatory responses in models of acute lung injury (ALI), arthritis, contact allergy, and allergic airway inflammation (136–139). During ALI, this was partially due to diminished expression of the GR-dimer target gene *Sphk1* (138) (see above). In models of allergic airway inflammation, contact hypersensitivity, antigen-induced arthritis (AIA) or serum transfer-induced arthritis (STIA), GR^{dim} mice failed to repress

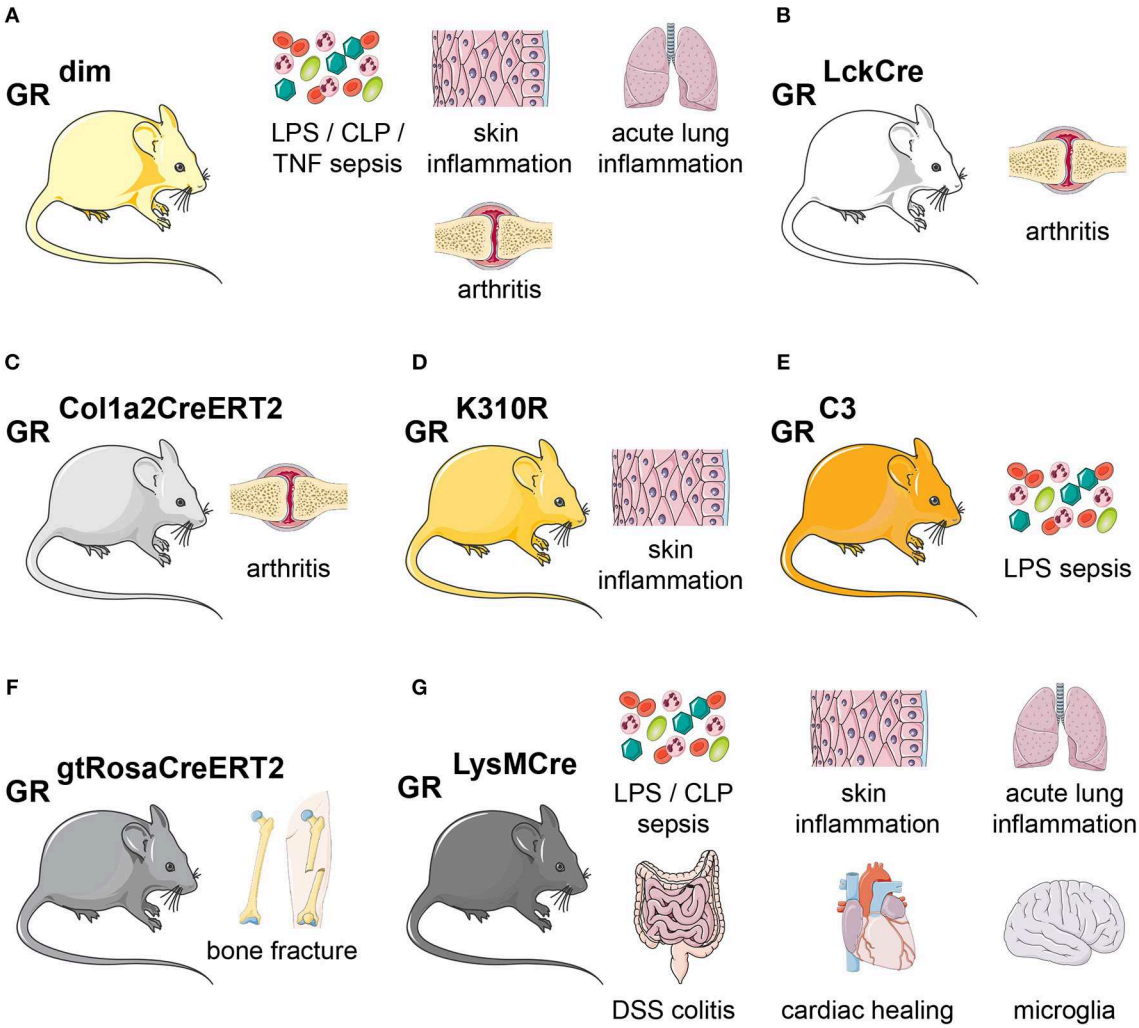

FIGURE 4 | Glucocorticoid receptor mutant mouse models of inflammation. Overview of the mouse lines discussed in this article. **(A)** GR^{dim} mice are more sensitive during LPS-, CLP-, or TNF inflammation. GR^{dim} mice are refractory to GC treatment in models of skin inflammation, acute lung injury and arthritis. **(B)** In GR^{LckCre} mice, GR is lacking in T-cells, making them refractory to GC treatment during arthritis. **(C)** $GR^{Col1a2CreERT2}$ (lacking GR in fibroblasts) show delayed GC-induced suppression in arthritis. **(D)** GR K310R mutant mice lack GR SUMOylation and show impaired control of skin inflammation. **(E)** GR-C3 mice, lacking the most active GR isoform C3, are more sensitive to LPS-induced endotoxic shock. **(F)** During fracture, GR is necessary in all cells, as shown by $GR^{gtRosaCreERT2}$ (tamoxifen-induced ubiquitous Cre-mediated recombination) for fracture healing. **(G)** $GR^{LysMCre}$ mice (GR is deleted in myeloid cells) show no proper healing in LPS- or CLP-sepsis, skin inflammation, acute lung injury, DSS colitis, cardiac healing, and Parkinson disease. The skin, lungs, bones, intestine, heart and brain cartoons were obtained from Servier Medical Art.

inflammation when given GC therapy (136, 137, 139, 140). In the model of AIA, GR dimerization was shown to be essential in T cells (GR^{LckCre} mice) to reduce inflammation [(137); **Figures 4A,B**]. More recently, GR^{dim} mice reconstituted with wild type hematopoietic stem cells failed to induce non-classical ($CD11b^{+}$, $F4/80^{+}$, $Ly6C^{-}$), non-activated ($CD11b^{+}$, $F4/80^{+}$ $MHCII^{-}$), anti-inflammatory (CD163, CD36, AnxA1, Axl, and MertK) macrophages during STIA, while cytokines were repressed normally (140). This strongly indicated that intact dimerization in stromal non-immune cells could contribute to the suppression of inflammation. More precise, the GR in fibroblast-like synoviocytes ($GR^{Col1a2CreERT2}$) was crucial to reduce STIA (140) (**Figure 4C**). GR^{dim} mice were also resistant to GC treatment during TNF-induced inflammation, and exhibited increased gut barrier leakiness, cell death of intestinal epithelial cells and cell death. An increased STAT1-responsive interferon-stimulated gene signature was observed in the gut of GR^{dim} mice (141).

Whereas, the GR^{dim} knock-in mice were intensively studied, less is known about other point mutations. The GRK310R mutation, which abrogates SUMOylation of the GR, failed to respond to GCs during skin inflammation. This was in part due to reduced SMRT/NCoR-co-repressor recruitment to GR/NF-κB/AP-1 repressive complexes [(42, 43); **Figure 4D**].

Finally, Cidlowski and colleagues published a knock-in mouse of the most active GR isoform C3. The lethality of these mice

could be overcome by antenatal GC administration, and adult mice were hypersensitive to LPS administration. This indicated that either the absence of other isoforms like the most abundant GR-A, or indeed the specific overexpression of GR-C3 might confer anti-inflammatory actions [(142); **Figure 4E**]. However, further studies are warranted to dissect these observations in more detail.

Taken together, GR point mutations introduced *in vivo*, namely the GR^{dim} mutation, but also the more recent mutations, have yield valuable insight into the molecular features of GR. With the emergence of CRISPR/Cas9 gene editing technology, more *in vivo* models for specific GR functions will help our understanding of GR in physiological processes in the future.

Glucocorticoid Action on Macrophages

GCs exert their immunosuppressive effects through many cells of the innate immune system, including dendritic cells, mast cells, neutrophils, and eosinophils (143, 144). GCs also play a major role in the regulation of adaptive immunity. For example, GCs decrease the proliferation of early B cell progenitors (145) and induce apoptosis in B cells and T cells (145–149). In this review, we will focus mainly on the effects of GCs in macrophages, since these innate immune cells are essential mediators of defense responses, beyond the mere removal of pathogens, and regulate tissue homeostasis in a myriad of ways (150).

Macrophages reside in many different tissues and are the first line of defense against pathogens (151). Depending on the activating stimulus, they can be categorized as M1-like and M2-like macrophages. The M1-like macrophages (classically activated macrophages) mediate pro-inflammatory actions. They are activated by exposure to LPS, INFγ, TNF-α, or pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively) (151–153). GCs suppress inflammatory responses downstream of TLRs, in part by interfering with the NF-κB- and AP-1-activated transcription of pro-inflammatory cytokines and chemokines (154, 155).

The M2-like macrophages on the other hand, are characterized by their anti-inflammatory potential and are activated by cytokines involved in inflammatory resolution, like IL-4, IL-10, and IL-13 (151, 153, 156). GCs can also polarize macrophages to an M2-like phenotype by regulating the expression of anti-inflammatory proteins (153, 156). A major, yet undervalued aspect of GC control of anti-inflammatory macrophage polarization is the regulation of efferocytosis. GCs enhance the clearance of apoptotic cells, which in itself can augment the development of an anti-inflammatory macrophage phenotype (157, 158).

In sum, GCs can modulate macrophage activity in a number of different and intricate ways, which include suppressing the production of pro-inflammatory proteins and inducing anti-inflammatory mediators.

Glucocorticoid Receptor Target Genes Mediating Immune Modulation

GC stimulated macrophages shift to an M2-like anti-inflammatory and inflammation-resolving phenotype (156). These effects are achieved by the repression of pro-inflammatory genes, the induction of gene products antagonizing pro-inflammatory signaling, and by synergism with pro-inflammatory signaling pathways to activate genes resolving inflammation.

While the mechanisms of gene repression have been extensively discussed [referring to interleukins, chemokines, matrix metalloproteinases, inducible nitric oxide synthase (iNOS), and other mediators], the activated anti-inflammatory genes have only recently received attention (**Table 1**).

Prominent examples are the induction of MAPK phosphatase 1 (Mkp1 or Dusp1), that interferes with the p38MAPK pathway; GC induced leucine zipper (GILZ/Tsc22d3), which binds to the NF-κB subunit p65; the induction of IκBα and β, which oppose NF-κB activity; the activation of kruppel like transcription factors (Klf), which are important for alternative macrophage polarization, and many others (**Table 1**). This upregulation of anti-inflammatory genes further emphasizes that both gene repression and activation are required for the immunomodulatory effects of GCs.

More recently, there were intriguing observations that GCs not only antagonize inflammatory signaling, but also synergize with pro-inflammatory signaling pathways (**Table 1**). GCs synergize with *Haemophilus influenzae* activated inflammatory pathways in macrophages, bronchial epithelial cells (BEAS-2B) and lung epithelial cells (A549) to induce IRAK-M, a negative regulator of TLR signaling (203). Mechanistically, this synergistic activation of *Irak-M/Irak-3* transcription is dependent on binding of both GR and p65 to its promoter, showing a cooperative induction by NF-κB and GR that limits inflammation (203). Similarly, GCs activate TLR2 expression synergistically with *H. influenza* signaling *in vitro* (194).

In ALI models, GR was shown to cooperate with LPS-induced p38MAPK-Msk1 to induce Sphingosine Kinase 1 (SphK1) expression in macrophages (138). SphK1 produces the active mediator Sphingosine-1-phosphate (S1P), that binds to the S1P receptor 1 (S1PR1) on endothelial cells to reduce vascular leakage and infiltration during lung inflammation (138, 204–208). In ALI, mice lacking SphK1 in macrophages were resistant to GC treatment and showed reduced S1P levels. Additional examples of synergistically regulated genes important for modulation of inflammation are acute phase proteins like Serpin A3 (α1-antichymotrypsin) (195) and Metallothioneins (Mt1 and Mt2) (196, 197).

The synergistic regulation of immune-modulating genes by GCs and pro-inflammatory pathways is an important component of their mechanism, but the underlying dynamics and time windows are still poorly understood.

Loss of Function Models of GC Signaling in Macrophages

Strong evidence for the role of GR during homeostasis and inflammation was derived from conditional loss-of-function studies in mice. Applying the Cre/LoxP system, GR tamoxifen-inducible mice ($GR^{gtROSACreERT2}$) could be used to determine the impact of GR deletion in adult animals, circumventing the lethality of global GR knockouts. For example, they have been useful to study GR during inflammation-dependent bone

TABLE 1 | GR target genes relevant for (anti-) inflammatory action.

GC-regulated genes	Targets	GC effect on immune responses	References
Cytokines	Il-1α, Il-1β, Il-6, Il-8, and Il-12	Repression of cytokine production	(114, 159, 160)
Chemokines	Ccl2, Ccl3, Ccl4, Cxcl9, and Cxcl11	Suppression of chemokine release	(77, 160–162)
Matrix metalloproteinases	Mmp12 and Mmp13	Reduction of extracellular matrix remodeling, proteolytic processing	(77, 161)
MAPK phosphatase 1	Induction of Mkp1	Suppression of Jnk and p38Mapk	(133, 163–169)
GC-induced leucine zipper (Tscd22d3)	Induction of Gilz	Inhibition of NF-κB	(170–177)
IκBα and IκBβ	Induction of IκBα and IκBβ	Trapping NF-κB in the cytoplasm, reduced NF-κB activity	(178, 179)
Kruppel-like factor 2	Induction of Klf2	Competition with AP-1 and NF-κB, reduction of inflammatory cytokines	(180–182)
Kruppel-like factor 4	Induction of Klf4	Inhibition of NF-κB	(180, 183)
A3 adenosine receptor	Upregulation of A3AR	Enhanced Erk1/2, anti-apoptotic and pro-survival	(184)
Annexin A1	Induction of Annexin A1	Induction of efferocytosis and monocyte recruitment	(185–189)
Pparγ	Upregulation of Pparγ	Reduced migration	(190)
Tristetraprolin	Induction of TTP	Destabilization of TNF-α	(191–193)
Irak-M	Irak-M induction through synergistic action of GC/GR and NF-κB	Suppression of pro-inflammatory mediators	(193, 194)
Sphingosine Kinase 1	Sphk1 induction through synergism of GC/GR and p38Mapk-Msk1	Reduced vascular leakage and infiltration during acute lung injury	(138)
Serpin A3	Serpin A3 induction through synergism GC/GR and TNFSR1	GR recruitment to Serpin A3 TSS by Dex and TNF-α treatment	(195)
Metallothioneins	Mt1 induction through synergism of Il-6 and GC/GR	Increased susceptibility in inflammatory model in the absence of Mts	(196–202)

repair after fracture (209). Overall, the mice displayed a mild increase in inflammation, with elevated serum IL-6 levels and increased IL-1β levels at the fracture hematoma, accompanied by increased $CD3^+$ and $CD8^+$ cells. Consequently, the lack of GR and potentially the elevated inflammation, caused a delayed endochondral regeneration and maturation of callus and a decreased healing response [(209); **Figure 4F**].

Since the publications of conditional GR alleles in 1999 (210), 2003 (211), and 2012 (212), many cell types have been targeted with specific Cre lines to characterize specific functions of the GR in numerous cell types in the brain, muscle, heart, T lymphocytes, and others.

Insights into the function of GR in macrophages *in vivo* mainly stems from Lysozyme 2 (LysM)–Cre mice crossed to GR floxed alleles, which causes deletion in the myeloid cell lineage (monocytes, mature macrophages, and granulocytes) [(135, 136, 163, 213); **Figure 4G**].

In both the LPS-induced endotoxic shock model and during CLP, myeloid GR is crucial for the repression of inflammatory cytokines and for survival (135, 163). Not only in LPS-induced inflammation, but also in dextran sodium sulfate (DSS)-induced colitis, the action of endogenous GCs in macrophages was essential to reduce intestinal inflammation (214). Mice deficient for macrophage GR had a higher disease score, with increased infiltration of neutrophils, T cells and macrophages in the colon, which was associated with enhanced serum IL-6 (214). Moreover, macrophages were shown to play an essential role for cardiac healing, tissue repair and hence survival in myocardial infarction (215). Deletion of GR in macrophages delayed cardiac healing 7 days after myocardial infarct, with impaired cardiac function, collagen scar formation and neovascularization, and larger myofibroblasts. Consequently, targeting macrophage GR during myocardial infarction might be a potential pharmacological intervention for tissue repair (215). In contrast, in a mouse model of atherosclerosis, macrophage GR deletion was beneficial and showed reduced levels of vascular calcification, due to reduced RANKL, BMP2, and Mx2 expression (216).

During skin inflammation in a model of contact hypersensitivity, the anti-inflammatory effects of GCs required GR in myeloid cells (136). Additionally, in a model of ALI, $GR^{LysMCre}$ mice were resistant to GC therapy, did not reduce cellular infiltration in the lung and did not induce the endothelial barrier stabilizing sphingosine-1-phosphate [(138); **Figure 4G**].

$GR^{LysMCre}$ mice were shown to efficiently express Cre in microglia, knocking out GR in brain resident macrophages. Studies on the function of microglial GR during acute inflammation demonstrated more cellular lesions, damage, demyelination in the corpus callosum, and increased neuronal degeneration. It also significantly increased pro-inflammatory cytokines after LPS injections (217). The activation of microglia induces secretion of pro-inflammatory proteins that contribute to dopaminergic neuronal death, a major a hallmark of Parkinson's disease. The absence of GR in microglia revealed that increased death of dopaminergic neurons in Parkinson's may contribute to neurodegenerative processes (218). Additionally, recent studies suggest that the absence of microglia GR facilitates TLR9 activation

of inflammatory processes and affects Parkinson's disease progression (219).

In summary, the genetic deletion of GR in myeloid cells in various inflammatory models demonstrated the pivotal role of this cell type for GC actions. However, one of the limitations of the LysMCre mouse is the recombination in other myeloid cells such as neutrophils, whose contribution cannot be excluded. Nonetheless, this wealth of data supports the concept that selective targeting of glucocorticoids to macrophages, while sparing other cell types, could be a promising approach to optimize therapy.

CONCLUSION

During the past decade, much has been learned about the immunomodulatory mechanisms employed by GR: analyzing various mouse models, creating distinct mutations, mapping GR target genes genome-wide, functionally characterizing individual proteins mediating GC responses, studying different inflammatory settings, identifying essential co-regulators, and applying novel molecular biology methods, have broadened our understanding of these steroids' intricate actions. Taken together, it becomes obvious how basic research is fundamental in enabling drug development. However, we now realize that GR's molecular mechanisms are very complex, cell-type, locus- and signal-specific, and much more sophisticated than we previously anticipated. Intra- and extra-cellular signals can control GR function on many levels, and these multi-layered machineries demand new interpretation of previous over-simplified models. In the future, the rapid advancement of high-throughput technologies such as machine learning, genomics, proteomics, genome engineering, etc. will be key to the development of safer immunomodulators or novel GR ligands.

AUTHOR CONTRIBUTIONS

LE-T, GC, AM, and SV wrote the manuscript with supervision of JT and NU. SV, JT, and NU secured funding.

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Implicating the Role of GILZ in Glucocorticoid Modulation of T-Cell Activation

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Glucocorticoid-induced leucine zipper (GILZ) is a protein with multiple biological roles that is upregulated by glucocorticoids (GCs) in both immune and non-immune cells. Importantly, GCs are immunosuppressive primarily due to their regulation of cell signaling pathways that are crucial for immune system activity. GILZ, which is transcriptionally induced by the glucocorticoid receptor (GR), mediates part of these immunosuppressive, and anti-inflammatory effects, thereby controlling immune cell proliferation, survival, and differentiation. The primary immune cells targeted by the immunosuppressive activity of GCs are T cells. Importantly, the effects of GCs on T cells are partially mediated by GILZ. In fact, GILZ regulates T-cell activation, and differentiation by binding and inhibiting factors essential for T-cell function. For example, GILZ associates with nuclear factor-κB (NF-κB), c-Fos, and c-Jun and inhibits NF-κB-, and AP-1-dependent transcription. GILZ also binds Raf and Ras, inhibits activation of Ras/Raf downstream targets, including mitogen-activated protein kinase 1 (MAPK1). In addition GILZ inhibits forkhead box O3 (FoxO3) without physical interaction. GILZ also promotes the activity of regulatory T cells (Tregs) by activating transforming growth factor-β (TGF-β) signaling. Ultimately, these actions inhibit T-cell activation and modulate the differentiation of T helper (Th)-1, Th-2, Th-17 cells, thereby mediating the immunosuppressive effects of GCs on T cells. In this mini-review, we discuss how GILZ mediates GC activity on T cells, focusing mainly on the therapeutic potential of this protein as a more targeted anti-inflammatory/immunosuppressive GC therapy.

Keywords: glucocorticoids, glucocorticoid-induced leucine zipper (GILZ), T-cell activation, T-cell, immune response, glucocorticoid receptor

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INTRODUCTION

Glucocorticoids (GCs) are the mainstay of current immunosuppressive and anti-inflammatory therapies (1). Decades of study have revealed that their primary mechanism of action involves GC binding to GC receptors (GRs) to modulate gene transcription (2–5). However, the biological effects of GCs are diverse and are likely controlled by several mechanisms. Given this functional diversity, identifying molecules that are transcriptionally induced by GCs, and can mediate specific GC effects presents a significant challenge.

One potential molecule is glucocorticoid-induced leucine zipper (GILZ), a ubiquitously expressed protein that is primarily under GR transcriptional control. GILZ was originally identified in 1997 when searching for genes that mediate GC-induced apoptosis (6). However, since that time, the roles of GILZ have expanded to include most of the anti-inflammatory, and immunosuppressive effects of GCs (7). Indeed, GILZ is now known to regulate cell apoptosis, proliferation, and differentiation by modulating transcription factors, and signaling pathways associated with host immunity, and inflammation (8–12).

GILZ has a high degree of homology with other members of the TSC22D family. The TSC22D family includes leucine zipper proteins that are differentially expressed and involved in the regulation of multiple biological processes (13). TSC22D isoform heterodimers regulate cell cycle entry and exit (14).

One mechanism by which GCs induce immunosuppression is through regulation of the T-cell response (15, 16). In this review, we discuss the literature concerning how GILZ mediates the effects of GCs on T cells. Regardless of the specific role of GILZ, we highlight information about GC-dependent, and GC-independent GILZ functions to expand the current understanding of the GC mechanism of action. Ultimately, such understanding is critical to improving GC clinical use.

GCS AND THE T-CELL RESPONSE

T-cell activation is an essential part of the adaptive, cell-mediated immune response. GCs modulate T-cell differentiation and activation regulating: (1) antigen-presenting cells (APCs); (2) T helper (Th) cell differentiation; and (3) T-cell receptor (TCR) signaling (**Figure 1**) (15). The GR acts through genomic and non-genomic mechanisms, regulating adhesion molecules, co-accessory molecules, and cytokines implicated in T-cell activation (17–19).

Acting directly on T cells, GCs function through different mechanisms, most of which involve GR/transcription factor interaction. GCs affect the activity of transcription factors downstream of TCR activation, including nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and nuclear factor of activated T cells (NF-AT) (15). GCs can also act through non-genomic mechanisms to limit kinase activity downstream of TCR activation, ultimately inhibiting the above-mentioned transcription factors and T-cell activation (20) (**Figure 1**).

Moreover, GCs can modulate T-cell activation indirectly through other cells such as dendritic cells (DCs), which are professional APCs. DCs have dual functionality, as they both orchestrate adaptive immune responses and also actively maintain peripheral specific tolerance against innocuous antigens (21). The balance between the activating and tolerogenic DC phenotypes is crucial to generating an efficient immune response while also preventing autoimmunity. GCs inhibit DC functions, reducing expression of MHC class II, and costimulatory molecules, decreasing proinflammatory cytokines and increasing anti-inflammatory cytokines such as IL-10 (22). Importantly, GCs can also increase the ability of DCs to capture antigens, suggesting that GCs drive DCs toward a tolerogenic phenotype (23). Tolerogenic DCs induce T-cell suppression and anergy and promote the generation of regulatory T cells (Tregs) (24). Therefore, GC modulation of DCs indirectly inhibits T-cell activation (**Figure 1**).

GCs can also modulate T cells by targeting tissue macrophages, mast cells, and stromal cells. Myeloid cells modulate T-cell function, acting as APCs and/or secreting inflammatory cytokines in response to stimulation of pattern recognition receptors (PRRs) (15). GCs can attenuate signals downstream of PRR activation, including the transcription factors AP-1, NF-κB, and the mitogen-activated protein kinase 1 (MAPK1) pathway (15, 25, 26). Those signaling changes alter the cytokine network, with important consequences for both inflammation, and T-cell responses. In fact, this mechanism may partially account for both GC inhibition of Th-1 and Th-17 differentiation and GC promotion of Th-2 differentiation and Treg production (27, 28) (**Figure 1**).

What is the role of GILZ in this context?

GILZ AND THE T-CELL RESPONSE

Similar to the GCs, GILZ inhibits innate, and adaptive immune responses, affecting T-cell function (activation, differentiation, and apoptosis) either directly or through APCs (7, 9, 10) (**Figure 2**).

GC-induced GILZ expression in T cells is involved in multiple GC effects (9); however, its endogenous expression in the naïve T cell suggests a GC-independent function (29).

GCs can modulate T-cell apoptosis, and GILZ can either induce or protect against apoptosis (6). The first studies on apoptosis were performed using the T-cell hybridoma 3DO, which overexpresses GILZ (6, 30). In this cell line, GILZ inhibits both NF-κB (30), and AP-1 (31), behaves as a GC by inhibiting CD3-mediated apoptosis and TCR-driven IL-2 production through Fas/FasL modulation (30). Furthermore, T cells from GILZ-knockout mice (GILZ-KO) show increased antigen-induced T-cell activation (32). These data indicate mutual antagonism between GILZ expression and T-cell activation, suggesting that T cells must inhibit GILZ expression to become activated (29, 33). Moreover, in T cells, GILZ expression mimics the antiproliferative effects of GCs by interacting with Ras and Raf and inhibiting Ras downstream signals, such as MAPK (33, 34) (**Figure 2**). Notably, IL-2 deprivation in T cells upregulates GILZ (35), whereas IL-2 treatment (35), and T-cell activation (29, 30, 33) decrease GILZ expression. Moreover, IL-2 withdrawal induces cell death and upregulates GILZ by promoting forkhead box O3 (FoxO3) transcriptional activity in GILZ promoter region. In turn, GILZ prevents FoxO3 transcriptional activity, promoting its nuclear exclusion through a mechanism involving the nuclear export receptor Crm1 (36), and inhibiting its own expression and that of the proapoptotic gene Bim. In this case, GILZ protects T cells from IL-2 withdrawal-induced apoptosis by regulating its own expression (35, 37). The role of GILZ in T-cell apoptosis has been further clarified using GILZ

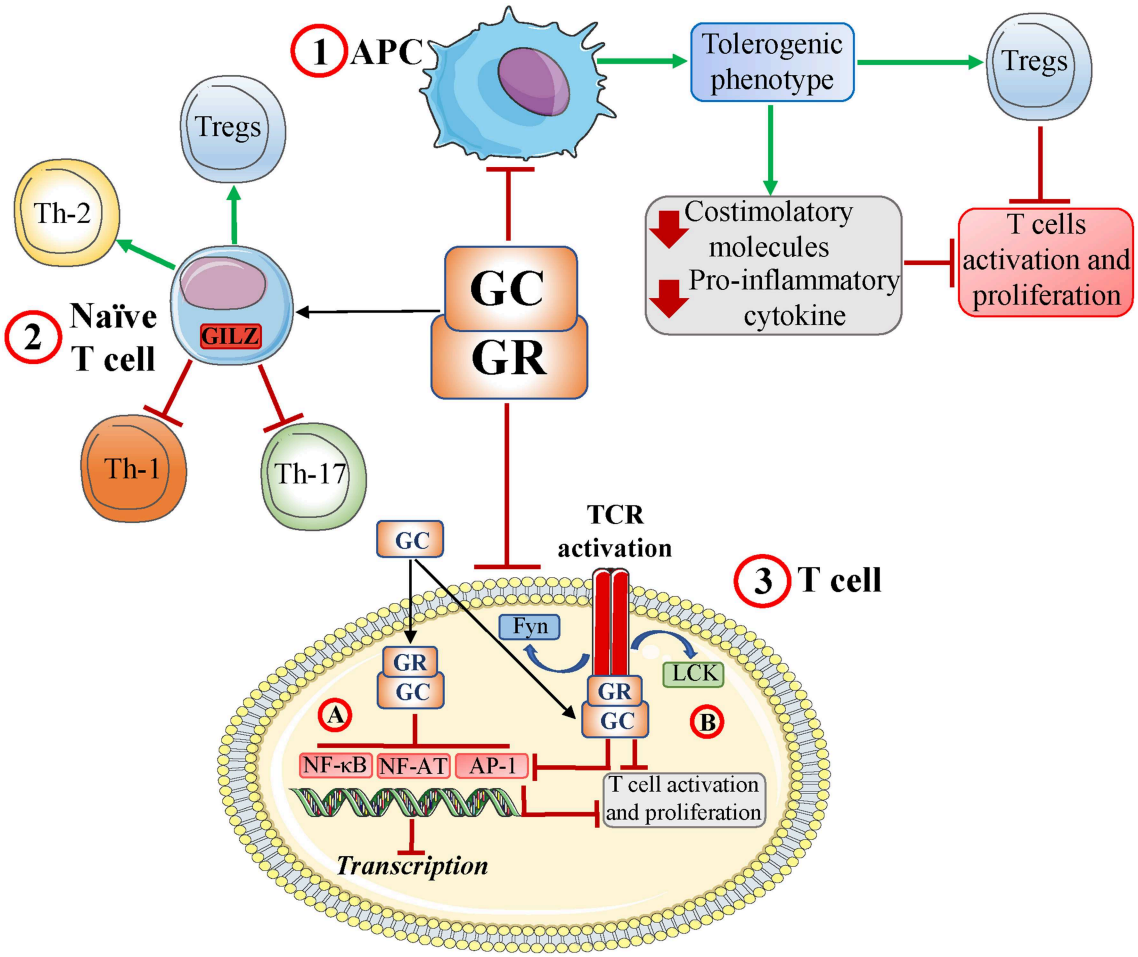

FIGURE 1 | Glucocorticoids and the T-cell response. Glucocorticoid (GCs) and glucocorticoid receptor (GR) interactions induce: (1) a tolerogenic antigen-presenting cell (APC) phenotype with decreased production of both proinflammatory chemokines and costimulatory molecules and development of regulatory T cells (Tregs). This subsequently inhibits T-cell activation; (2) a modulation of naïve T-cell differentiation, including inhibition of Th-1 and Th-17 cell development and induction of Th-2 cells and Tregs; and (3) inhibition of T-cell receptor (TCR) signaling by inhibiting (genomic effects) key transcription factors such as NF-AT, AP-1, and NF-κB (3A) and disruption of TCR-associated multiprotein complexes containing GR, LCK, and FYN (rapid, non-genomic effects) with inhibition of NF-AT, AP-1, and NF-κB (3B). Ultimately, these interactions impair TCR signaling and T-cell activation/proliferation. Red T-headed leaders indicate inhibition; green arrow-headed leaders indicate activation.

transgenic mouse models (GILZ-TG). Thymocytes from GILZ-TG mice undergo apoptosis through caspase-8 activation and Bcl-xL downregulation (38), regulating the thymic repertoire similar to GCs. However, these cells are rescued by TCR-induced apoptosis, suggesting a GC-like mechanism of mutual exclusion (39). In contrast, GILZ does not induce apoptosis in peripheral mature mouse T lymphocytes (40). The ability of GCs to induce the apoptosis of lymphoid cells supports their inclusion in protocols for the treatment of lymphohematopoietic malignancies. GILZ upregulation may underlie these effects of GCs. For example, in multiple myeloma, for which GCs are used, decreasing GILZ levels by siRNA knockdown inhibited GC-induced apoptosis (41).

Constitutive expression of GILZ in naïve T cells (29) plays a major role in their differentiation (**Figure 2**). GILZ promotes Treg differentiation by activating transforming growth factor-β (TGF-β) signaling (42) and is partly responsible for GC-mediated effects on Tregs (15). In fact, dexamethasone (DEX) treatment augments the frequency of splenic Tregs in WT, but not GILZ-KO, mice (42).

Moreover, GILZ overexpression in CD4+ lymphocytes from GILZ-TG mice promotes Th-2 and inhibits Th-1 differentiation (43); thus, GILZ behaves like GCs (27, 44). As a consequence, GILZ-TG mice are less susceptible to Th-1-mediated diseases, such as experimental dinitrobenzene sulfonic acid- (DNBS-) colitis (45), and spinal cord injury (46). In these models, GILZ-TG mice exhibit an attenuated immune response, which may be explained by GILZ-mediated inhibition of NF-κB, which is crucial for Th-1 cytokine production, in T cells of the intestinal lamina propria, and in spinal cord lesions, respectively (45, 46). Accordingly, injection of mice with either the transactivator of transcription (TAT)-glutathione-*S*-transferase (GST)-GILZ

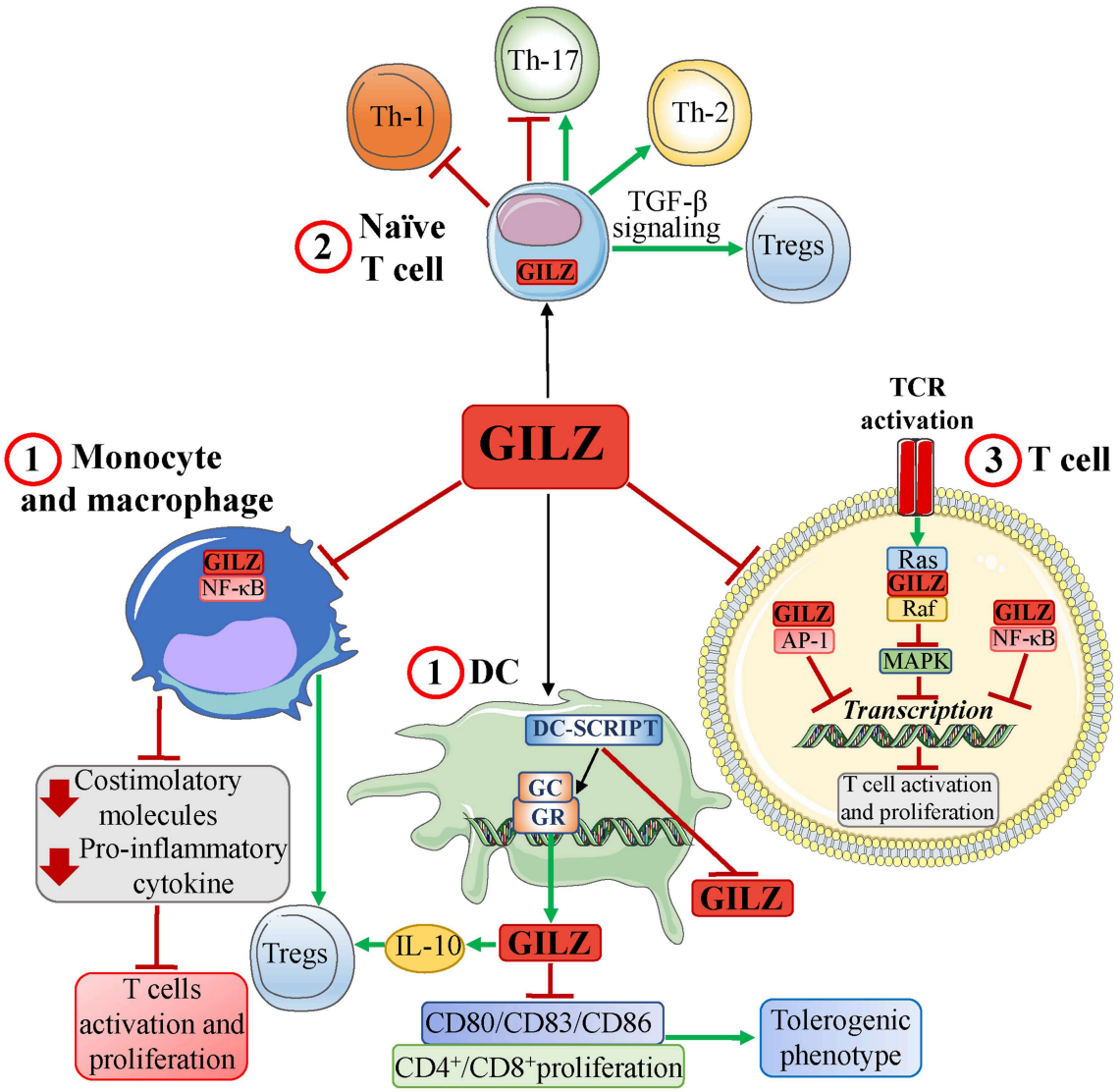

FIGURE 2 | GILZ and the T-cell response. GILZ, expressed basally and/or in response to GCs, induces: (1) a tolerogenic dendritic cell (DC) phenotype and inhibition of human monocyte and mouse macrophage activation via inhibition of NF-κB, thereby limiting production of both proinflammatory chemokines, and costimulatory molecules. In DCs, GILZ expression is regulated by a GR corepressor, DC-specific transcript (DC-SCRIPT), whose recruitment inhibits GILZ expression. GILZ-induced inhibition of APC functions promotes development of Tregs and ultimately inhibits T-cell activation; (2) a modulation of naïve T-cell differentiation (expressing endogenous GILZ) that includes induction of Th-2 cells and Tregs (favoring TGF-β signaling), inhibition of Th-1 cells, and inhibition or development of Th-17 cells; (3) an inhibition of TCR signaling by inhibiting pathways, such as MAPK, and transcription factors, such as AP-1, and NF-κB, through protein-protein interactions. Red T-headed leaders indicate inhibition; green arrow-headed leaders indicate activation.

(TAT-GST–GILZ) fusion protein or high doses of DEX, which upregulates GILZ in mucosal T lymphocytes, rescues mice from Th-1-mediated experimental colitis, again by inhibiting NF-κB (45). GILZ, in this model, is crucial for effects on Tregs cells. In fact, in GILZ-KO mice, the severity of DNBS-colitis is increased compared with WT due to impaired generation of Tregs cells. Transfer of WT Treg cells reverses the augmented vulnerability. DEX ameliorates the symptoms of DNBS-colitis in WT, but not GILZ-KO, through Treg augmentation. Therefore, GC anti-inflammatory activities in this model may be mediated by GILZ expression in T lymphocytes (45), and GILZ-induced Treg generation (42). However, in other murine models of inflammation, GILZ does not appear to be involved in the anti-inflammatory activity of GCs. For example, endogenous GILZ is detectable in the synovia of mice with collagen-induced arthritis (CIA), and in patients with active rheumatoid arthritis, and is upregulated by GC therapeutic doses (47). Moreover, GILZ reduction by RNAi worsens the symptoms of CIA, suggesting a role for GILZ as an endogenous inhibitor (47). However, its deletion does not impair the effects of exogenous GCs in CIA

and does not affect the severity of antigen-induced or K/BxN serum–transfer arthritis (32). In fact, no difference in arthritis severity was found between GILZ-KO and WT mice, although antigen-induced T-cell proliferation was higher in GILZ-KO mice. However, injection of adeno-associated virus expressing GILZ (GILZ-rAAV) in CIA mice results in joint GILZ expression and attenuation of joint inflammation without affecting T-cell proliferation (32). These data suggest different roles for GILZ in inflammation, again as a brake for T-cell proliferation, an endogenous natural anti-inflammatory protein, and as a drug.

A pharmacological use of the GILZ protein was also shown in experimental autoimmune encephalomyelitis (EAE), an inflammatory model for human multiple sclerosis. GILZ peptide (GILZ-P) binds to and inhibits NF-κB, suppresses T-cell activation, and shows therapeutic efficacy when administered in EAE mice. Specifically, GILZ-P inhibits NF-κB, Th-1 cytokines, and T-Bet transcription but increases expression of GATA-3 and Th-2 cytokines, mimicking GILZ (48), and GC activity (17, 27).

GILZ negatively modulates Th-17 development by binding to IL-21 and Irf4 sites, as demonstrated via ChIP-seq analysis of Th-17 cells. These sites overlap the binding sites of major transcription factors involved in Th-17 polarization. Therefore, GILZ may act as a transcriptional repressor, inducing displacement of Th-17 transcription factors from their sites with inhibitory effects on Th-17 development (49). Consistently, GILZ downregulation in naïve CD4+ T cells is required for development of Th-17 (29). GILZ expression in T cells is protective against several pathologies, including psoriasis, a disease commonly treated with GCs, and myocardial infarction, in which Th-17 lymphokines are pathogenetic (29, 50). However, conflicting data were obtained *in vivo* with imiquimod (IMQ), a murine model for IL23-, and IL17-dependent psoriasis. Some researchers demonstrate that IMQ-induced psoriasis is more serious in GILZ-deficient mice, with upregulation of Th-17 cytokines and Th-17 proliferation (29). In contrast, other researchers show that IMQ-induced psoriasis is more severe in GILZ-TG mice, with increased Th-17 cytokines (51) (**Figure 2**). Thus, based on this model, GILZ can be proinflammatory, similar to the effects of prolonged GC treatment (51), or anti-inflammatory (29).

As mentioned, GILZ can modulate T-cell activity indirectly through its actions on APCs. Its effects on myeloid cells have a broad spectrum of action on all cells of the immune system (9). DC subsets constitutively express GILZ at different levels depending on DC functional status (52). Endogenous GCs appear to regulate constitutive DC GILZ expression, whereas exogenous GCs upregulate DC GILZ *in vivo* and *in vitro*. Thus, by mediating the effects of GCs, GILZ can regulate the balance between activating and tolerogenic DCs (53, 54). GILZ expression is transcriptionally regulated by the GR, which can either induce or inhibit GILZ by recruiting its corepressor, DC-specific transcript (DC-SCRIPT) (**Figure 2**). Importantly, neutralizing DC-SCRIPT augments GR-induced GILZ expression (55). This suggests that the tolerogenic-promoting effects of GILZ in DCs are so crucial that a biological brake on its expression is required. Indeed, GILZ overexpression induces a DC tolerogenic phenotype comparable to that induced by GCs (56), downregulating the costimulatory molecules CD86, CD83, and CD80 (57, 58), and reducing CD4+ T-cell proliferation (53) (**Figure 2**). Knocking down GILZ in activated monocyte-derived DCs (Mo-DCs) promotes more efficient CD8+ T-cell secondary responses (59). *In vitro* GC treatment of human Mo-DCs induces GILZ expression, driving a DC tolerogenic phenotype that prevents efficient antigen presentation (57), and induces IL-10-promoting Tregs. Together, these changes inhibit the T-cell response (58). This effect is reproduced by GILZ overexpression (60) and abolished by GILZ silencing (57, 59). Finally, GILZ expression in tumor-infiltrating DCs drives a tolerogenic DC phenotype, and T-cell tolerance against the tumor (54). This suggests that tumor cells may "learn" to secrete GCs to induce GILZ as an escape mechanism against the immune system. These results may explain how GCs, both endogenous and exogenously administered, can either block or worsen tumor progression (especially epithelial tumors) through GILZ expression (61).

Similar to DCs, human monocytes and mouse macrophages constitutively express GILZ. GCs further upregulate GILZ expression, which, via inhibition of NF-κB, mediates GC activity in these cells (62–64) (**Figure 2**). In fact, transfecting GILZ into THP-1 macrophages mimics the effects of GCs and inhibits the production of chemokines, and costimulatory molecules (62). Correspondingly, GILZ is downregulated by Toll-Like agonists, leading to macrophage activation (65). Moreover, GILZ expression is decreased during neuroinflammation, inversely correlating with the development of innate immune responses (66), and in white blood cells from patients with sepsis (67). These findings confirm the immunosuppressive role of GILZ in myeloid cells and the biological necessity of GILZ downregulation for efficient natural or adaptive immune responses.

Furthermore, expression of GILZ, as with GC (15), limits Th-17 differentiation, and induced Treg cell activity by modulating cytokine production by DCs and mesenchymal cells (68, 69). In a mouse model of rheumatoid arthritis, GILZ expression in mesenchymal stem cells (MSCs) is required for therapeutic effectiveness of MSCs in arthritis (68) and inhibition of transferred- Th-1, and Th-17 cells in immunized mice (70). In a model of acute kidney injury, TAT-GST-GILZ fusion protein conferred renoprotection by regulating cross-talk between T cells and neutrophils, reducing proinflammatory type 1 neutrophils and Th-17 cells, and increasing anti-inflammatory type 2 neutrophils and Tregs (71).

The role of GILZ in T-cell activation is even more complex if we consider the effects on its expression following accessory molecule triggering. Indeed, blocking the co-accessory molecule, CD80, enhances GILZ expression in activated CD4+ T cells (72). However, this field of investigation remains unexplored.

PERSPECTIVE AND EXPECTATIONS

Based on our critical review of the literature, we suggest that GILZ has at least three different functions in T cells: (1) endogenous; (2) mediator of GC activity; and (3) as a drug.

As discussed above, basal endogenous GILZ expression in immune cells has a predominant role in T-cell activation, the

development of CD4+ naïve T cells, and the physiological control of inflammation. The latter is demonstrated by the many murine models of inflammation, in which the absence of GILZ aggravates inflammatory pathologies (12, 29, 32, 42, 47). However, GILZ expression in T cells underlies many of the effects of GCs established in experimental *in vivo* and *in vitro* models. These models demonstrate that a lack of GILZ inhibits the activity of GCs, and overexpression may mimic GC effects (8–10, 43).

The use of GILZ as a drug is a great challenge given the potential side effects on metabolism. However, many experimental models support and encourage this possibility. Experiments with fusion proteins TAT-GST-GILZ, and HHph-GILZ, viral constructs GILZ-rAAV expressing GILZ, and GILZ-peptide GILZ-P provide examples of achieving pharmacokinetic, pharmacodynamic, and therapeutic efficacy using GILZ *in vivo* as a drug (29, 32, 45, 47, 73). Many of the experimental models discussed above involve pathologies due to an imbalance of the development of naïve CD4+ cells, demonstrating how the therapeutic activity of GILZ is related to actions on T cells (11, 12, 45, 47).

GCs inhibit T-cell activation through genomic and non-genomic mechanisms. GR-mediated genomic regulation induces immunosuppressive molecules, including GILZ (8, 15, 74–76). GCs also modulate T-cell activity through non-genomic mechanisms that occur immediately after drug exposure (77, 78). In T cells, the GR physically associates with the TCR in a multiprotein complex with LCK, and FYN. Short-term treatment with DEX induces the non-genomic destruction of this complex, thereby limiting TCR activation (20) (**Figure 1**). Is it possible to hypothesize that GCs regulate GILZ function and/or expression through both genomic and non-genomic mechanisms? The regulation of GILZ by GC non-genomic effects would lay the groundwork for several future lines of study. In particular, because GC-induced GILZ transcription in T cells interacts with and inhibits TCR-triggered signaling pathways and transcription factors, it is likely that there is a GC-induced non-genomic effect on constitutive GILZ expression. This would reveal another mechanism by which GCs regulate the T-cell response. Such a mechanism might provide further explanation for the basal level of GILZ in immune cells (63, 79). Therefore, it would be interesting to investigate whether GC/GR interactions induce rapid changes in the cytoplasmic basal pool of GILZ, as such GILZ expression may have alternative functions compared to those of peak GILZ activation induced by GR-mediated transcription. Ultimately, building on our understanding of the molecular mechanisms involving GCs and GILZ may improve the use of GCs as clinical therapeutics and limit treatment-related side effects.

AUTHOR CONTRIBUTIONS

EA wrote the review and suggested the general topic. SA prepared the figures. LC and DD discussed and reviewed the manuscript. CR suggested the general topic and discussed and reviewed the manuscript.

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Mechanisms Underlying the Functional Cooperation Between PPARα and GRα to Attenuate Inflammatory Responses

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Glucocorticoids (GCs) act via the glucocorticoid receptor (NR3C1, GRα) to combat overshooting responses to infectious stimuli, including lipopolysaccharide (LPS). As such, GCs inhibit the activity of downstream effector cytokines, such as tumor necrosis factor (TNF). PPARα (NR1C1) is a nuclear receptor described to function on the crossroad between lipid metabolism and control of inflammation. In the current work, we have investigated the molecular mechanism by which GCs and PPARα agonists cooperate to jointly inhibit NF-κB-driven expression in A549 cells. We discovered a nuclear mechanism that predominantly targets Mitogen- and Stress-activated protein Kinase-1 activation upon co-triggering GRα and PPARα. *In vitro* GST-pull down data further support that the anti-inflammatory mechanism may additionally involve a non-competitive physical interaction between the p65 subunit of NF-κB, GRα, and PPARα. Finally, to study metabolic effector target cells common to both receptors, we overlaid the effect of GRα and PPARα crosstalk in mouse primary hepatocytes under LPS-induced inflammatory conditions on a genome-wide level. RNA-seq results revealed lipid metabolism genes that were upregulated and inflammatory genes that were additively downregulated. Validation at the cytokine protein level finally supported a consistent additive anti-inflammatory response in hepatocytes.

Keywords: PPARα, GRα, crosstalk, molecular mechanism, inflammation, MSK1

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INTRODUCTION

Glucocorticoid hormones (GCs) are the mainstay of treatment for most inflammatory and autoimmune diseases (1, 2). GCs also regulate glucose and fat homeostasis, however a long-term therapeutic treatment with exogenous GCs causes hyperglycaemia, insulin resistance and disturbed fat profiles as clinically worrying drawbacks (3). A reduction in adverse effects related to glucose and fat regulation would be highly desirable in clinical GC applications.

Therapeutic activities of GCs are mediated by the glucocorticoid receptor (NR3C1) (4), belonging to the superfamily of ligand-inducible transcription factors (4). Unliganded GR

predominantly resides in the cytosol in an inactive state associated with heat shock proteins (HSPs) and immunophilins (4, 5). Upon GC binding, GR translocates to the nucleus and binds to GR binding sequences (GBSs), widely dispersed throughout the genome (6). These may include enhancers, hot spots, as well as GC-response elements (GREs) within the promoter regions of target genes, hereby regulating their transcriptional activity (7–10). Additionally, transcriptional regulation mediated by the GR also encompasses inhibitory effects on the activity of pro-inflammatory transcription factors driving the onset of inflammation, such as nuclear factor-κB (NF-κB), resulting in pro-inflammatory gene suppression (11–13). Throughout the years, many different mechanisms have been proposed explaining how GR inhibits pro-inflammatory gene expression, including direct mechanisms as well as feedback loop mechanisms by GC-induced anti-inflammatory proteins (14, 15). Suggestive of conserved mechanisms among nuclear receptors, the fibrate ligand-activated transcription factor peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear hormone receptor superfamily, may also exert anti-inflammatory actions by down-regulating the activity of NF-κB and other pro-inflammatory transcription factors via multiple mechanisms, with some reminiscent of the ones GR is deploying (16, 17).

In addition, both GR and PPARα exhibit overlapping and complementary roles in liver with regard to carbohydrate and fat metabolism (13, 18) and co-ordinately control key genes involved in the maintenance of blood glucose levels, cooperatively support fatty acid β-oxidation during fasting, and stimulate immune suppression (19–21).

We previously reported that GRα and PPARα, when co-activated, physically interact *in vitro* and *in cellulo*, in the nucleus (22), paving the way for an extra level of gene regulatory mechanisms apart from triggering their own cognate gene programs. PPARα activation further enhanced GR-triggered suppression of TNF-induced NF-κB-driven gene expression and pro-inflammatory cytokine production in fibroblast (L929sA) cells (22). PPARα activation also suppressed GR-induced upregulation of *G6PC* (22), one of the metabolic genes responsible for adverse effects related to glucose metabolism upon chronic GC therapy. Mice subjected to a 7-week high fat diet and that received a daily administration of the synthetic GC Dexamethasone (DEX) for another 7 days instead of solvent, demonstrated a worsened glucose intolerance which coincided with enhanced hyperinsulinemia. Oppositely, high fat diet fat mice receiving the PPARα agonist fenofibrate (FENO) for 7 days supported clear glucose tolerance. Remarkably, the latter phenotype was also observed when combining DEX with FENO, indicating crosstalk and a potential advantage at the glucose metabolism level when combining two nuclear receptor ligands for which anti-inflammatory actions had been demonstrated (22). Collectively, these results justify further mechanistic exploration of a combination of GCs with PPARα agonists in a context of inflammation, starting with simple cell models to understand first the cell-autonomous crosstalk modes in more detail.

Mitogen- and Stress-activated protein Kinase-1 (MSK1) is a kinase that acts, among others, in the TNF-signaling pathway. It promotes inflammatory gene transcription by phosphorylating NF-κB, which facilitates association of p65 with cofactors, and by phosphorylating histone H3 (23–25). We previously reported that GCs counteract MSK1 recruitment at inflammatory gene promoters and partially drive MSK1 to the cytoplasm, as a contributory mechanism to inhibit NF-κB transactivation (23).

Crosstalk between GCs and MAPK signaling pathways was considered before as a valid mechanism to effectively inhibit NF-κB-driven inflammatory gene promoters (26). PPARα agonists have also been shown to modulate MAPK activities, indirectly suppressing inflammatory responses (27, 28). As we previously observed no significant inhibitory effect of GCs on p38 and ERK MAPK activation in L929sA mouse fibroblasts (29) and A549 human epithelial cells (23), we explored whether in A549 human epithelial cells combined treatment of GCs and PPARα agonists might target the more downstream kinase MSK1 and thus might contribute to the additive transrepression of NF-κB-driven inflammatory genes observed when triggering both receptors.

In the present research we overlaid a mechanistic study of the effect of GR and PPARα crosstalk under TNF-induced inflammatory conditions in A549 human epithelial cells as a first cellular model system for inflammatory responses, with a genome-wide impact of combined ligand treatment in metabolic effector cells using LPS-induced primary hepatocytes as a second, complementing, model system. RNA-seq results in primary hepatocytes revealed inflammatory genes that were synergistically downregulated and lipid metabolism genes that were additively upregulated following the activation of both nuclear receptors. In addition, our data reveal that, upon co-triggering of GRα and PPARα, a nuclear anti-inflammatory mechanism may follow from a hampering at the level of TNF-activated kinase MSK1 activation in a lung epithelial cell line. Taken together, our findings unveil novel molecular aspects of the PPARα-GR-mediated NF-κB-targeting anti-inflammatory mechanism.

MATERIALS AND METHODS

Cytokines, Plasmids, and Reagents

Dexamethasone (D4902) (DEX) and GW7647 (G6793) (GW) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Anti-GR, anti-PPARα, anti-RNA pol II and anti-p65 antibodies were obtained from Santa Cruz. Phospho-specific rabbit antibodies to p38 (Thr-180/Tyr-182), p42/44 ERK (Thr202/Tyr204), MSK1 (Thr581) and IKKα/β (Ser180/S181) were used to detect the respective phosphorylated forms and purchased from Cell Signaling. Anti-p38, anti-ERK, anti-MSK1, and anti-IκBα antibodies were purchased from Cell Signaling. Anti-tubulin and anti-actin were used as loading control and obtained from Santa Cruz. Anti-phospho-65 was obtained from Santa Cruz. Recombinant murine TNFα was produced and purified as described (30). TNFα was used at a final concentration of

2,000 IU/ml. p(IL6κB)$_3$50hu.IL6P-luc+ (hereafter renamed NF-κB-Luc), PPARα, GR, and 5HT7 control plasmids were described previously (21, 31–33). LPS was purchased from Invivogen.

Cell Culture

A549 cells were grown in DMEM plus 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were maintained in a 5% CO_2-humidified atmosphere at 37°C.

Transfection and Reporter Assays

A549 cells were transiently transfected using Lipofectamine and PLUS reagents, as described by the manufacturer (Invitrogen, Life Technologies). In short, cells within each well of a 24-well plate were transfected using 400 ng DNA, 1.2 μl lipofectamine and 0.8 μl PLUS reagent. After 5 h incubation with the transfection reagent, the medium was refreshed with standard culture medium (see above). After transfection, cells were left to rest for another 24 h before inductions. Cells were induced as indicated in the figure legends, after which luciferase assays were carried out according to instructions of the manufacturer (Promega). Luciferase measurements were performed at least in triplicate and normalized by measurement of β-galactosidase levels using the Galacto-Light kit (Tropix). Results presented are from 3 independent biological replicates.

Western Analysis

Total cell lysates were prepared using 1 × SDS sample buffer (50 mM Tris pH 6.8; 2% SDS; 10% glycerol; bromophenol blue and 100 mM DTT, freshly added). Samples were incubated at 95°C for 5 min and separated on a SDS-PAGE gel and subsequently blotted onto a Nitrocellulose membrane (Whatman, Dassel, Germany). Immunoblotting was performed according to the standard protocol of Santa Cruz (Santa Cruz, CA, USA). Imaging of antibody-tagged protein signal was obtained via Western Lightning (PerkinElmer, Waltham, MA, USA). To quantify bands obtained via Western analysis, we applied band densitometric analysis via ImageJ software (http://rsb.info.nih.gov/ij/). The area under curve (AUC) of the specific signal of the protein of interest as indicated in the figure legend was corrected for the AUC of the loading control, indicated in the figure legend. Results representative of 2 independent biological repeats are shown.

Immunofluorescence

Indirect immunofluorescence was performed as previously described (34). In short, A549 cells, seeded on coverslips and serum-deprived for 48 h, were induced as indicated in the figure legends. After fixation, endogenous p65 and MSK1 were visualized using the corresponding rabbit antibodies followed by Alexa Fluor 488 or Alexa Fluor 568 anti-rabbit IgG (Molecular Probes, Invitrogen). Endogenous PPARα was visualized using the corresponding goat antibody followed by Alexa Fluor 488 anti-goat IgG (Molecular Probes, Invitrogen). Endogenous GRα was visualized using the corresponding mouse antibody followed by Alexa Fluor 568 anti-mouse IgG (Molecular Probes, Invitrogen). Cell nuclei were stained using DAPI DNA staining (300 nM, Invitrogen).

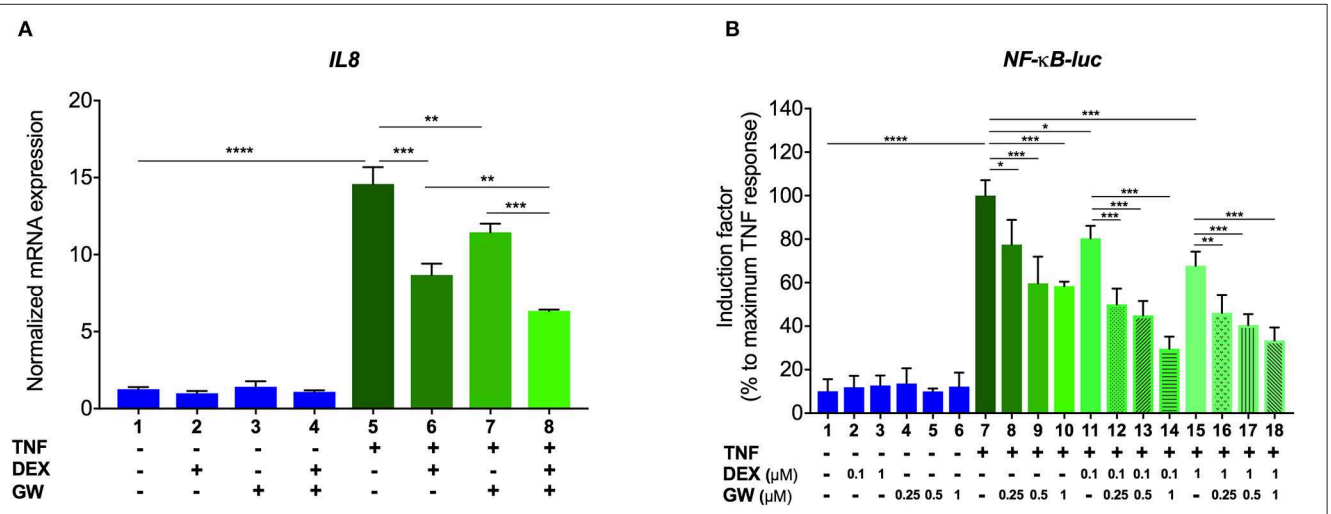


FIGURE 1 | GCs and PPARα agonists inhibit pro-inflammatory gene expression in A549 cells. **(A)** A549 cells were pre-incubated with solvent, DEX (1 μM), GW (0.5 μM) or various combinations thereof, for 1 h, before TNF (2000 IU/ml) was added, where indicated, for a total induction time of 6 h. mRNA was isolated, reverse transcribed, and subjected to QPCR using primers to detect IL8. qPCR measurements were performed in triplicates. qPCR results, normalized to expression of household genes, are shown ± SD. **(B)** A549 cells were transiently transfected with NF-κB-Luc using Lipofectamine/Plus reagents, as described (Invitrogen, Carlsbad, CA, USA). 24 h after transfection, cells were incubated with solvent, DEX (0.1 or 1 μM), GW (0.25, 0.5, or 1 μM) or various combinations thereof, for 1 h, before TNF (2000 IU/ml) was added, where indicated, for a total induction time of 6 h. Cell lysates were assayed for luc activities and normalized with β-gal activities. Promoter activities are expressed as relative induction factor calculated as percentage of maximal TNF response. Results in **(A,B)** are from three independent biological replicates ($n = 3$) with measurements in triplicate. Statistical analysis was done using ANOVA with Tukey's multiple comparison post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

In vitro Protein-Protein Interaction Assay (GST Pull-Down)

GST-fusion proteins with PPARα and 5HT7 were expressed in BL21 bacterial cells and purified with glutathione-agarose beads. GRα and p65 proteins were transcribed and translated *in vitro* using the TNT T7-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. GST pull-down was carried out by incubating the equivalent of 2 μg of GST-PPARα beads with 10 μl of *in vitro* translated [^{35}S]-methionine labeled GRα with increasing amounts of non-labeled GRα, or by incubating the equivalent of 2 μg of GST-PPARα beads with 10 μl of [^{35}S]-methionine labeled p65 with increasing amounts of [^{35}S]-methionine labeled GRα or finally, by incubating the equivalent of 2 μg of GST-PPARα beads with 10 μl of [^{35}S]-methionine labeled GRα with increasing amounts of [^{35}S]-methionine labeled p65. All of these interaction studies were performed in a total volume of 200 μl of incubation buffer [20 mM Tris-HCl (pH 8), 300 mM NaCl, 6 mM $MgCl_2$, 8% glycerol, 0.05% Nonidet P-40, 0.1% dithiothreitol]. The mixture was gently rotated for 2 h at 4°C. After centrifugation, the beads were washed five times with incubation buffer supplemented with NaCl up to a final concentration of 500 mM, next resuspended in 25 μl of 1x Laemmli buffer, boiled for 3 min, and centrifuged. After GST-mediated purification and extensive washes, proteins were separated on polyacrylamide gels and visualized by autoradiography. GST-5HT7 was used as a negative control.

Primary Hepatocyte Isolation

Primary hepatocytes were isolated from 10 to 12 week-old male C57BL/6 mice by collagenase perfusion (35). The procedure was modified by excluding insulin and DEX supplementation in the William's medium (Sigma, W1878), but keeping 0.1% free-fatty acids and 1% glutamine. After isolation cells were seeded on collagen-coated 6-well plates at a density of 0.75×10^6 cells. After 2 h of attachment medium was refreshed and ligands were introduced, as indicated in the figure legends.

qPCR and ChIP-qPCR

RNA was isolated with the RNeasy purification kit (Qiagen) according to the user manual. cDNA was synthesized with a PrimeScript kit (Takara). qPCR was performed using Light Cycler 480 SYBR Green I Master Mix (Roche). The primer list is provided in **Table S1**. qPCR data were normalized and quantified relative to the 2 most stable reference genes with qbase+ (36). ChIP assays were performed as previously described (37). The relative amount of the precipitated target sequence was determined via normalization to the "input", i.e., the purified total gDNA levels. The primers for IL8, encompassing −121/+61, have been described earlier (38).

RNA-Seq Analysis

RNA-seq was done in three biological replicates. Each replicate was obtained by pooling cells from 3 to 4 mice and then performing induction in three technical replicates. RNA was isolated with the RNeasy purification kit (Qiagen) according to the user manual. Library preparation and sequencing was prepared by the VIB Nucleomics Core facility. 75 bp long sequenced reads were generated with Illumina NextSeq 500 and were mapped to the mm10 genome using tophat (version 2.0.11). Gene counts were calculated with htseq-count (0.6.1) using "intersection-strict" mode. Gene level differential expression analysis was performed with the aid of the R package "DESeq2" by applying the following contrasts (p adjusted < 0.05): LPS

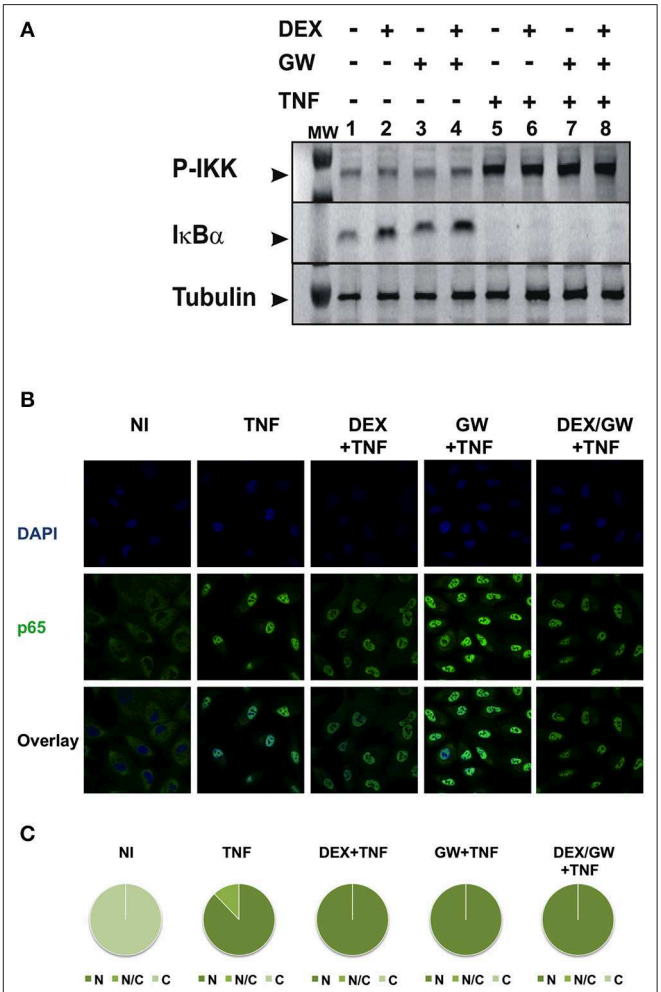


FIGURE 2 | Co-activation of GRα and PPARα does not affect pathways influencing the nuclear accumulation of activated p65. **(A)** A549 cells, starved for 48 h in DMEM devoid of serum, were pretreated with solvent, DEX (1 μM), GW (0.5 μM) or various combinations for 1 h, before TNF (2000 IU/ml) was added, where indicated, for 30 min. Cell lysates were subjected to western blotting using anti-phospho-IKK or anti-IκBα antibodies, and using anti-tubulin as a loading control, as indicated. A representative blot of $n = 2$ is shown. **(B)** A549 cells were treated with DEX (1 μM) and/or GW (0.5 μM) and/or TNF (2000 IU/ml). Indirect immunofluorescence was performed using an anti-p65 antibody. Endogenous p65 was visualized (green), DAPI staining indicates the nuclei of the cells (blue) and "Overlay" shows a merged image with both stainings combined. Representative images of $n = 2$ are shown. **(C)** Per induction, minimally three random fields of minimally 5 cells/field were scored. Scored cells are categorized into three groups according to the subcellular distribution of p65, i.e., C, mainly cytoplasmic; N, mainly nuclear; N/C, equally distributed (nuclear/cytoplasmic) with % distribution presented as pie charts.

vs. DEX+LPS, LPS vs. GW+LPS, LPS vs. DEX/GW+LPS, DEX+LPS vs. DEX/GW+LPS and GW+LPS vs. DEX/GW+LPS. Differentially expressed genes were combined into a single list and re-ordered using a K-mean clustering (6 clusters). Gene ontology analysis of gene clusters 2, 3, and 5 was performed using "goseq" R package.

ELISA

CCL2 and IL6 ELISA was performed on media from primary hepatocytes after 19 h induction with compounds DEX and/or GW in combination with 100 ng/ml LPS by using the ELISA MAX Standard (BioLegend, 432702, 430502), in according with the manual.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software (version 7.02 or 8). Significant differences between groups were evaluated using two-way (2 factors) ANOVA with Dunnett's test for multiple comparison, which was found to be appropriate as groups displayed a normal

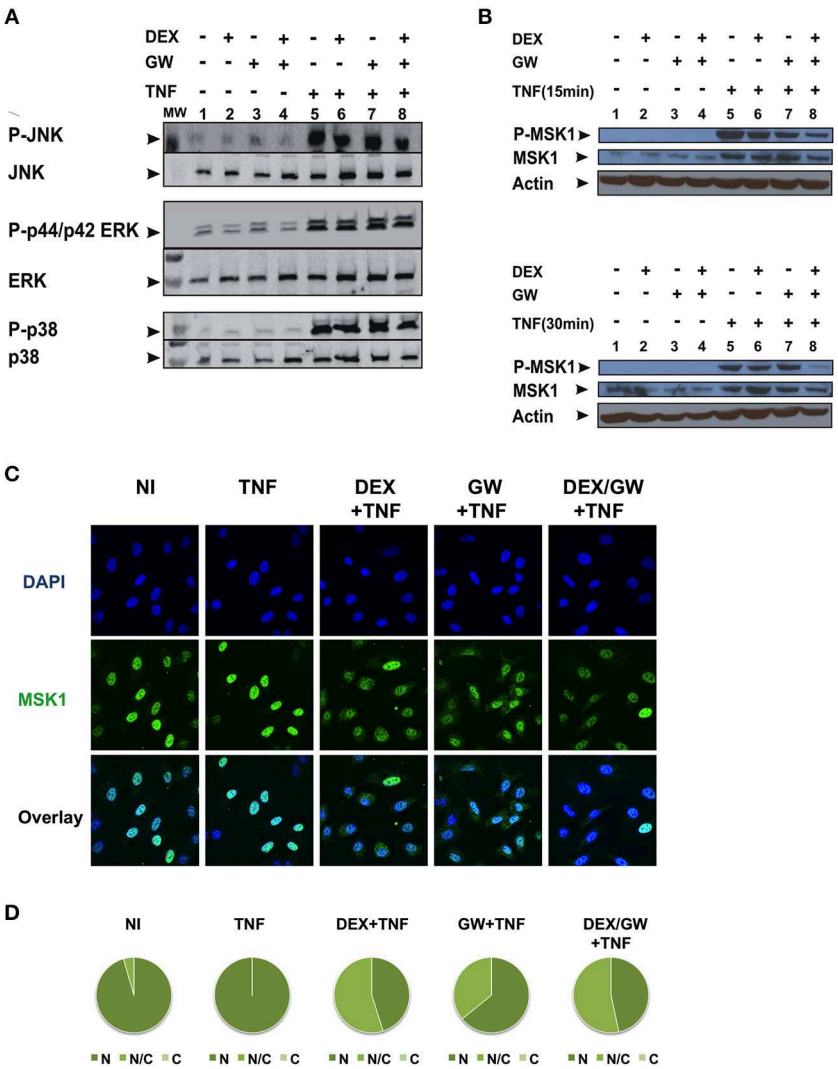


FIGURE 3 | Co-activation of GRα and PPARα efficiently lowers levels of phospho-MSK-1 in A549. **(A)** A549 cells, starved for 48 h in DMEM devoid of serum, were pretreated with solvent, DEX (1 μM), GW (0.5 μM) or various combinations for 1 h, before TNF (2000 IU/ml) was added, where indicated, for 30 min. Cell lysates were subjected to western blotting with anti-phospho-MAPK and the corresponding non-phospho antibodies; for this re-probed blot the same overall loading control applies as shown in **Figure 2A**. A representative blot of $n = 2$ is shown. **(B)** A549 cells, starved for 48 h in DMEM devoid of serum, were pretreated with solvent, DEX (1 μM), GW (0.5 μM) or various combinations for 1 h, before TNF (2000 IU/ml) was added, where indicated, for 15 min and 30 min. Cell lysates were subjected to western blotting with anti-phospho-MSK1, anti-MSK1 and anti-actin as a loading control, as indicated. A representative blot of $n = 2$ is shown. **(C)** A549 cells were treated with DEX (1 μM) and/or GW (0.5 μM) and/or TNF (2000 IU/ml) for 30 min. Indirect immunofluorescence was performed using an anti-MSK1 antibody. Endogenous MSK1 was visualized (green), DAPI staining indicates the nuclei of the cells (blue) and Overlay indicates an image of both stainings combined. Representative images of $n = 2$ are shown. **(D)** Per induction, minimally three random fields of minimally 5 cells/field were scored. Scored cells are categorized into three groups according to the subcellular distribution of MSK1, i.e., C, mainly cytoplasmic; N, mainly nuclear; N/C, equally distributed (nuclear/cytoplasmic) with % distribution presented as pie charts.

distribution. Normality was tested with the D'Agostino-Pearson normality test. When variances across groups were not equal, logarithmic transformation was applied prior to statistical analysis. Values are expressed as mean + SEM, and error bars were derived from biological replicates rather than technical replicates. $p < 0.05$ was considered statistically significant.

RESULTS

GCs and PPARα Agonists Inhibit Pro-inflammatory Gene Expression in a Concentration-Responsive Manner

We first verified, using A549 lung epithelial cells, that the single PPARα agonist GW7647 (hereafter GW) and the single

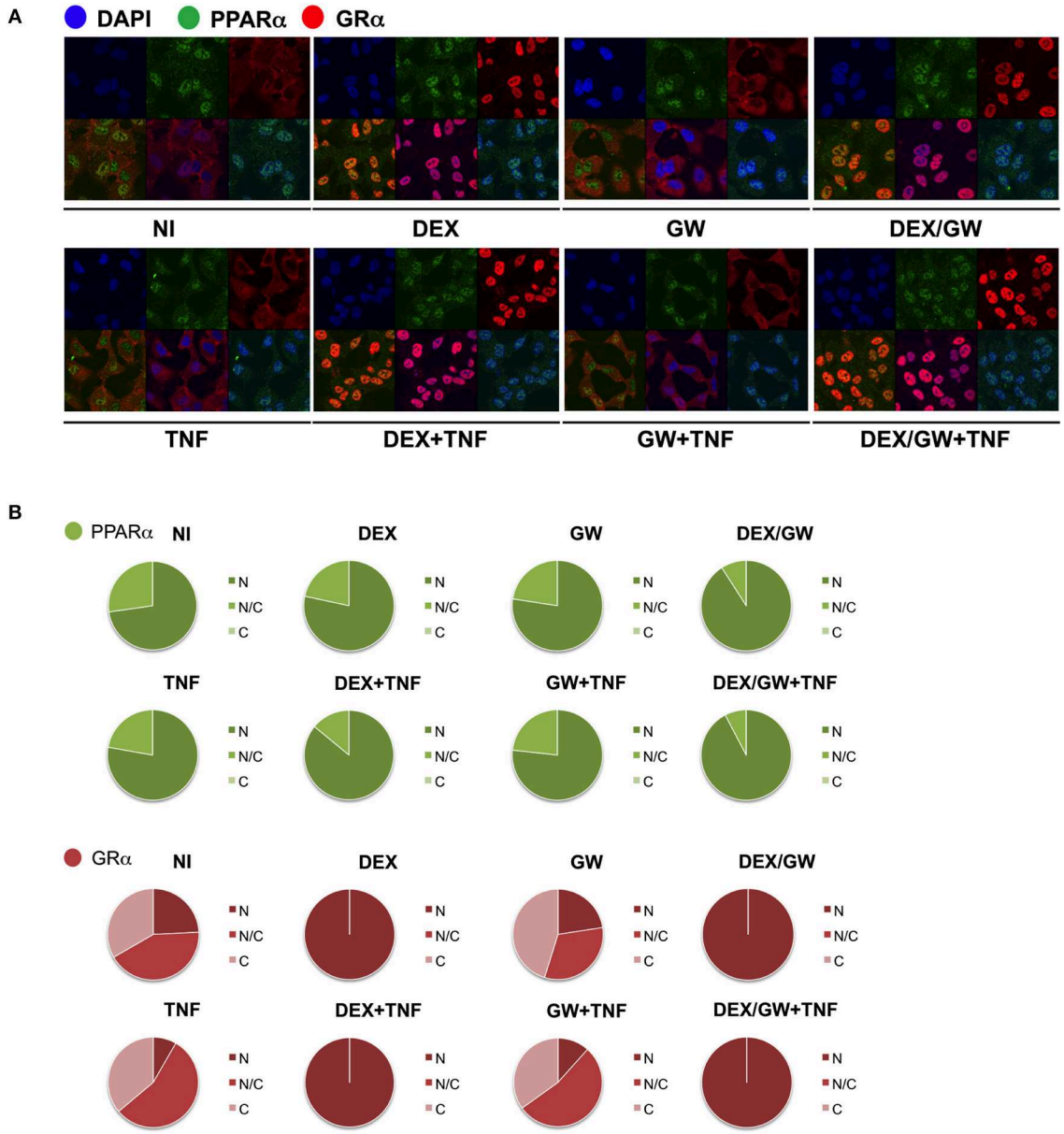


FIGURE 4 | Ligand-activated GRα and PPARα are both localized in the nucleus in TNF-stimulated cells. **(A)** A549 cells, starved for 48h in DMEM devoid of serum, were pretreated with solvent, DEX (1 μM), GW (0.5 μM) or various combinations for 1h, before TNF (2000 IU/ml) was added, where indicated, for 30 min. Localization of PPARα (green) and GRα (red) was assessed by confocal analysis. DAPI staining indicates the nuclei of the cells (blue). Immunofluorescence of representative cell fields are shown ($n = 1$). **(B)** Per induction, minimally three random fields of minimally 5 cells/field were scored. Scored cells are categorized into three groups according to the subcellular distribution of PPARα (green) and GRα (red), i.e., C, mainly cytoplasmic; N, mainly nuclear; N/C, equally distributed (nuclear/cytoplasmic).

synthetic GR agonist dexamethasone (DEX) are both able to inhibit TNF-induced gene expression (**Figure 1A,** lanes 6 and 7 compared to lane 5). We go on to show that an additive anti-inflammatory effect can be observed for a complex NF-κB-driven promoter in its endogenous promoter context, i.e., TNF-induced IL-8 mRNA expression (**Figure 1A,** lane 8 compared to lanes 6 and 7). Results from A549 cells transiently transfected with a recombinant NF-κB-driven promoter construct as a direct transcriptional read-out (**Figure 1B**) confirm TNF-induced NF-κB as a relevant nuclear receptor target and show anti-inflammatory effects by single DEX and GW, in a concentration-responsive manner (**Figure 1B**, lanes 8 to 10 and lanes 11 and 15 compared to lane 7). Combined DEX/GW treatment results in an additive repression of TNF-induced recombinant NF-κB promoter activity when compared to compound alone (**Figure 1B**, lanes 12 to 14 compared to lane 11 and lanes 16–18, compared to lane 15) even when using saturating amounts of DEX. Taken together, these data support our previous findings in L929sA where the additive anti-inflammatory effect of DEX and GW also converged on NF-κB (22). Collectively, these results raise the question whether combined ligand treatment may act differently on components of the upstream cascade leading toward NF-κB or may differently impinge on NF-κB binding or activity.

Co-activation of GRα and PPARα Does Not Affect the Upstream TNF-Induced IKK Activation Pathway or the Nuclear Accumulation of Activated p65

To first test whether the TNF-induced kinase cascade upstream of the activity of p65 can be a target of a GRα and PPARα-mediated inhibition, we evaluated levels of activated IKK and the inhibitory protein of NF-κB. IκBα is known to be degraded following activation of IKK and subsequent phosphorylation upon an inflammatory stimulus, e.g., TNFα. This was confirmed in **Figure 2A** (for quantification please see **Figure S1**). No significant effect of DEX, GW or the combination hereof was apparent on TNF-activated IKK (**Figure 2A**). In line with these results, DEX and GW also did not affect the TNFα-induced nuclear translocation of the p65 subunit of NF-κB as shown by indirect immunofluorescence analysis (**Figure 2B**). Based on these results, the cooperative anti-inflammatory activity of GCs and PPARα agonists most likely operates within the cellular nucleus.

Co-activation of GRα and PPARα Does Not Affect MAPK Activation but Efficiently Lowers Levels of Phospho-MSK-1 in A549

As we observed no significant inhibitory effect of combined DEX/GW treatment on the above-mentioned kinases in **Figure 2**, we further explored whether combined treatment of GCs and PPARα agonist might target TNF-induced phospho-ERK, phospho-JNK and phospho-p38 or the downstream nuclear kinase MSK1 (**Figure 3**). As shown in **Figure 3A**, none of the TNF-activated MAPK is differentially affected comparing GC/PPARα co-treatment with single treatments (for quantification please see **Figure S2A**). However, compared to each compound alone, co-treatment with the PPARα agonist GW and DEX clearly reduces the TNF-induced MSK1 phosphorylation, apparent at 15 min (**Figure 3B**, upper panel) and at 30 min (**Figure 3B,** lower panel) (for quantification please see **Figure S2B**). In line with our previous results (23), DEX is able to partially extrude TNF-induced MSK1 from the nucleus (**Figure 3C**). Both GW alone as well as the combination DEX/GW yields a similar result when combined with TNF, as

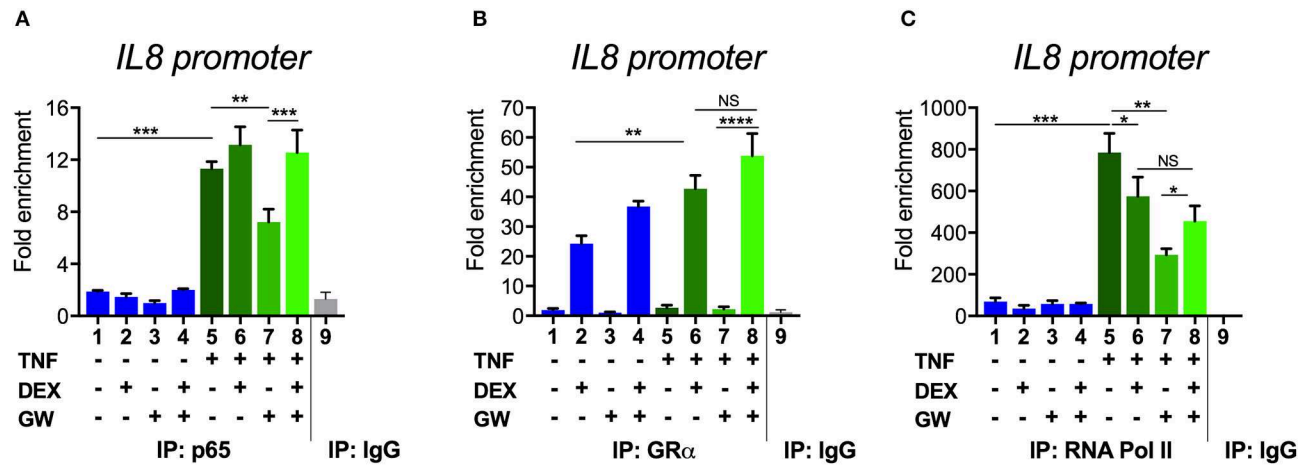


FIGURE 5 | Combined DEX and PPARα agonist treatment maintains chromatin recruitment of TNF-activated p65. Following serum starvation for 48 h, A549 cells were pre-incubated with solvent, DEX (1 μM), GW (0.5 μM) or various combinations for 1 h, before TNF (2000 IU/ml) was added, where indicated, for 30 min. Cross-linked and sonicated cell lysates were subjected to ChIP analysis against p65 **(A)**, GR **(B)** or RNA pol II **(C)**. qPCR was used to assay recruitment at the IL8 gene promoter. The quantity of p65, GR or RNA pol II detected at the IL8 promoter is shown with a correction of the SYBR green qPCR signal for input control. Lanes 1–8 contain data derived from DNA pulled with specific antibody-prepared ChIPs, as indicated in the graph; lane 9 includes the IgG control. The reaction was performed in triplicate. Results are compiled from three independent biological replicates ($n = 3$). Statistical analysis was done using ANOVA with Tukey's multiple comparison post-test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

compared to TNF alone (**Figure 3C**). From the cell counts it is clear that combined DEX/GW with TNF recapitulates the same phenotype as observed for DEX/TNF (**Figure 3D**). Still, in all combinations a predominant nuclear MSK1 signal remains. Taken together, these results suggest that the combined inhibitory effect of GCs and PPARα agonists on phosphorylated MSK1 may contribute to the additive transrepression of NF-κB-driven inflammatory genes triggered by activated GR and PPARα.

Ligand-Activated GRα and PPARα Are Both Localized in the Nucleus in TNF-Stimulated A549 Cells

We next wondered whether the activated nuclear receptors would remain nuclear in absence and presence of TNF. Endogenous co-immunolocalization analyses show that under conditions in which p65 is activated upon TNF (**Figures S3, S4**) and under conditions when both GRα and PPARα are activated, the latter proteins effectively reside predominantly in the nuclear compartment (**Figure 4**).

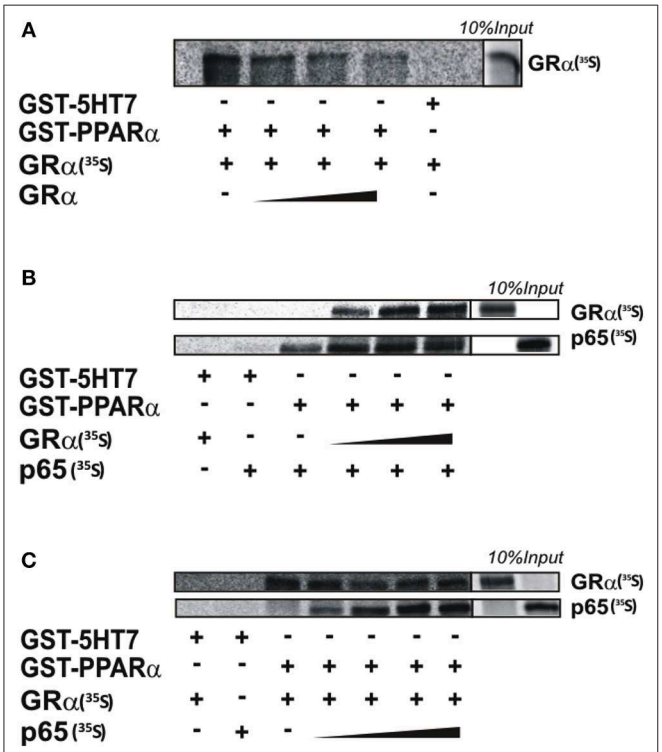


FIGURE 6 | PPARα and GRα interact with NF-κB p65, in a non-competitive manner *in vitro*. GST-fusion proteins PPARα and 5HT7 were expressed in BL21 bacterial cells and purified with glutathione-agarose beads. [S35]-methionine labeled GRα and or p65 products were generated with TNT reaction, using rabbit reticulocyte lysates. **(A)** [^{35}S]-methionine labeled GRα was incubated with Glutathione-Sepharose 4B beads loaded with GST-PPARα or GST-5HT7 as control with increasing amount of non-labeled GRα. **(B)** [^{35}S]-methionine labeled p65 was incubated with Glutathione-Sepharose 4B beads loaded with GST-PPARα or GST-5HT7 as control with increasing amount of [^{35}S]-methionine labeled GRα. **(C)** [^{35}S]-methionine labeled GRα was incubated with Glutathione-Sepharose 4B beads loaded with GST-PPARα or GST-5HT7 as control with increasing amount of [^{35}S]-methionine labeled p65. Representative images of $n = 2$ are shown.

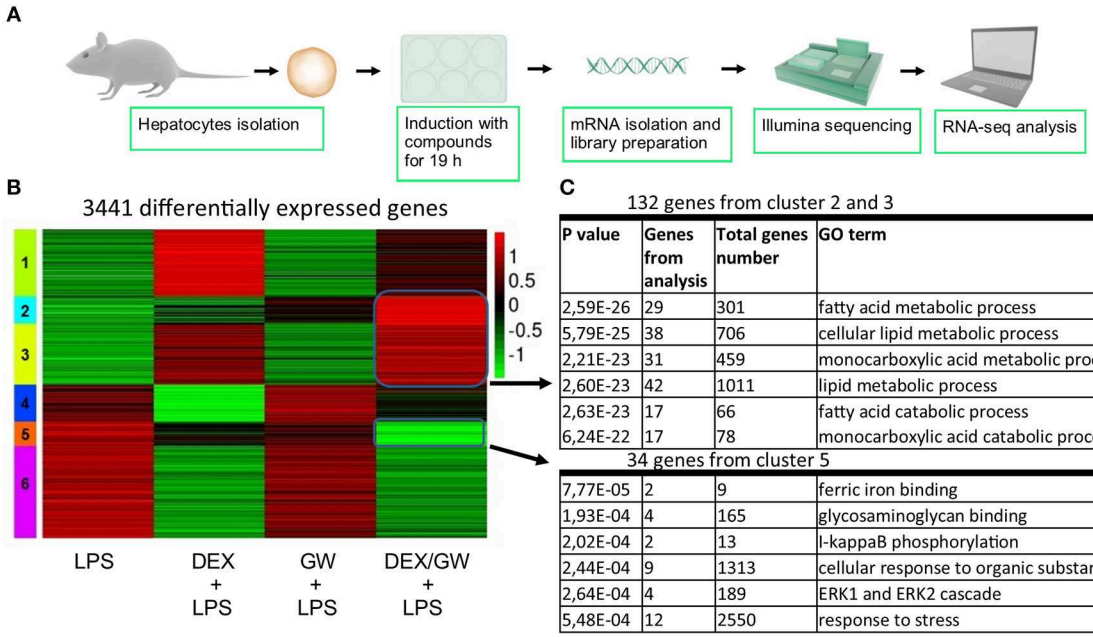
Combined DEX and PPARα Agonist Treatment Maintains Chromatin Recruitment of TNF-Activated p65

To next study the impact of single vs. combined ligand treatment on the subsequent binding behavior of NF-κB we analyzed the IL8 promoter nearby the promoter proximal NF-κB binding site, using chromatin immunoprecipitation (ChIP) analysis. The results in **Figure 5A** show that the PPARα agonist GW alone reduces the TNF-induced p65 recruitment at this inflammatory promoter, however, single DEX or combined DEX/GW treatment clearly does not affect TNF-induced promoter occupation of p65. When analyzing concomitant GR occupancy under the same conditions, DEX treatment consistently increases GR recruitment at the IL8 promoter (**Figure 5B**). When combined with TNF, DEX supports even more GR recruitment (**Figure 5B**, compare lanes 2 and 6). Of note, additional GW treatment does not further affect GR recruitment (**Figure 5B,** lane 8). In concordance with the results on gene repression (**Figure 1**), we detect lower IL8 promoter occupancy of RNA polymerase II (RNA pol II) when combining DEX, GW or DEX/GW as compared to TNF alone (**Figure 5C**). The combination of DEX/GW with TNF did however not result in a lower IL8 promoter occupancy of RNA pol II as compared to DEX/TNF, or GW/TNF alone. Lower levels of RNA pol II recruitment upon GW/TNF (**Figure 5C**) nicely correlate with a lower level of p65 recruitment upon GW/TNF (**Figure 5A**), yet again the effect of DEX, and additional presence of GR (**Figure 5B**) is dominant. Taken together, these results show that even though MSK1 activation is reduced (**Figure 3B**), still, p65 is not dissociated from the IL8 promoter under conditions of a maximal pro-inflammatory gene inhibition by DEX and PPARα agonists.

PPARα and GRα Interact With NF-κB p65 in a Non-competitive Manner *in vitro*

The underlying mechanism as suggested by the transcriptional data (**Figure 1**) and the ChIP results (**Figure 5**) may involve either tethering events or independent DNA binding events. Direct interactions between single GR or single PPARα with the p65 subunit of NF-κB were previously reported to contribute to the inhibition of NF-κB-dependent pro-inflammatory gene expression and were described to involve (a) the DNA binding domain of either GRα or PPARα and (b) the Rel Homology Domain (RHD) of p65 (33, 39, 40). To obtain further insight into the molecular basis of the additive anti-inflammatory effect observed upon combining GR and PPARα agonists, we tested whether GRα and PPARα are able to bind p65 simultaneously or instead in a competitive and mutually exclusive manner. Since both receptors have been described to interact with largely similar domains within p65 (AA 22-248 and 12-378 for GRα and PPARα, respectively (33, 39, 40), the possibility of a competitive and independent binding was considered.

GST-pull down experiments show that binding between GST-PPARα and *in vitro* produced GRα (^{35}S) can be outcompeted by



P value	Genes from analysis	Total genes number	GO term
2,59E-26	29	301	fatty acid metabolic process
5,79E-25	38	706	cellular lipid metabolic process
2,21E-23	31	459	monocarboxylic acid metabolic process
2,60E-23	42	1011	lipid metabolic process
2,63E-23	17	66	fatty acid catabolic process
6,24E-22	17	78	monocarboxylic acid catabolic process
7,77E-05	2	9	ferric iron binding
1,93E-04	4	165	glycosaminoglycan binding
2,02E-04	2	13	I-kappaB phosphorylation
2,44E-04	9	1313	cellular response to organic substance
2,64E-04	4	189	ERK1 and ERK2 cascade
5,48E-04	12	2550	response to stress

FIGURE 7 | Co-activation of GRα and PPARα enhances lipid metabolism gene subsets and lowers stress response gene subsets in LPS-induced primary hepatocytes. **(A)** Schematic overview of the RNA-seq experiment ($n = 3$). **(B)** Heatmap using K-mean clustering of 3,441 differentially expressed genes from contrasts (p adjusted < 0.05): LPS (100 ng/ml) vs. DEX+LPS, LPS vs. GW+LPS, LPS vs. DEX/GW+LPS, DEX+LPS vs. DEX/GW+LPS and GW+LPS vs. DEX/GW+LPS. Color scale represents gene counts. **(C)** Gene ontology analysis of differentially upregulated (clusters 2 and 3) and downregulated (cluster 5) genes by DEX/GW vs. LPS, DEX and GW.

cold GRα (**Figure 6A,** quantification see **Figure S5A**), illustrating the feasibility to detect competitive binding in a GST-pull down assay and supporting our previous findings, via co-IP, that PPARα and GRα indeed physically interact (22). The interaction between GST-PPARα and *in vitro* produced p65 (^{35}S) is however not affected by increasing amounts of GRα (^{35}S) (**Figure 6B,** quantification see **Figure S5B**). Similarly, adding increasing amounts of p65 (^{35}S) also does not affect the binding between GST-PPARα and *in vitro* produced GRα (^{35}S) (**Figure 6C,** quantification see **Figure S5C**). Altogether, our GST-pull down experiments support that GR and PPARα may interact with the RHD of p65 in a non-competitive manner, supporting the hypothesis of complex formation between all three transcription factors.

The *in vitro* experiments cannot take into account the possibility that the single ligand treatments and/or co-treatments may additionally affect receptor protein expressions in a cellular environment. To address this extra parameter, A549 cells were pretreated with solvent, DEX (1 mM), GW (0.5 μM) or various combinations for 1 h, before TNF (2000 IU/ml) was added for a total induction time of 6 h (to match the time points in **Figure 1**). Interestingly, the results from **Figure S6** show that in inflamed cells (last 4 lanes, with TNF added) the combined ligand treatment DEX/GW is capable of lowering not only protein levels of the pro-inflammatory protein p65, but concomitantly also of both receptor levels. Strikingly, GW/DEX alone largely recapitulated the effect observed of both ligands in presence of TNF. Similar data were found for a shorter time point (1.5 h) (**Figure S7**), albeit not as outspoken. These findings nevertheless support the validity of the findings presented in **Figure S6**.

GR and PPARα Co-regulate Lipid Metabolism and Inflammatory Gene Expression in Opposite Manners in Inflamed Murine Hepatocytes

When looking at the broader picture of possible target cells, GCs and PPARα will not only regulate genes in immune or structural cell types coping with an inflammatory insult (e.g., synovial fibroblasts, macrophages, T-cells, or lung epithelial cells as studied here), but will also trigger gene programs in metabolic tissues, such as hepatocytes. Activated GR and PPARα have been described before to additively upregulate a vast subset of key genes of the lipid metabolism pathway in naïve murine primary hepatocytes (21). Combined ligand treatment was shown to exhibit anti-inflammatory capacities in lung epithelial cells as typical effector cells contributing to an inflammatory response (**Figure 1**), but it remained uncertain whether primary hepatocytes would behave in a similar manner, given a dominant role of GR/PPARα in glucose and fat metabolism in this cell type. To address this question, we performed RNA-seq following DEX and GW co-treatment for 19 h in presence of LPS to additionally mimic an inflamed state (**Figure 7A**). K-means clustering following the differential expression analysis revealed 992 genes (**Figure 7B**, cluster 2 and 3) upregulated by the combination of DEX/GW with LPS treatment compared to

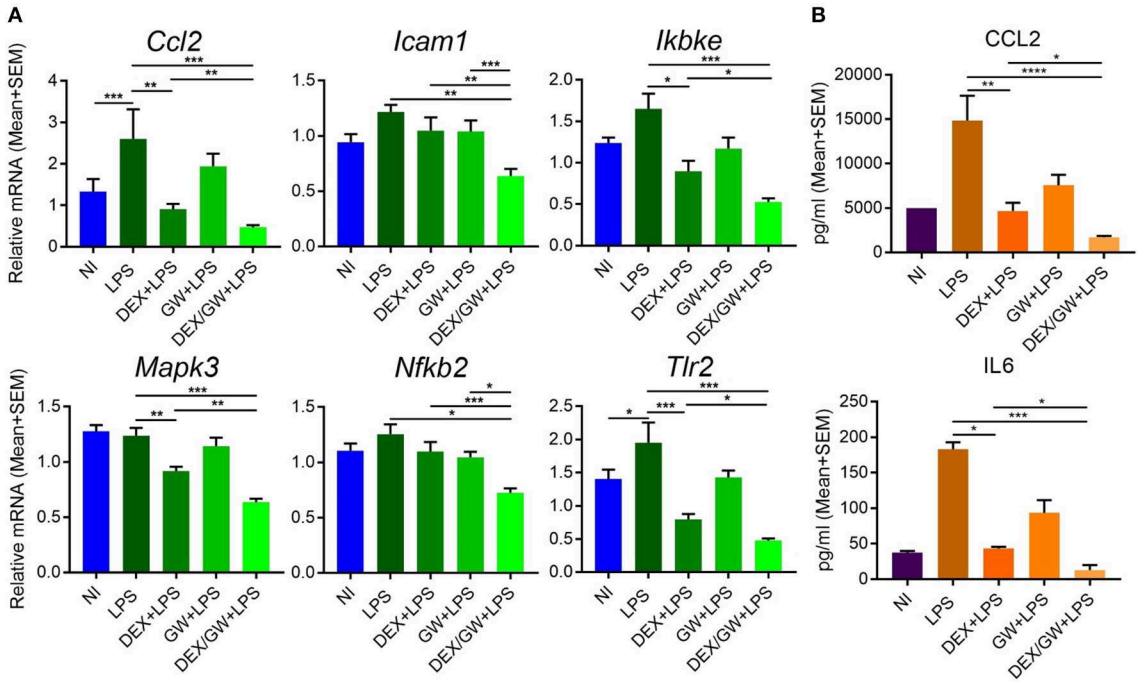


FIGURE 8 | Co-activation of GRα and PPARα additively lowers inflammatory gene and protein expression in LPS-induced primary hepatocytes. Results are shown for mRNA **(A)** and protein **(B)** levels. **(A)** Following the treatment of primary hepatocytes as described in the legend of **Figure 6**, mRNA was isolated followed by qPCR analysis. Gene expression levels were normalized to Ppia/cyclophilin and Gapdh reference gene expression using qbase+ ($n = 4$–5). **(B)** CCL2 and IL6 ELISA from the media of primary hepatocytes after 19 h treatment with DEX (1 μM) and GW (0.5 μM) in combination with 100 ng/ml LPS ($n = 3$). Statistical analysis was done using 1-way ANOVA and Dunnett's test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). NI, non-induced.

LPS alone. Among those, 132 genes were significantly more upregulated when compared to each compound alone (DEX + LPS or GW + LPS). Gene ontology analysis of these 132 genes attributed them to the lipid metabolism pathway (**Figure 7C**). This was consistent with previous results obtained in a basal state (21). LPS treatment did not influence DEX/GW-co-regulated gene expression in primary hepatocytes of one of the key co-controlled genes, *Angptl4*; a result that was independently validated by qPCR (**Figure S8**). We detected also 279 genes downregulated by DEX/GW + LPS treatment compared to LPS (**Figure 7B**, cluster 5). Only 34 of those were significantly more repressed upon comparing with either DEX + LPS or GW + LPS treatment alone. Some of these genes are inflammatory markers such as *Icam1, Ikbke, Nfkb2, Mapk3, Tlr2*.

GR and PPARα Cooperate to Downregulate Inflammatory Genes and Proteins in Inflamed Murine Hepatocytes

The results were next validated using qPCR in independently isolated murine primary hepatocytes (**Figure 8A**). We also determined mRNA levels of the classic inflammatory marker *Ccl2*. Similar to mRNA results, the protein levels of CCL2 were suppressed by combined DEX/GW treatment in presence of the inflammatory stimulus when compared to each compound alone (**Figure 8B**). Although the overall expression levels of IL6 in LPS-induced hepatocytes were almost two orders of magnitude lower than of CCL2 levels, we still observed a similar regulation (**Figure 8B**). Taken together, in analogy with the TNF-induced lung epithelial cell model, simultaneous GR and PPARα activation also supports additive anti-inflammatory effects in the LPS-inflamed primary hepatocyte model.

DISCUSSION

The activation of PPARα was shown before to suppress the induction of liver gluconeogenic *G6PC* and *PEPCK* genes that were activated by GR in mice subject to a high fat diet (22). As such, combined PPARα and GRα agonist treatment might hold a promise of therapeutic benefit when able to cooperatively enhance anti-inflammatory effects, while circumventing (at least) the side effect of GC-induced glucose intolerance. In the current research we studied the GRα-PPARα crosstalk paradigm and its putative role in the transcriptional regulation of inflammatory genes comparing two cell types in which both GRα and PPARα are well-expressed and functional, i.e., hepatocytes and lung epithelial cells. We demonstrated that simultaneous GRα-PPARα activation additively suppresses inflammation both in LPS-treated murine primary hepatocytes and TNF-induced human lung epithelial cells. In the latter cell type, we went on to show via Western analysis using phospho-specific antibodies,

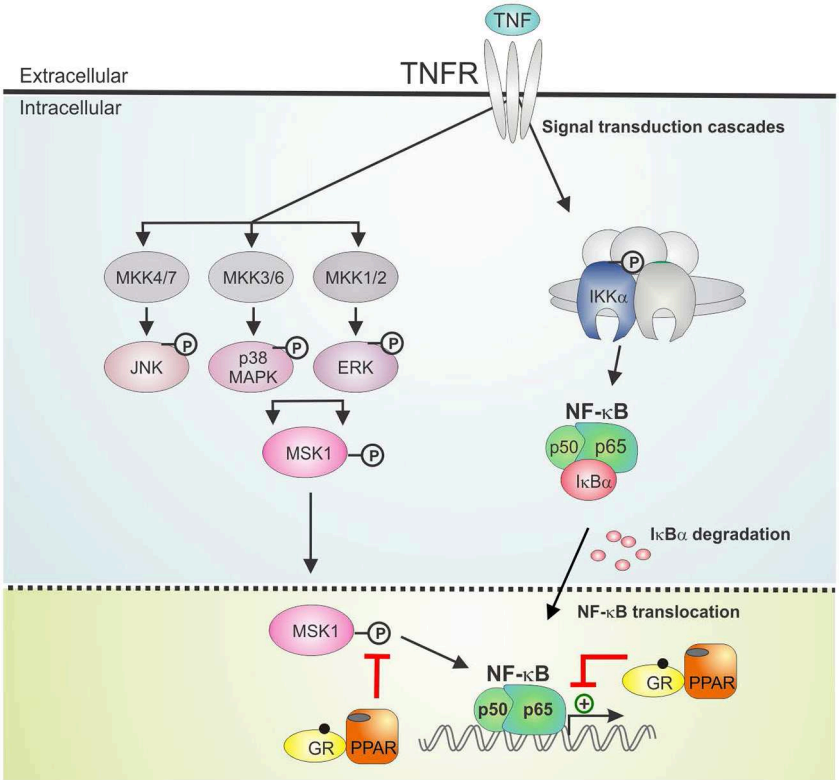


FIGURE 9 | GRα/PPARα controlled points of interference with the TNF signaling pathway. Graphical abstract demonstrating activated GRα/PPARα efficiently inhibits TNF-driven gene expression in A549 cells primarily by interfering with the phosphorylation status of MSK1. Signaling components of the NF-κB pathway that have been studied in the manuscript are shown in non-gray colors. GR, Glucocorticoid Receptor α; IKK, IκB kinase, MAPK, Mitogen-activated protein kinase; MKK, MAP kinase kinase; PPAR, peroxisome proliferator activated receptor α; TNF, tumor necrosis factor; TNFR, TNF receptor.

that GR-PPARα crosstalk may block inflammatory cytokine gene expression in the nucleus by mitigating the activity of a kinase upstream of NF-κB, MSK1, but not its upstream MAPK activators. This mechanism seems in contrast with a recently described mechanism in macrophages, explaining anti-inflammatory effects of single GCs not solely via gene suppression but through cooperative actions with p38 MAPK- and MSK1-dependent pathways, culminating in the upregulation and activation of another kinase, Sphingosine kinase 1 (SphK1) (41). However, these mechanisms do not necessarily exclude each other and are likely complementary. Indeed, it is not unreasonable to infer that different GC-assisted mechanisms may come in at different phases of the inflammatory response, or that in different cell types GCs may preferentially impact at different levels to establish a net anti-inflammatory effect. From our data, both GCs and PPARα agonist alone are able to partially drive MSK1 kinase from the nucleus, confirming earlier findings for GCs (23). At any rate, the finding that the subcellular distribution of MSK1 upon DEX/GW/TNF is similar to DEX/TNF implies that extrusion by itself is probably not a main mechanism explaining the additive gene repression. Rather, inhibition of MSK1 activation, which will hamper MSK1 activity, and interference at the level of NF-κB further downstream seem sufficient mechanisms to achieve additive cytokine gene repression (model in **Figure 9**). Taken together, it is clear that anti-inflammatory pathways that jointly tackle pathways leading to NF-κB activity will have an added advantage, as also found before in a study combining GCs with MSK1 inhibitors (42). Of interest from a clinical perspective, increased levels of activated MSK1 were detected in circulating blood CD14+ cells from patients with steroid-resistant asthma as compared to samples from steroid-sensitive asthma patients, linking a potential involvement of MSK1 in the regulation of cellular steroid responses (43). In a recent study in support of combination strategies, the team of Goleva showed benefit upon combining GCs with vitamin D, by demonstrating anti-inflammatory and GC-enhancing effects in monocytes of patients not only in steroid-sensitive asthma but also to some extent in steroid-resistant asthma (44).

We found that combined DEX/GW was able to reduce not only GRα and PPARα protein levels but also p65, in absence and presence of TNF. Regardless, inflammatory gene repression by combined GRα-PPARα agonists (studied here at the human IL8 promoter) was found to still involve maintaining the p65 subunit of NF-κB as well as GRα and PPARα at the chromatin (model in **Figure 9**). This finding apparently contrasts a study

in macrophages showing GR activation, on its own, results in genome-wide blockade of NF-κB interaction with chromatin, as a late GC-induced event when inflammatory responses are allowed to fully mount (45). Again, this is not necessarily in conflict, as our study rather brings forward mechanisms likely to occur when GCs are ahead of a full-blown inflammatory response. In support of our data, in another recent study on mouse macrophages GR was rather shown to suppress pro-inflammatory gene expression by targeting distinct temporal events and components of transcriptional machinery in a gene-dependent manner, yet, the mechanism consistently involved a rapid GR tethering to p65 at NF-κB-binding sites (46). Our findings, adding PPARα to the equation, make it tempting to suggest a tripartite physical interaction mechanism may be possible. In line herewith, we retrieve all activated proteins (p65, GR, and PPARα) in the nuclear compartment, when performing pairwise indirect immunofluorescence of endogenous proteins in A549. Support for a physical interaction between p65, GRα and PPARα, at least *in vitro*, was found through non-competitive associations in GST-pull down analyses. Our data only shed light on a little piece of the anti-inflammatory mechanism following combined action of GRα and PPARα. Combined GRα/PPARα treatment reduces MSK1 kinase activation and appears to change the balance between nuclear vs. cytoplasmic MSK1, perhaps by preventing the accessibility of the kinase to the NF-κB target. Although these events clearly do not affect promoter recruitment of p65 or of pol II, at least not for IL8, a change in the activity status of NF-κB may well change coregulator associations, leading to a negative impact on gene expression. The *in vitro* interaction data, involving bacterial proteins and *in vitro* translated protein, suggest GR/PPAR/p65 complex formation, at least *in vitro*, might not be dependent on phosphorylation events, which is supported by the finding from the cell data that activated p65 remains efficiently recruited in presence of co-activated GRα/PPARα. It remains to be studied however, how frequent GRα and PPARα may co-localize in the cell models we have presented here, when subject to an inflammatory stimulus. In addition, direct proof of *in cellulo* complex formation at relevant promoter regions awaits firm evidence, for instance upon using re-ChIP experiments. Also the nature of the predominant binding sites remains to be investigated (half-site or palindromic GRE vs. PPRE vs. NF-κB response elements). In line with a previously recognized role for GRIP1 acting as a corepressor contributing to the suppressive action of GR (47–49), it is of current also unclear which cofactors may differentially associate with the GRα/PPARα co-suppressed inflammatory promoters as compared to either stimulus alone. On the physiological side, follow-up studies will have to demonstrate a predicted improved therapeutic benefit may take place, when co-administering GCs and PPARα agonists in an animal model of chronic inflammation (e.g., multiple sclerosis, arthritis, or asthma). Such study will allow simultaneous evaluation of the anti-inflammatory activity in relevant inflammatory target cells (depending on the animal model) with a metabolic impact addressing responses of the liver, regulating glucose and fat metabolism, when allowed to communicate with the other endocrine tissue within a complex organism under chronic inflammatory pressure.

DATA AVAILABILITY

RNA-seq data have been submitted to the ArrayExpress tool (https://www.ebi.ac.uk/fg/annotare/) under the accession numbers E-MTAB-7296.

ETHICS STATEMENT

Experiments were approved by the animal ethics committee of the Faculty of Medicine and Health Sciences at the University of Ghent (code dossiers 14/84 and 17/13).

AUTHOR CONTRIBUTIONS

NB and VM conducted experiments, analyzed data, and wrote parts of the manuscript. DR conducted experiments and contributed to the data analysis. IB revised the manuscript and advised on some experiments. LDC and JTh conducted experiments. JTa, BS, and CL contributed to the discussion section. KDB designed and supervised the research, conducted experiments, analyzed data, and wrote parts of the 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.2019.01769/full#supplementary-material

Figure S1 | (Quantification **Figure 2A**). Western blot densitometric analysis. The P-IKK band (upper panel) and IκBα band (Lower panel) visualized via Western blot analysis in **Figure 2A** were subjected to band densitometric analysis using Image J. The amount of specific signal for P-IKK and IκBα was corrected to the respective tubulin loading control.

Figure S2 | (Quantification **Figures 3A,B**). Western blot densitometric analysis. **(A)** The phospho-MAPK bands visualized via Western blot analysis in **Figure 3A** were subjected to band densitometric analysis (Image J). The amount of specific signal for phospho-MAPK was corrected to the respective corresponding non-phospho-MAPK signal. **(B)** The phospho-MSK1 bands visualized via Western blot analysis in **Figure 3B** were subjected to band densitometric analysis using Image J. The amount of specific signal for the phospho-MSK1 was corrected to the respective non-phospho-MSK1.

Figure S3 | Ligand-activated GRα and p65 are both localized in the nucleus in TNF-stimulated cells. **(A)** A549 cells, starved for 48 h in DMEM devoid of serum, were pretreated with solvent, DEX (1 μM), GW (0.5 μM) or various combinations for 1h, before TNF (2000 IU/ml) was added, where indicated, for 30 min. Localization of p65 (green) and GRα (red) was assessed by confocal analysis. DAPI staining indicates the nuclei of the cells (blue). Immunofluorescence of representative cell fields is shown ($n = 1$). **(B)** Per induction, minimally three random fields of minimally 5 cells/field were scored. Scored cells are categorized into three groups according to the subcellular distribution of p65 (green) and GRα (red), i.e., C, mainly cytoplasmic; N, mainly nuclear; N/C, equally distributed (nuclear/cytoplasmic).

Figure S4 | Ligand-activated PPARα and p65 are both localized in the nucleus in TNF-stimulated cells. **(A)** A549 cells, starved for 48 h in DMEM devoid of serum,

were pretreated with solvent, DEX (1 μM), GW (0.5 μM) or various combinations for 1h, before TNF (2000 IU/ml) was added, where indicated, for 30 min. Localization of PPARα (green) and p65 (red) was assessed by confocal analysis. DAPI staining indicates the nuclei of the cells (blue). Immunofluorescence of representative cell fields is shown ($n = 1$). **(B)** Per induction, minimally three random fields of minimally 5 cells/field were scored. Absolute amounts of cells that were scored per induction were between 30 and 65 cells and categorized into three groups according to the subcellular distribution of PPARα (green) and p65 (red), i.e., C, mainly cytoplasmic; N, mainly nuclear; N/C, equally distributed (nuclear/cytoplasmic).

Figure S5 | (Quantification **Figures 6A–C**). GST pull down analysis. **(A)** [^{35}S]-methionine labeled GRα pull down and **(B,C)** [^{35}S]-methionine labeled p65 and [^{35}S]-methionine labeled GRα pull down visualized via autoradiography in **Figure 5** were quantified using ImageJ analysis. Signals were normalized against respective inputs.

Figure S6 | Combined PPARα/GRα activation diminishes p65 levels as well as nuclear receptor levels following 6h inductions, in absence and presence of TNF. **(A)** A549 cells, starved for 48 h in DMEM devoid of serum, were pretreated with solvent, DEX (1 μM), GW (0.5 μM) or various combinations for 1h, before TNF (2000 IU/ml) was added, where indicated, for a total induction time of 6h. Cell lysates were subjected to western blotting to detect GRα, PPARα or p65. Detection of β-actin served as a loading control. $n = 1$. **(B)** The bands visualized via Western blot analysis were subjected to band densitometric analysis using Image J. The amount of specific signal for was corrected to the respective actin loading control.

Figure S7 | Combined PPARα/GRα activation already diminishes p65 levels as well as nuclear receptor levels following 1.5 h inductions, in absence and presence of TNF. **(A)** A549 cells, starved for 48 h in DMEM devoid of serum, were pretreated with solvent, DEX (1 μM), GW (0.5 μM) or various combinations for 1 h, before TNF (2000 IU/ml) was added, where indicated, for a total induction time of 1.5 h. Cell lysates were subjected to western blotting to detect GRα, PPARα or p65. Detection of β-actin served as a loading control. $n = 1$. **(B)** The bands visualized via Western blot analysis were subjected to band densitometric analysis using Image J. The amount of specific signal was corrected to the respective actin loading control.

Figure S8 | Co-activation of GRα and PPARα enhances the lipid metabolism gene *Angptl4*. Gene counts for *Angptl4* upon DEX (1 μM), GW (0.5 μM) and LPS (100ng/ml) treatment of primary hepatocytes, from the experiment described in **Figure 7**. $n = 1$. Bars represent mean+SEM. NI, non-induced.

Table S1 | List of qPCR primers.

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Glucocorticoids and Glucocorticoid-Induced-Leucine-Zipper (GILZ) in Psoriasis

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Psoriasis is a prevalent chronic inflammatory human disease initiated by impaired function of immune cells and epidermal keratinocytes, resulting in increased cytokine production and hyperproliferation, leading to skin lesions. Overproduction of Th1- and Th17-cytokines including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-23, IL-17, and IL-22, is a major driver of the disease. Glucocorticoids (GCs) represent the mainstay protocol for treating psoriasis as they modulate epidermal differentiation and are potent anti-inflammatory compounds. The development of safer GC-based therapies is a high priority due to potentially severe adverse effects associated with prolonged GC use. Specific efforts have focused on downstream anti-inflammatory effectors of GC-signaling such as GC-Induced-Leucine-Zipper (GILZ), which suppresses Th17 responses and antagonizes multiple pro-inflammatory signaling pathways involved in psoriasis, including AP-1, NF-κB, STAT3, and ROR-γt. Here we review evidence regarding defective GC signaling, GC receptor (GR) function, and GILZ in psoriasis. We discuss seemingly contradicting data on the loss- and gain-of-function of GILZ in the imiquimod-induced mouse model of psoriasis. We also present potential therapeutic strategies aimed to restore GC-related pathways.

Keywords: glucocorticoids (GCs), glucocorticoid-induced-leucine-zipper (GILZ/TSC22D3), skin inflammation, psoriasis, keratinocytes, immune cells, signaling

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INTRODUCTION

Endogenous glucocorticoids (GCs) regulate development, metabolism, and immune responses in mammals (1, 2). In healthy individuals, GCs are synthesized by the adrenal glands and released to circulation as the final step of a complex cascade governed by the central hypothalamic–pituitary–adrenal (HPA) axis, with key roles in basal, and stress-related homeostasis (2). In addition, GCs can be produced locally by multiple tissues including the nervous system, thymus, and epidermis (3, 4). Synthetic GC counterparts are widely used as the first and most effective treatment to combat acute and chronic inflammatory pathologies. Both endogenous and exogenous GCs exert their actions through binding to the GC receptor (GR/*NR3C1*), a protein of the superfamily of nuclear hormone receptors that act as ligand-regulated transcription factors (5).

A main mechanism of GR action involves binding to genomic regulatory sequences called GR response elements to induce or repress target gene expression. GR induces genes encoding for anti-inflammatory mediators such as GC-Induced-Leucine-Zipper (GILZ), Dual-Specificity protein Phosphatase 1 (DUSP1), Inhibitor of kappaB alpha (IκBα), and Zinc Finger Protein 36/TrisTetraProlin (ZFP36/TTP) (6). Also, GR represses pro-inflammatory genes induced by the

NF-κB and Mitogen Activated Protein Kinase (MAPK)/AP-1 pathways through protein-protein interactions that do not require GR binding to DNA. These two mechanisms classically referred to as transactivation and transrepression, respectively, coexist and are required for the optimal anti-inflammatory actions of GCs. Notwithstanding their effectivity, GC-based therapy is accompanied by side-effects of variable severity (the most extreme including metabolic syndrome, osteoporosis, and impairment of childhood growth), which may advise to discontinue treatment (7).

GILZ (encoded by the *TSC22D3* gene) was identified more than 20 years ago as anti-apoptotic in dexamethasone-treated thymocytes (8). Since then, GILZ expression has been reported in cell types of immune, and non-immune lineages. Multiple GILZ isoforms, resulting from alternative transcriptional initiation and splicing, have been identified with differential activities, and tissue specific expression patterns (9, 10). As of now, the majority of studies regarding therapeutic applications have been centered on the *GILZ1* isoform (referred to as *GILZ* hereafter). GILZ plays an anti-inflammatory role in macrophages, is crucial to regulate proliferation, survival, and differentiation in regulatory T (Treg) and dendritic cells; and contributes to regulation of phagocytosis in neutrophils and macrophages, thus putting an additional brake on chronic inflammation (11–14). GILZ is also expressed in airway epithelial cells (15), as well as in epidermal keratinocytes. In keratinocytes, GILZ is rapidly induced by GCs although its role in this cell type is not yet clarified (16–18).

GC immunosuppressive effects are exerted upon almost all immune cells including distinct effector lineages of T helper (Th) cells: Th1, Th2, Th17, or regulatory T (Tregs) (19). GCs inhibit Th1 development and induce differentiation of Th2 and Treg cells that limit immune response (20, 21). Th17 cells, producing interleukin 17 (IL-17) as their signature cytokine, are critical mediators of immune and inflammatory diseases including rheumatoid arthritis, asthma, and psoriasis (22). One key finding was the demonstration that GILZ increased Treg cell production by enhancing the transforming growth factor (TGF)-β/SMAD2 signaling pathway leading to induction of Foxp3, a lineage specific transcription factor responsible for development and function of these cells (21). GILZ has been shown to limit pro-inflammatory Th17 cell differentiation by binding to promoter regions and inhibiting expression of key cytokines, and classic Th17 transcription factors, like STAT3, and the master regulator of this cell lineage, retinoic acid-related orphan receptor (ROR)-γt (23).

Other anti-inflammatory GILZ actions are mediated through protein-protein interactions with NF-κB and AP-1 transcription factors precluding nuclear translocation, DNA binding, and regulation of gene expression (24, 25). Also, GILZ can bind to RAS/RAF, and thus suppress the MAPK pathway by inhibiting MAP2K/ERK1/2 phosphorylation (26).

In vitro studies in various cell types, including keratinocytes, showed GILZ downregulation upon treatment with pro-inflammatory mediators that activate toll-like receptors (TLRs) or cytokines such as tumor necrosis factor (TNF)-α, IL-1-β, or interferon (IFN)-γ (12, 15, 16, 27). In several chronic inflammatory diseases, GILZ expression inversely correlates with disease severity, suggesting that lower levels may aggravate these diseases and/or may be part of the pathogenesis [reviewed in (25, 28)] For instance, GILZ expression negatively correlates with disease severity in lupus patients, and murine models of this disease (29, 30). Moreover, *GILZ* mRNA was downregulated in white blood cells of sepsis patients (14), in activated macrophages of individuals with Crohn's disease (31), in patients with chronic rhinosinusitis where more pronounced decreases of *GILZ* associated with poor response to surgery (32), and in human psoriatic lesions (33, 34). However, in other instances, such as in the synovium of patients with active rheumatoid arthritis, GILZ levels were increased relative to healthy subjects; nevertheless, among patients being treated with therapeutic GCs, those able to induce GILZ showed improved disease activity (35). Overall these data underline that GILZ levels and activity are likely dependent on the disease type and tissue context.

MOUSE MODELS OF INFLAMMATION TO ASSESS GILZ FUNCTION

GILZ was initially postulated as an alternative to GC therapies that could mediate GC immune-suppressive actions and anti-inflammatory effects without producing GC-associated side effects (11, 12, 25, 36). GILZ-deficient mice were viable and featured alterations that included male infertility due to impaired spermatogenesis, and electrolyte alterations (37–41). The lack of GILZ neither altered the immune response in several diseases (including arthritis and LPS-induced sepsis) nor decreased the anti-inflammatory effects of GCs in these models (37, 39, 42). Given that global GILZ-deficient mice had increased levels of endogenous GCs and other anti-inflammatory mediators, it is feasible that these compensatory mechanisms account for the observed results *in vivo* (28, 39, 43). In turn, the use of cell-type specific GILZ KO mouse models, such as macrophage-specific GILZ KO, which did not exhibit differences in their serum corticosteroid levels, represent a more adequate setting to investigate the impact of ablating endogenous GILZ (44).

However, in other settings, downregulation of GILZ during inflammation led to enhanced pro-inflammatory responses (44). For instance, the administration of GILZ siRNA enhanced disease progression in a mouse model of rheumatoid arthritis (45) and conversely, injection of GILZ-adeno-associated virus into the joints inhibited disease development to a similar extent as GC treatment (39). GILZ knockdown also resulted in increased disease severity in a mouse model of colitis due to pronounced granulocytic infiltrates and enhanced inflammation (13). GILZ-deficient macrophages showed increased responsiveness toward LPS, with augmented expression of pro-inflammatory cytokines

Abbreviations: GC, glucocorticoid; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; GILZ, Glucocorticoid-Induced-Leucine-Zipper; MAPK, mitogen-activated protein kinase; TGF-β, transforming growth factor beta; TNF-α, Tumor necrosis factor alpha; IFN, interferon; ROR-γ, retinoic acid-related orphan receptor gamma; ZFP36/TTP, Zinc Finger Protein 36/TrisTetraProlin.

due to ERK activation, and reduced desensitization to LPS, i.e., endotoxin tolerance (28).

In most mouse models of disease, higher levels of GILZ were protective against inflammation although with a variable degree of efficacy. The increased expression of GILZ in the SPRET/Ei mouse strain was shown to be the cause of its resistance to LPS-induced endotoxemia (46). GILZ overexpression with a T cell lineage specific promoter induced an anti-inflammatory Th2-type response in naive CD4 T cells (47), and these mice were less susceptible to a spinal cord injury model (48). Moreover, the use of GILZ peptides suppressed inflammation in a mouse model of autoimmune encephalomyelitis (24). Similarly, mice with generalized overexpression of GILZ (GILZ-Tg) had better survival rates in the cecal ligation and puncture sepsis model relative to controls (14). However, in this model, the protective effects of GILZ were not due to a decrease in systemic inflammation but linked to increased bacterial clearance due to more efficient phagocytosis by $CD45^+$ peritoneal cells. Overall GILZ gain- or loss-of-function in mouse models of inflammation does not always result in opposite phenotypes. The cell-type specific mechanisms by which GILZ modulates tissue function both in normal homeostasis as well as in inflammatory settings need to be considered. The pleiotropic effects of GCs are mediated by numerous downstream targets in addition to GILZ; this biological redundancy likely accounts for the findings that GILZ deficiency does not always cause major inflammatory phenotypes.

PSORIASIS

The epidermis is composed of keratinocytes which terminally differentiate to form a permeability barrier essential for survival. The balance between keratinocyte proliferation and differentiation is tightly regulated, with alterations that affect barrier function leading to common inflammatory skin pathologies (49). One such disease, psoriasis, is a chronic relapsing inflammatory condition identified in 1–2% of the population, whose clinical presentation includes different symptoms and severity, age of onset, and location of skin lesions (50). Psoriatic patients typically develop reddish scaly plaques, and one-third of patients also have affected joints, which may lead to severe joint destruction (psoriatic arthritis). In addition, this disease shows high comorbidity with other inflammatory conditions such as metabolic and cardiovascular diseases (51).

Psoriasis pathophysiology is complex and includes both genetic and environmental risk factors. Dysregulation of Th1 and Th17 lineages leads to overproduction of various cytokines including IFN-γ, TNF-α, IL-23, IL-17, and IL-22 resulting in epidermal hyperproliferation and skin immune infiltrates (52). ROR-γt is induced during early Th17 differentiation and is a central driver of the later stages of this process (53). ROR-γt is present in IL-17-producing Th17 cells in a mouse model of psoriasis, indicating involvement in the disease, and is currently being investigated as a therapeutic target for drug design (54, 55). Both keratinocyte and lymphocytes can mediate psoriasis due to alterations in pro-inflammatory signaling pathways and transcription factors AP-1 [loss of function; (56–58)], as well as NF-κB, STAT3, and TGF-β [gain of function; (59, 60)].

Histopathological characterization of psoriatic lesions reveals epidermal thickening, abnormal epidermal differentiation, and increased epidermal protrusions (rete-ridges), along with intra-epithelial neutrophil infiltrates (Munro-like abscesses), and pronounced immune infiltrates consisting of T cells and dendritic cells (52). A widely used mouse model of psoriasis consists of topical applications of imiquimod, a TLR7 agonist, which induces the IL-23–Th17-cell axis and closely recapitulates the histopathological, and molecular characteristics of the human disease (57, 61, 62).

Therapeutic Actions of Glucocorticoids

The symptoms of psoriatic patients can be treated systemically, topically, or by ultraviolet (UV) phototherapy (63). Classic treatments include synthetic compounds (GCs, retinoids, vitamin D derivatives, methotrexate, and cyclosporine) while novel therapies use antibodies targeting major cytokines associated with the disease (TNF-α, IL-17, and IL-23). As psoriasis is a relapsing disease, most patients require long-term management, which represents an important limitation for many of these treatments due to poor tolerability and/or cumulative toxicity (methotrexate and cyclosporine), or increased risk of non-melanoma skin cancer (phototherapy or TNF inhibitors) (64). These issues—age, specific symptoms, extent of lesions, and previous records of diseases—need to be addressed in the clinical practice to design efficient and safe treatments. While TNF-α and IL-17 inhibitors avoid many adverse effects of classic drugs, there are also concerns as these therapies can increase the risk of systemic infections, and their long-term use may represent an economic burden (52, 63).

GCs still represent the mainstay protocol for treating psoriatic patients with mild disease severity, and are preferably administered topically to minimize adverse side effects, including skin atrophy, loss of skin barrier function, increased susceptibility to infections, and delayed wound healing (65). However, in the long term, even topical GCs can cause Cushing's syndrome, and adrenal insufficiency with serious consequences (66). In addition, psoriatic patients with initially good responses to GCs can experience flares due to insensitivity to topical steroids (67). Downstream anti-inflammatory GC effectors such as ZFP36 and GILZ are attractive therapeutic candidates (36, 42, 68). Indeed GILZ is ideal as it interferes with multiple levels of pro-inflammatory signaling, including pathways involved in psoriasis like AP-1, NF-κB, STAT3, and ROR-γt. However, given the tissue- and cell type-specific differences in GILZ action it is important to decipher the impact of therapeutic doses of GILZ not only on skin immune cells, but also on epidermal keratinocytes, and dermal fibroblasts.

Impaired Glucocorticoid-Signaling in Psoriasis

GCs limit skin inflammation by signaling through GR (69). Consistent with this, $GR^{-/-}$ mice featured dramatically impaired epidermal differentiation, with decreased expression of differentiation markers, common features in human psoriasis

(49, 70). Also, late embryos and newborn mice with epidermal-specific inactivation of GR featured phenotypic and molecular alterations similar to those observed in psoriasis, including enhanced expression of pro-inflammatory markers (71). However, these alterations resolved spontaneously by yet uncharacterized mechanisms, and adult GR epidermal KO mice showed only mild skin defects (71). These data indicate that besides being the target of a treatment for psoriasis, keratinocyte-specific loss of GR is involved in the etiopathogenesis of the disease.

In control adult mouse skin, treatment with imiquimod strongly downregulated *Nr3c1* as well as the closely related mineralocorticoid receptor (MR/*Nr3c2*), which also plays anti-inflammatory roles in this tissue and can be activated by GCs (69, 72–74). Accordingly, GR- or MR- epidermal KO adult mice displayed increased susceptibility to imiquimod-induced psoriasis, and the loss of both receptors had significantly higher impact on disease severity (72). In the absence of epidermal GR and/or MR, regulation of downstream targets, like *Gilz*, is affected. In cultured keratinocytes, *Gilz* was induced by GCs in a GR-dependent manner (16, 17), consistent with a GR-ChIP sequencing experiment that identified GR-binding sites downstream of the *Tsc22d3* gene (17). Importantly, full induction of *Gilz* in response to GCs requires the presence of both GR and MR and GC-induced binding of GR to the genomic binding site near *Tsc22d3* was diminished in the absence of MR (17, 73).

In agreement with mouse models, expression of GR, MR, and GILZ (33, 34, 75, 76) was downregulated in human psoriatic lesions (**Figure 1**). Also, it has been reported that GR nuclear translocation was reduced in psoriatic skin (77, 78). Importantly, *GILZ* expression negatively correlated with levels of pro-inflammatory cytokines IL-17A, IL-23, and IL-22; and STAT3 in psoriatic lesions (33). In mice and humans, the expression of other GC-target genes such as *ZFP36*, *FKBP51,* and *ZBTB16* was also decreased in psoriasis (34, 72), likely aggravating disease severity. The findings that ZFP36 destabilizes *GILZ* mRNA suggests a mechanism by which GILZ levels are fine-tuned following exposure to GCs or cytokines that regulate these genes (44).

Defective Cutaneous Glucocorticoid Signaling in Psoriasis

Healthy skin is able to synthesize and release GCs through its own local HPA axis analog (**Figure 1**); however, the pathological relevance of local GC production had not been addressed until recently (4, 75, 76, 79, 80). In line with the observation that GC-target genes are downregulated in psoriasis, metabolomics and transcriptomic profiling demonstrated that cortisol was amongst the most decreased compounds in psoriatic vs. non-lesional skin (76). It was also shown that *de novo* synthesis of GCs was strongly decreased in psoriatic skin lesions (**Figure 1**) due to reduced expression of steroidogenic enzymes including steroidogenic acute regulatory protein (StAR), 3β-Hydroxysteroid dehydrogenase (3bHSD1), and the cytochrome P450 proteins CYP11A1, and CYP17 (75). 11-beta hydroxysteroid dehydrogenases type 1 and 2 (HSD11B1/HSD11B2) are responsible for cortisol to cortisone interconversion (81). Their expression ratio and activity is important for modulating epidermal differentiation, and have been reported to be altered in lesional tissue [**Figure 1**; (75, 76)]. Consistent with this, treatment with TNF-α, IL-17A, and IL-22 cytokines suppressed *HSD11B1* and *HSD11B2* expression in human keratinocytes in a reconstituted skin model (76).

Importantly, psoriatic patients that received topical GCs treatments not only normalized epidermal differentiation and skin inflammation but also restored endogenous GC biosynthesis in this tissue (76). Strikingly, mice exposed to clinically relevant doses of UVB showed induction of the systemic steroidogenic pathway, including GC production, indicating communication between the skin, and central HPA axes (82). This could explain at least partially why UVB therapy is beneficial for psoriatic patients and indicates that systemic and local GC levels are vital for cutaneous homeostasis. Altogether, these findings support that defective GC signaling in the skin (by keratinocytes and likely other cell types) is involved in the etiopathogenesis of psoriasis as it interferes with epidermal differentiation, eliciting sustained inflammatory responses. In this scenario, restoration of normal GC signaling represents one major objective, underscoring the relevance of elucidating the specific role of GILZ in psoriasis.

GILZ AND MOUSE MODELS OF PSORIASIS

The role of GILZ in the imiquimod-model of psoriasis was evaluated using gain- and loss-of-function mouse models (**Figure 2**). In control mice, besides the cutaneous phenotype, topical imiquimod also induces systemic effects including increased circulating cytokines and splenomegaly (61). While detailed histological evaluation of GILZ$^{-/-}$ skin has not been published, GILZ$^{-/-}$ mice treated with imiquimod showed increased severity in disease parameters, including the macroscopic skin phenotype of scaling and swelling; pro-inflammatory cytokine production; splenomegaly, and draining lymph node cellularity (33). The higher susceptibility to imiquimod-induced inflammation in GILZ$^{-/-}$ mice was explained by the augment of Th17-inducing cytokines by dendritic cells (IL-1, IL-23, and IL-6), and increased proliferation of Th17 cells (33). However, it is important to note that untreated GILZ$^{-/-}$ mice have increases in IL-17A and IL-22 producing lymphocytes and that the contribution of these basal alterations to the disease elicited in the psoriasis model is unclear. Importantly, while addition of IL-6 to Th17-promoting cytokines IL-1β/23 increased T cell proliferation and expression of Th17 genes *in vitro*, exogenous delivery of GILZ restored regulation of Th17 cell proliferation (33). These data confirm that GILZ is key to restrict pathogenic Th17 responses, which may be relevant for psoriasis treatments (23, 33).

Given the role of GILZ in suppressing Th17 responses and its downregulation in psoriatic lesions (33, 34), it was expected that transgenic mice with generalized overexpression of GILZ

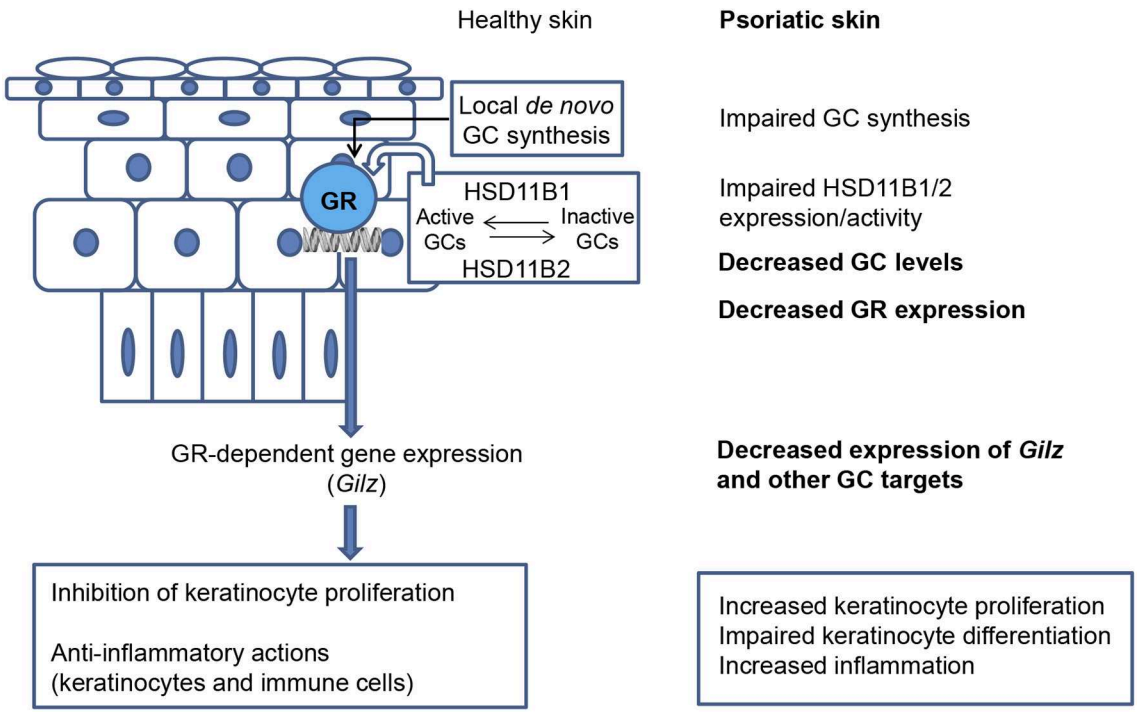


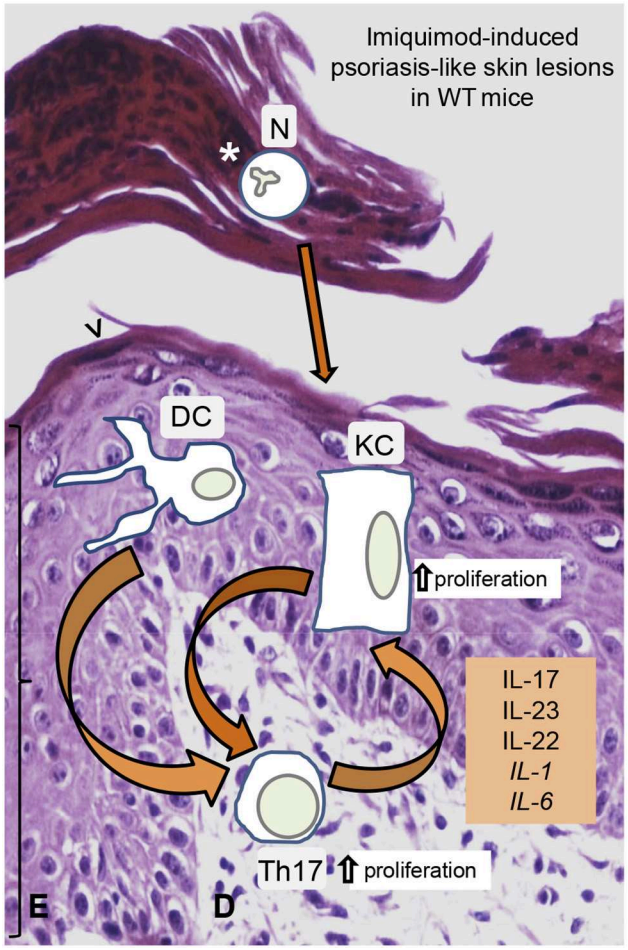
FIGURE 1 | Defective cutaneous GC signaling in psoriasis. Healthy skin is able to synthesize and release GCs *de novo* by a hypothalamic–pituitary–adrenal axis analog. The interconversion between inactive and active GCs by the enzymes 11-beta hydroxysteroid dehydrogenases type 1 and 2 (HSD11B1/HSD11B2) provides another source of corticosteroids. When local steroidogenesis is stimulated, GC-activated GR regulates gene expression, including that of *Gilz*. The actions of GCs in skin limit proliferation and inflammation. In psoriatic skin, *de novo* synthesis of GCs is strongly decreased and the expression/activity of HSD11B1/2 is impaired; decreased GC levels have an overall negative impact on epidermal differentiation. The downregulation of GR and downstream anti-inflammatory mediators in psoriatic lesions likely aggravates disease severity, including increased keratinocyte proliferation, impaired keratinocyte differentiation, and increased inflammation.

[GILZ-Tg mice (18)] would be protected from imiquimod-induced inflammation. Surprisingly, these animals showed a dramatic increase relative to controls in many disease parameters, including splenomegaly, and increased number and severity of skin lesions. GILZ-Tg mice showed increased scaling, abnormal keratinocyte differentiation, neutrophil infiltrates, and increased induction of molecules associated with the human disease (*Il-17*, *Il-22*, *Il-23*, *Il-6*, and *Stat*3). However, the systemic response to imiquimod was similar in GILZ-Tg and control mice (as was also the case in the cecal ligation, and puncture sepsis protocol in GILZ-Tg mice; (14), and there were not significant differences in the composition of skin neutrophil or T cell infiltrates of GILZ-Tg vs. controls (18).

Also, the pro-inflammatory actions of GILZ overexpression were specific to skin as neither intestine nor spleen showed increases in Th17-dependent cytokines relative to controls. The deleterious effects of GILZ in the psoriasis model were likely due to its overexpression in epidermis, rather than immune cells, as TGF-β1 signaling via SMAD2/3 was constitutively activated in GILZ-Tg keratinocytes. Moreover, GILZ overexpression in cultured keratinocytes enhanced the induction of the psoriatic marker *S100a*8 in response to IL-17A (18). Similar to human disease, imiquimod-treated control skin showed reduced *Gilz* expression (18). In contrast, as *Gilz* was not downregulated in GILZ-Tg skin, it is feasible that the resolution of inflammation requires reduced levels of GILZ, and that continuous expression, and/or relatively high levels of this GC-target gene can exert pro-inflammatory actions.

CONCLUSION

Despite their efficacy, topical administration of GCs to psoriatic patients is accompanied by adverse effects including loss of skin barrier function and increased susceptibility to inflammation and infections. Also, later stages of inflammatory diseases are characterized by a vicious circle of decreased response to GCs, resulting in lower production of anti-inflammatory mediators like GILZ and further loss of control of inflammation. Given these limitations, there is need of improving GC-based therapies for psoriasis and the delivery of GILZ appears as an attractive possibility. There is an inverse correlation between GILZ expression and psoriatic lesions; however, it is unclear whether lower levels aggravate the disease or are part of the pathogenesis. Also, the findings in mice that both gain- and loss of function of GILZ result in higher susceptibility to imiquimod-induced psoriasis raise questions about the therapeutic potential of exogenous GILZ for this skin pathology.



GILZ$^{-/-}$ mice

Untreated
- Increased IL-17A, IL-22 in immune cells

Imiquimod-treated
- Increased severity of psoriatic skin lesions
- Splenomegaly and increased draining lymph node cellularity
- Increased IL-1, IL-23, IL-6 in dendritic cells
- Increased proliferation of Th17 cells

GILZ-Tg mice

Untreated
- Normal cytokine profiles in immune cells
- Constitutive SMAD2/3 activation in keratinocytes

Imiquimod-treated
- Increased severity of psoriatic skin lesions
  - Abnormal keratinocyte differentiation
  - Neutrophil infiltrates in skin
- Splenomegaly
- Increased *Il-17a*, *Il-23*, *Il-22*, *Stat3*, *S100a8* in skin
- SMAD2/3 overactivation in keratinocytes

FIGURE 2 | Impact of GILZ in skin psoriatic lesions: Phenotypes of GILZ$^{-/-}$ and GILZ-overexpressing mice. **(Left)** Cell type-specific contributions to cutaneous alterations in psoriatic lesions induced by the imiquimod mouse model in WT mice. Epidermal thickening (bracket), abnormal differentiation of keratinocytes (arrowhead), and intra-epidermal neutrophil infiltrates (asterisk) are indicated. Dysregulation of both immune cells and keratinocytes leads to cytokine overproduction, resulting in immune infiltrates, epidermal hyperproliferation, and abnormal epidermal differentiation. Arrows represent communication between cell types. **(Right)** Summary of phenotypes in GILZ$^{-/-}$ (34) and GILZ-overexpressing (GILZ-Tg; 18) mice. Briefly, while untreated GILZ$^{-/-}$ mice had increased IL-17A and IL-22 in immune cells, both GILZ$^{-/-}$, and GILZ-Tg treated mice showed increased severity of imiquimod-induced psoriatic lesions. GILZ-Tg keratinocytes had constitutively increased phosphorylation of SMAD2/3, which was further increased by imiquimod. E, epidermis; D, dermis; N, neutrophils; DC, dendritic cells; KC, keratinocytes.

Apparent discrepancies may derive from yet uncharacterized cell-type specific functions of GILZ such as recently reported effects on neutrophil and macrophage phagocytosis modulating bactericidal activity. Also, as an exon common to all isoforms of *Tsc22d3* was deleted in GILZ$^{-/-}$ mice, it is plausible that other GILZ isoforms play differential roles. Above threshold effects from overexpression in GILZ-Tg mice may also explain these seemingly controversial results. Until the physiological role of GILZ in all skin compartments is better understood, therapies based on generalized delivery of GILZ seem premature. Based on the relevance of cutaneous GC-signaling, one may speculate on future strategies of local delivery of GILZ specifically to immune cells. It is also feasible that in psoriasis, the ability to produce GILZ in response to GCs could be used to stratify patients into two groups: those who upregulate this GC target would be good candidates for GC therapy and those who do not could be candidates for GILZ delivery to bypass the resistance.

AUTHOR CONTRIBUTIONS

LS and PP wrote the manuscript and approved this version for publication.

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Steroids, Pregnancy and Fetal Development

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Maternal glucocorticoids critically rise during pregnancy reaching up to a 20-fold increase of mid-pregnancy concentrations. Concurrently, another steroid hormone, progesterone, increases. Progesterone, which shows structural similarities to glucocorticoids, can bind the intracellular glucocorticoid receptor, although with lower affinity. Progesterone is essential for the establishment and continuation of pregnancy and it is generally acknowledged to promote maternal immune tolerance to fetal alloantigens through a wealth of immunomodulatory mechanisms. Despite the potent immunomodulatory capacity of glucocorticoids, little is known about their role during pregnancy. Here we aim to compare general aspects of glucocorticoids and progesterone during pregnancy, including shared common steroidogenic pathways, plasma transporters, regulatory pathways, expression of receptors, and mechanisms of action in immune cells. It was recently acknowledged that progesterone receptors are not ubiquitously expressed on immune cells and that pivotal features of progesterone induced- maternal immune adaptations to pregnancy are mediated via the glucocorticoid receptor, including e.g., T regulatory cells expansion. We hypothesize that a tight equilibrium between progesterone and glucocorticoids is critically required and recapitulate evidence supporting that their disequilibrium underlie pregnancy complications. Such a disequilibrium can occur, e.g., after maternal stress perception, which triggers the release of glucocorticoids and impair progesterone secretion, resulting in intrauterine inflammation. These endocrine misbalance might be interconnected, as increase in glucocorticoid synthesis, e.g., upon stress, may occur in detriment of progesterone steroidogenesis, by depleting the common precursor pregnenolone. Abundant literature supports that progesterone deficiency underlies pregnancy complications in which immune tolerance is challenged. In these settings, it is largely yet undefined if and how glucocorticoids are affected. However, although progesterone immunomodulation during pregnancy appear to be chiefly mediated glucocorticoid receptors, excess glucocorticoids cannot compensate by progesterone deficiency, indicating that additional und still undercover mechanisms are at play.

Keywords: glucocorticoids, progesterone, hormone receptors, pregnancy pathophysiology, fetal programming

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INTRODUCTION

In order to support mammalian pregnancies, a myriad of adjustments in maternal physiology takes place. For example, maternal immune responses are tightly regulated to prevent inflammatory responses and rejection of alloantigens expressed on fetal tissues (1, 2). The maternal immune adaptations to pregnancy are pivotally modulated by endocrine signals. These signals include the pronounced rise of sex hormones such as progesterone and estradiol. Progesterone is essential for the establishment and continuation of pregnancy (3). Progesterone not only plays multiple immunomodulatory functions (4), but also it supports uterine receptivity and quiescence (3, 5). Additionally to sex steroids, maternal glucocorticoids dramatically increase over the course of pregnancy in order to meet the increasing energy demands (6). Glucocorticoids are potent activators of GR, and this activation has pleiotropic effects on immune cells (7, 8). However, the molecular mechanisms underlying how glucocorticoids contribute to the maternal immune adaptation to pregnancy and the interplay between glucocorticoids and sex hormones such as progesterone remain largely unclear.

Intriguingly, although progesterone is generally acknowledged to promote maternal immune tolerance to alloantigens derived from the conceptus, progesterone receptors are not ubiquitously expressed on immune cells (9). Light was shed into this enigma only very recently, when it was identified that pivotal features of progesterone induced- maternal immune adaptations to pregnancy are mediated via the glucocorticoid receptor (9, 10). Hence, in the present review manuscript, we aim to revisit the current evidence about the synthesis and interplay between glucocorticoids and progesterone during pregnancy, their impact on the immune system and consequences for pregnancy maintenance and fetal development.

PROGESTERONE AND GLUCOCORTICOID SYNTHESIS, REGULATION AND RECEPTORS DURING PREGNANCY

Progesterone and Glucocorticoid Receptors in Immune Cells

Both, progesterone and glucocorticoids, are significantly involved in the regulation of immune responses (4, 7, 11). The structural similarities between glucocorticoids and progesterone raise the intriguing concept of mutual, interrelated as well as individual pathways elicited by these hormones. This concept gains relevance in the context of pregnancy, where disequilibrium between these steroids is related to altered maternal immune responses and pathological pregnancy outcomes (2, 7).

The genomic effects of progesterone and glucocorticoids are mediated by the intracellular progesterone and glucocorticoid receptors (PR and GR), which belong to a subfamily of the nuclear receptor superfamily (4, 7, 12). Upon binding to ligands, PR and GR translocate to the cell nuclei, where they interact with specific regions of the DNA to act as transcription factors that modulate gene expression (7, 11, 12). Despite the high amino-acid identity between PR and GR (12), their steroid binding affinities, expression patterns, and target genes differ remarkably, as summarized in **Table 1**.

The Nr3c1 gene encoding for GR is expressed in most tissues of the organism, and virtually in all cells of the immune system (31, 32). Glucocorticoids can bind the GR with high affinity to elicit genomic but also non-genomic pathways in immune cells (7, 33). Importantly, promiscuous binding of progesterone to GR has also been observed in a number of settings, particularly in *in vitro* models (9, 14). Due to alternative splicing and alternative translation initiation sites, many isoforms of the GR have been described (7, 13). These isoforms are also present in immune cells and associated with diverse translational activities or binding to glucocorticoids (7, 34). However, it remains unknown whether GR isoforms are affected during pregnancy or if they have differential affinity for progesterone. Indeed, as detailed in **Table 1** most progestogens have only very limited affinity to glucocorticoid receptor compared to glucocorticoids (14–16, 34).

The Nr3c3 gene encodes for two PR isoforms, PRA and PRB (35). Both PR isoforms have differential transcriptional activity and are predominantly found in mammary gland and in the female reproductive tissues, such as the ovary and uterus (23, 35). Overall, the presence of PR in immune cells is a matter of controversy. Although a direct effect of progesterone on e.g., T cells during pregnancy has long been proposed (36–39), recent findings based on RT-qPCR approaches aiming to detect PR on distinct immune cell subsets failed to confirm the expression of PR in e.g., T and NK cells (9, 20, 40, 41). Promiscuous binding of PR by glucocorticoids has been reported, although there is no consensus on the reported relative binding affinities compared to progesterone (14, 15).

Besides the PR, progesterone can elicit non-genomic actions by binding to G-protein coupled membrane progestin receptors (membrane progesterone receptors: mPR) and the so-called progesterone receptor membrane components (PGRMC) [reviewed in (4)]. Among them, mPRalpha/PAQR7 and mPRbeta/PAQR8 as well as PGRCM1 and 2 are present in T cells (20, 29) and mPRalpha is expressed in particular fractions of circulating Tregs (42). Hence, these pathways may explain some of the effects of progesterone on immune cells. Of note, information on glucocorticoid binding to mPRs is ambiguous [(18), **Table 1**), whilst glucocorticoid binding to PGRMCs has been described, albeit with low affinity (19).

Taken together the close structural similarities and the cell-restricted expression of receptors, progesterone and glucocorticoids may act on immune cells via non-genomic pathways as well as by likely binding to GR rather than to PR. Due to their high levels during pregnancy, it seems plausible that both progesterone and glucocorticoids act on GR to trigger immunoregulatory signals. This will depend on the bioavailability of the steroids, which varies across pregnancy according to their synthesis, the amount of carrier proteins limiting the free steroids reaching the tissues as well as from the metabolism or exclusion of these steroids from the target cells.

TABLE 1 | Comparison between general features of the progesterone and glucocorticoid receptors.

	Progesterone receptor	Glucocorticoid receptor	Membrane progestin receptors (mPR)	Progesterone receptor membrane components (PGRMC)
Genes	NR3C3	NR3C1	PAQR 5-9 (progestin and adipoQ receptor)	PGRMC1 and PGRMC2
Isoforms/subtypes	PRA and PRB isoforms	Multiple isoforms, including variants of GRα, GRβ, GRγ, GRA, GRB and GRP (13)	mPRα (PAQR7), mPRβ (PAQR8), mPRγ (PAQR5), mPRδ (PAQR6) and mPRε (PAQR9)	PGRMC1 and PGRMC2
Relative binding affinity*	Progesterone: 100% (14–16) other progestogens: 1–46% (16)	Progesterone and other progestogens: 1–6% (14) or 40% (15)	progesterone: 100% (17)	progesterone: 100%
	Corticosterone: 2.6% (16) Dexamethasone: 0.2% (15)	Corticosterone: 85% (16) Dexamethasone: 100% (16)	glucocorticoids: 0–26% (17, 18)	glucocorticoids: low affinity (19)
Expression in immune cells	Limited to specific cell lineages (9, 20, 21)	+++ (9, 20)	++ (20, 22) or undetermined	++ (22) or undetermined
Uterus	+++ (23)	++ (23, 24)	++ (22, 25)	+++ (26)
Genomic pathways	Dimers act as transcriptions factors by binding progesterone response elements	Gene transactivation or transrepression through DNA and/or transcription factor binding (27)	–	–
Non-genomic pathways	Monomers activate MAPK pathways through Src-kinase (28)	Binding to membrane receptors (27) and signaling through cytoplasmic ligand-bound GR and chaperone proteins (8)	Still controversial. Pathways may involve G-proteins and modulation of adenylyl cyclase activity (4, 18, 29)	Multiple intracellular signaling pathways, e.g., interacts with EGFR, ERK1, casein kinase 2, and PDK (30)

** Compared to the respective ligand with higher affinity.*

Bioavailability of Progesterone and Glucocorticoids During Pregnancy

Steroid synthesis such as in the case of progesterone and glucocorticoids consists of the conversion of cholesterol as a substrate through a series of enzymatic reactions, to produce structurally interrelated products. This process is tightly regulated by the tissue- and cell-specific expression of steroidogenic enzymes (43).

For example after ovulation the ovarian follicular cells that support the maturation of the oocyte undergo the so-called luteinization process to form the corpus luteum. During luteinization, the expression of genes and proteins that mediate progesterone synthesis is prominently upregulated (44). In mice and other mammals, the corpus luteum largely accounts for the significant *de novo* synthesis of progesterone during the entire duration of pregnancy. Here, progesterone concentration in the blood increases until mid-late pregnancy, when it gradually starts decreasing (45). This progesterone deficiency is considered as an upstream event triggering parturition in mice (46). In humans, the placenta expresses the enzymes involved in progesterone production and commences steroidogenic synthesis at gestation weeks 7–9, following the initial ovarian progesterone synthesis (47). Progesterone levels continuously rise until reaching a plateau in the last weeks of pregnancy (48). A progesterone decline at late gestation does not occur in humans and it has been suggested that parturition results from a functional progesterone deficiency occurring at myometrial and other uterine tissues (4, 49). Here, differential expression of progesterone receptor isoforms may allow for progesterone-induced cervical relaxation during parturition (49), hereby promoting the delivery of the human fetus (50, 51).

It is well-known that glucocorticoids are largely produced in the adrenal cortex, where they exhibit circadian and ultradian rhythms (4). Maternal glucocorticoids rise dramatically during pregnancy, e.g., during late murine pregnancy, glucocorticoids reach an ∼20-fold increase compared to mid-pregnancy concentrations (6). In humans, cortisol, the main glucocorticoid, also increases dramatically during pregnancy, reaching ∼350 ng/ml serum on week of gestation 26 (52). Thereafter, cortisol remains relatively stable until parturition, when it is strongly upregulated (52). In women, corticotrophin releasing hormone (CRH) is produced by the placenta to further stimulate adrenal glucocorticoid production (53) pinpointing the critical relevance of glucocorticoid synthesis during pregnancy.

The actions of these high levels of progesterone and glucocorticoids are limited by their binding to plasmatic carrier proteins (54). Only the "free" fractions of progesterone and glucocorticoids are considered to be able to bind receptors to exert biological functions, e.g., after diffusing inside the target cells (54). Corticosteroid-binding globulin (CBG) transports around 75–80% of plasma glucocorticoids, thereby critically limiting the abundance of free glucocorticoids available to cells (55). Despite a pronounced increase of CBG levels and binding capacity throughout pregnancy (6), 5–6% of the total cortisol remains free (56). Hence, the absolute concentration of free glucocorticoids increases during pregnancy (56). In contrast,

both the fraction of free progesterone and its total concentration increase throughout pregnancy (57). Progesterone only partially binds CBG with four times lower affinity as glucocorticoids. Instead, approximately the 80% of plasma progesterone primarily binds albumin (54).

The availability of steroid hormones can be additionally reduced by their intracellular metabolism. However, physiological expression of 11β-hydroxysteroid dehydrogenase type 2 capable of metabolizing glucocorticoids into inactive forms (6) is largely negligible in human or mouse immune cells (58) and potential modulation during pregnancy remains to date unexplored. Moreover, the progesterone-metabolizing enzyme 20α-hydroxysteroid dehydrogenase (Akr1c18) was shown to be highly expressed in thymocytes and initially considered as a marker for mature T cells (59, 60). However, data available to date seem ambiguous, as Akr1c18 is not listed when searching gene-expression database for immune cells (32). Hence, the significance or role of the expression of 20α-HSD or 11β-HSD in lymphocytes and possibly also myeloid cells is still unknown.

Moreover, Abcb1a and Abcc1 efflux transporters, members of the ATP binding cassette (ABC) transmembrane transporters family can actively exclude intracellular glucocorticoids hereby limiting their activity e.g., in mouse placenta (6). Abcb1a and Abcc1 (also known as Mdr1 and Mrp1) are differentially expressed in immune cells such as T lymphocytes (61) and Abcb1a deficiency was associated to decreased generation of Tregs *in vivo* and *in vitro* mouse models (62). Remarkably, progesterone and other progestogens are potent inhibitors of Abcb1a function (63), mechanism that may act synergistically with the high levels of glucocorticoids to further promote glucocorticoids actions during pregnancy.

Taken together existing published data on progesterone and glucocorticoids levels as well as their binding to plasma proteins during human pregnancy, it becomes evident that early pregnancy consists in a period of high progesterone and low glucocorticoid availability. In contrast, both free progesterone and glucocorticoids increase throughout pregnancy and are found at comparable concentration ranges in late pregnancy (48, 57). Hence, while a large body of evidence supports that steroid driven immune modulation relies mainly on progesterone at the beginning of pregnancy it is tempting to hypothesize that in later stages, glucocorticoids with high affinity for GR gain relevance in sustaining maternal immune tolerance. In this context, the regulation of progesterone and glucocorticoids bioavailability by expression of specific metabolizing enzymes and exclusion transporters in immune cells during pregnancy remains still unknown.

Modulation of Steroids by External Factors

The availability of steroid hormones during pregnancy, but also unrelated to reproduction, can be dramatically modulated by external factors. One key example is the exposure to stress, commonly described as a high perception of stress. It is well-established that stressful stimuli trigger the activation of the hypothalamic–pituitary–adrenal (HPA) axis, which results in secretion of glucocorticoids by the adrenal glands (**Figure 1**). Although this neuroendocrine response is gradually attenuated across pregnancy (53), stressful stimuli can still elicit the secretion of glucocorticoids in mouse and humans (6, 64). Concomitantly, stress challenges reduce progesterone levels during pregnancy in mammals (65–68). This could result from impaired steroidogenesis in the ovary, e.g., due to poor stimulation by placental lactogens (68). Stress-induced glucocorticoids may directly influence progesterone synthesis, as GR is also expressed in the ovary, where depending on the experimental conditions they have been shown to stimulate or inhibit steroidogenesis (69, 70).

Moreover, progesterone and glucocorticoids share common steroidogenic pathways and precursors, such as *cholesterol-derived pregnenolone* (**Figure 1**). Hence, a hypothesis for the depletion of progesterone as a result of glucocorticoid production in response to high stress perception has been proposed (71). This hypothesis is referred to as "pregnenolone steal" (71) and supports that the elevated synthesis of cortisol caused by stress depletes ("steals") the availability of pregnenolone for the

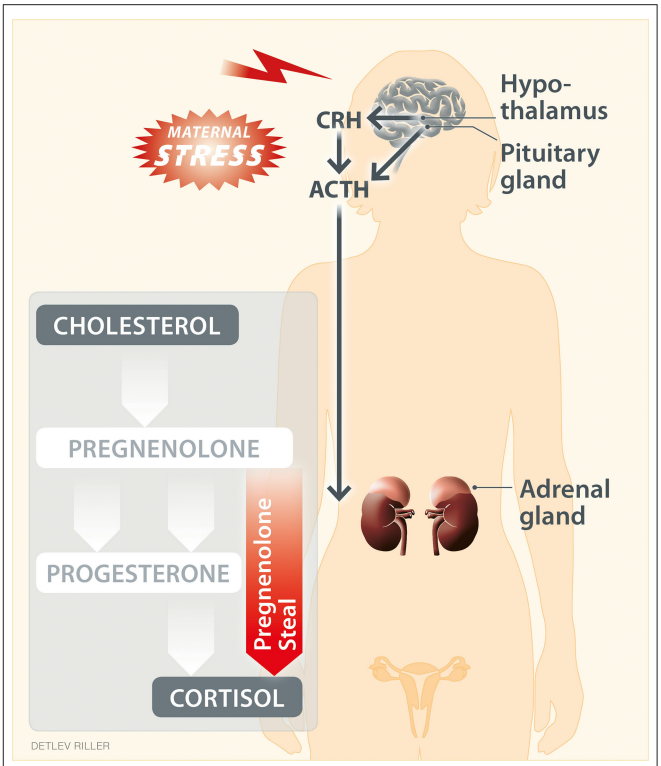

FIGURE 1 | "Pregnenolone steal" or how high stress perception may drive the depletion of progesterone. High stress perception activates the hypothalamic–pituitary–adrenal axis, resulting in the respective secretion of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol, the main glucocorticoid in humans. Moreover, stress can affect steroidogenesis in peripheral tissues. Steroidogenesis refers to the transformation of cholesterol into steroid hormones through a serious of steps. Here, the intermediate pregnenolone is a precursor of most steroid hormones, including progesterone and cortisol. Upon stress, the elevated synthesis of cortisol may reduce ("steal") the pregnenolone available for the synthesis of down-stream hormones other than cortisol. This hypothetical scenario provides an explanation for the impaired progesterone production in response to stress.

synthesis of down-stream hormones other than cortisol, which subsequently may also impede the synthesis of progesterone. This hypothesis requires confirmation also in the context of pregnancy. The conversion of cholesterol to cortisol occurs in the mitochondria of steroidogenic tissues, best described for the adrenal cortex, but also for various other tissues, including primary lymphoid organs, intestine, skin and brain (72, 73). If cortisol synthesis could also be induced e.g., by stress in tissues such as the ovaries and placenta, it could theoretically result in a reduction of the precursors available to produce progesterone during pregnancy. Interestingly, in ovary, the main site of progesterone synthesis in early human pregnancy, the specific enzymatic machinery for glucocorticoid production has already been described (74), and it remains to be confirmed whether the pregnenolone steal may indeed impede ovarian progesterone synthesis in response to stress.

IMMUNE PATHWAYS MEDIATED BY PROGESTERONE AND GLUCOCORTICOIDS

Antigenic disparity between the mother and the fetus is not only tolerated by the maternal immune system, but also promotes placental and fetal growth in mice (75). Understanding the mechanisms through which maternal immune tolerance toward fetal antigens is maintained is not only critical to decipher how survival of species is ensured. Such insights also allow shedding light on the pathogenesis of pregnancy complications. The collapse of maternal immune tolerance can become evident as cytotoxic responses at the feto-maternal interface and subsequent fetal loss (21, 76, 77) or impaired placental and fetal development (68, 78).

To date, a wealth of data highlights that high levels of progesterone are critically required to switch the maternal immune responses toward tolerance [e.g., discussed at length in (4)]. Progesterone promotes a tolerogenic profile on innate immune cell subsets, such as macrophages and dendritic cells, which is essential for successful uterine tissue remodeling and pregnancy maintenance (1–3). For example, *in vitro* stimulation with progestogens induces maturation of macrophages with M2 profile (79), and prevents the differentiation of dendritic cells toward a mature phenotype (80). A progesterone-mediated modulation of the adaptive immune responses has also been investigated in *in vivo* and *in vitro* models. Here, progesterone supports the expansion and suppressive function of Tregs during pregnancy, the skew toward an anti-inflammatory cytokine profile and suppression of $CD8^+$ T cell cytotoxicity (20, 68, 81–83).

Despite the availability of PR and GR specific pharmacological agonists and antagonists (**Table 1**), experimental interventions during pregnancy employed most often progesterone as agonist or the antagonist RU486, both of which can bind PR and GR. Hence, these approaches do not allow differentiation between the individual effects of progesterone or glucocorticoids on distinct immune cell subsets, which greatly limits to understand the individual role of hormones or cell subsets in maintaining pregnancy. Such limitation can now be easily overcome by the use of mice with targeted deletion of certain hormone receptors on distinct immune cell subsets. In fact, recent evidence revealed that the targeted deletion of PR on dendritic cells in mice promotes a non-tolerogenic, mature phenotype of dendritic cells, along with the failure to generate $CD4^+$ Treg and $CD8^+CD122^+$ Treg cells and impaired placental and fetal development (78). Also targeted gene deletion of the GR on T cells in mice pinpoints that GR and not PR is an upstream promotor of Treg expansion during pregnancy. *In vitro* approaches further support that GR mediates the expansion of T regulatory cells by selective induction of apoptosis in conventional T cells (9, 10). These mechanisms are at play during pregnancy, as in a mouse model of experimental autoimmune encephalomyelitis, GR deletion in T cells prevented pregnancy-induced expansion of T regulatory cells, as well the corresponding mitigation of autoimmunity (9).

In this context, functional analyses of the contribution of progesterone signaling through mPRs and PGRMC to immune regulation during pregnancy remain still largely elusive. To date, accumulating *in vitro* evidence highlights the importance of these non-genomic pathways e.g., on T cell responses (20, 29, 84).

Besides the direct hormone-steroid receptor interaction, progesterone can indirectly affect immune responses. Uterine and placental expression of the PR promotes the local expression of immunomodulatory molecules, such as progesterone-induced blocking factor (PIBF), galectin-1 (Gal-1) (41, 83), and heme oxygenase 1 (Hmox1) (68). These potent immunomodulators are critical for the establishment and continuation of pregnancy, as shown in mouse models and human pregnancies (41, 68, 83, 85, 86). For example, PIBF can enhance the synthesis of Th2 cytokines and dampens NK cell cytotoxicity (41) whereas Gal-1 induces a tolerogenic phenotype in dendritic cells, which results in Treg expansion (81). In turn, the enzyme Hmox1 supports the generation of $CD8^+CD122^+$ regulatory T cells that during pregnancy promote placental vascularization and fetal growth (68). Pathways involved in progesterone-mediated promotion of pregnancy maintenance may also include the epigenetic silencing of key T cell-attracting inflammatory chemokine genes in decidual stromal cells, as observed in mice upon progesterone stimulation (87). This epigenetic silencing of chemokine genes can subsequently suppress the accumulation of anti-fetal effector T cells in the decidua, hereby reducing the risk for fetal loss.

Some of progesterone-induced pathways in the uterus could also be mediated by GR. In fact, although glucocorticoids seem to be dispensable during early pregnancy (88) uterine GR expression is critical to ensure successful pregnancy. Evidence arising from transgenic mice shows that a targeted deletion of GR in the uterus results in subfertility, excessive inflammation and altered immune cell recruitment during decidualization (23).

In the light of these recent observations, an upstream role of GR in pregnancy induced immune tolerance is underscored, while new questions on the roles of progesterone and glucocorticoid non-genomic pathways appear. These concepts challenge previous notions on processes taking place during pregnancy and invite not only to revisit former data but also to advance in the research of these endocrine-immune mechanisms from this novel perspective. Of note, a number of technical

TABLE 2 | Salient technical tools available to discriminate steroid receptor-specific pathways.

	Progesterone receptor	Glucocorticoid receptor	Membrane progestin receptors (mPR)	Progesterone receptor membrane components (PGRMC)
Selective agonists	20α-dihydrodydrogesterone (DHD) (89)	Dexamethasone, betamethasone (15), ZK209614 (90)	progesterone conjugated to BSA (50)	
Antagonist	non-selective: RU-486		–	–
	selective: ZK98299 (91), Ulipristal acetate (92), Org31710 (93)	selective: RU-43044 (94)		
Mouse models for cell specific depletion	$Pr^{fl/fl}$ (21)	$Gr^{fl/fl}$ (9, 10)	–	$Pgrmc1^{fl/fl}$ and $Pgrmc2^{fl/fl}$ (30)

tools to discriminate the receptor-specific pathways are to date available (**Table 2**) and promise exciting progress in the research in the field.

IMPACT OF PROGESTERONE AND GLUCOCORTICOIDS ON PREGNANCY OUTCOME AND MATERNAL IMMUNE RESPONSE

Given the shared steroidogenic pathways and transport of progesterone and glucocorticoids as well as their widespread crosstalk in immune cells and reproductive tissues, it is tempting to speculate that a tight equilibrium between these steroids underlies healthy pregnancy and fetal development (**Figure 2**). As discussed below, this equilibrium can be disrupted with consequences for the establishment or continuation of pregnancy or affecting the developing offspring (**Figure 2**). Hence, progesterone and glucocorticoids appear as attractive pharmacological treatments, e.g., that could restore maternal immunotolerance, and they are often supplemented to women at risk for pregnancy complications.

Progesterone, Infertility, and Early Pregnancy Loss

Worldwide, around 10% of couples experience fertility problems, whereby male and female factors almost equally account for these incidences. Interestingly, the overall burden of female infertility has remained similar over the last 2 decades, despite the progress in assisted reproductive techniques (95). Besides infertility, early pregnancy loss clinically defined as spontaneous miscarriage before the week 20 of gestation occurs in 10–15% of healthy women (96). A large fraction of spontaneous miscarriages is due to unknown etiologies, in which immune maladaptations, e.g., in response to environmental factors (97), are suspected to play a critical role.

Progesterone insufficiencies and related inability to mount an appropriate immune response favoring embryo implantation has been frequently put forward to explain these incidences. However, to date, the high variability in progesterone secretion and the limitation to measure glucocorticoids in clinical routine hinder the diagnosis of progesterone deficiency or glucocorticoid imbalances during normally progressing pregnancies as well as pathologies such as infertility and spontaneous miscarriage (98, 99). Given the soaring levels of steroid hormones occurring during pregnancy, endocrine interventions have been frequently used in couples suffering from infertility or pregnancy losses. Infertile women orally treated with the progestogen Dydrogesterone, which shows a high affinity for the PR, had higher birth rates compared to treatment with vaginal micronized progesterone (100). However, the potential modulation of the maternal immune response by these treatments has not been tested.

Similar to the infertility trial described above, treatment with oral Dydrogesterone also reduced the risk in women with a history of recurrent pregnancy loss, whereas treatment with vaginal micronized progesterone failed to reduce the abortion risk (101). In this study, cytokine levels were tested and significantly differed between women with recurrent pregnancy loss who were assigned to the different treatment arms, which limits the analyses of treatment effects on immune responses. Comparably, progesterone withdrawal or blockage results in fetal loss in mammals (83, 102, 103) and the PR and GR antagonist RU486 is effectively employed to terminate human pregnancies (104, 105).

Insights into the mechanisms underlying the pregnancy protective effects induced by oral progestogens are highly desirable. Considering that vaginal administration of micronized progesterone did not improve implantation success in infertile patients and failed to reduce the abortion rate, it can be speculated that the oral route of application increase systemic progestogen levels to the degree required in order to initiate the pregnancy-protective effects on the maternal immune system.

Additional evidence for an upstream role of progesterone in ameliorating the risk for pregnancy pathologies arise from more recent studies on progestogens supplementation during early pregnancy (3, 106, 107). Reduced progesterone, e.g., due to luteal insufficiency or stress may influence maternal tolerance toward fetal antigens and result in fetal loss (108, 109). Despite the wealth of information on the interaction between progesterone and the immune response, very little insights into the causal relationship between altered hormones levels, collapse of the maternal immune tolerance and subsequent pregnancy loss are available to date, which should be addressed in future trials.

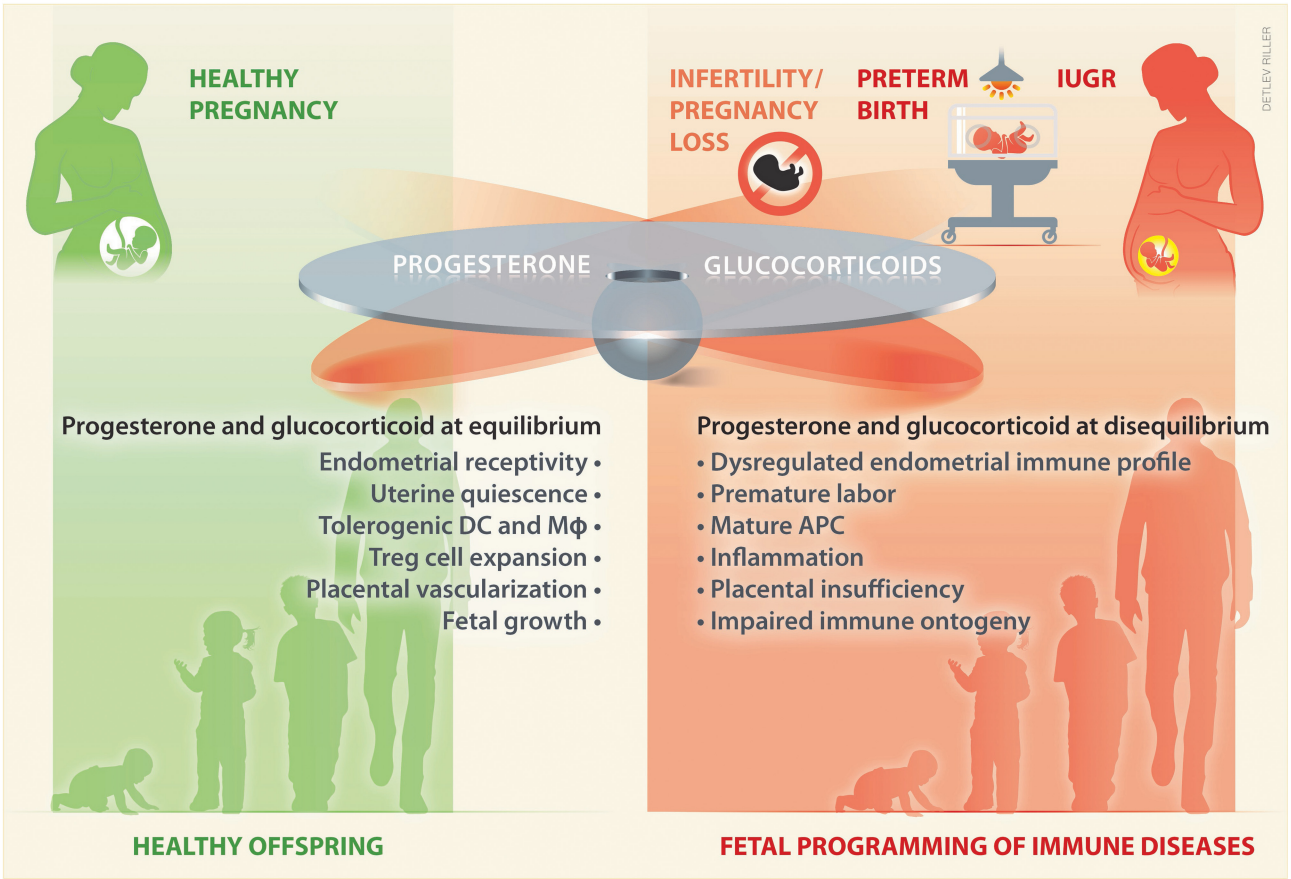

FIGURE 2 | During pregnancy a tight balance between glucocorticoids and progesterone may take place. An equilibrium between these hormones ensures adequate levels to sustain uterine receptivity and quiescence, as well as a tolerogenic immune profile, which pivotally promotes placental vascularization and a healthy fetal growth. In contrast, a disequilibrium in progesterone and/or glucocorticoids may fail to sustain pregnancy, and underlie an altered intrauterine immune profile, prone to inflammation, which leads to placental insufficiency and poor fetal growth. Such a disequilibrium may play an upstream role in women suffering from infertility or from pregnancy complications, such as early pregnancy loss, preterm birth, and IUGR. Impaired fetal growth and altered prenatal exposure to glucocorticoids influences the fetal immune ontogeny, which may result on fetal programming of immune disease in the offspring. DC, dendritic cells; Mϕ, macrophages; APC, antigen presenting cells; IUGR, intrauterine growth restriction.

Due to their potent immune regulatory capacity, glucocorticoids appear as a potential therapeutic option in women suffering from with repeated idiopathic embryo implantation failure. Corticoid therapy is becoming an important medication for patients with history of repeated implantation failures (RIF) after IVF/ICSI and at least a proportion of the patients respond to such intervention (110). Indeed, emerging data accumulated in small group of patients with increased numbers of NK cells in the endometrium suggests potential beneficial effects of corticosteroid therapy (111) as intrauterine perfusion of dexamethasone reduced NK cell frequencies and resulted in successful pregnancy (112). Of note, the safety of glucocorticoid administration during pregnancy has not yet been completely clarified (111, 113) and concerted efforts need to be devoted to identifying patients that can specifically benefit from corticosteroid therapies (114).

Preterm Labor

Rates of prematurity are currently on the rise, not only in developing countries or countries in transition to development, but also globally (115). Consecutively, preterm birth is the main reason for newborn death worldwide and a major contributing factor to poor offspring's health. Progress has been made to predict the risk for preterm birth, but its etiology is still enigmatic. In the context of preterm birth, the importance of the maternal immune system is increasingly recognized. Term labor is initiated by complex pathways, which include the up-regulation of inflammatory signals (116). Pilot data suggest that the collapse of maternal immune adaption and a premature activation of inflammatory pathways trigger labor prematurely (117). Here, it remains to be demonstrated whether the up-regulation of inflammatory signals follows a functional progesterone withdrawal. In fact, vaginal progesterone application has been demonstrated to decrease the risk of preterm birth and to

improve perinatal outcomes in singleton gestations with a short cervix in humans, suggesting that progesterone ensures uterine quiescence in cervical tissue (115). Very recently, it has been demonstrated that treatment with progesterone may be a strategy to prevent preterm labor/birth and adverse neonatal outcomes by attenuating the proinflammatory responses at the maternal-fetal interface and cervix induced by T cell activation (24).

Similar to PR, the myometrium expresses GR, although at lower levels (118), and some of anti-inflammatory progesterone actions in this tissue, e.g., COX-2 or IL-1β repression may be also mediated by GR (23, 93). At term labor glucocorticoids are potently triggered (52). However, there are no reports on beneficial effects of glucocorticoids on the maternal outcomes, e.g., on women that received antenatal steroid therapy for fetal lung maturation. Altogether, potential implications of maternal glucocorticoids on the modulation in preterm labor are not yet clearly established.

Intrauterine Growth Restriction

Intrauterine growth restriction (IUGR) refers to suboptimal fetal growth, a condition that affects 3–10% of pregnancies (119). IUGR may result from placental insufficiency, e.g., due to impaired uterine or placental vascularization. Progesterone can promote uterine and placental vascularization by diverse pathways. For example, progesterone upregulates the VEGF homolog placental growth factor (PlGF) (120), which is expressed by trophoblast and uterine NK cells (121, 122). PlGF promotes NK cytokinesis and consequently decidual spiral arteries remodeling during early pregnancy and labyrinth vascular branching in mid to late murine pregnancy (122). Indeed, it is well-accepted that uterine NK cells (122) promote pregnancy related uterine vascular changes through pathways including the secretion of cytokines such as IFN-γ and IL-17. IFN-γ affects uterine vasculature and stromal gene expression, which leads to vessel instability and facilitates remodeling of decidual arteries (123). Recently, it was also proposed that progesterone and estradiol trigger apoptosis in neutrophils, which transfer proteins to T cells. These "neutrophil-induced T" (niT) cells upregulate regulatory markers and promote vessel growth *in vitro* through IL-17 and VEGF expression (124).

Moreover, in a mouse model of mid-gestational stress we observed that reduced progesterone was associated to epigenetic changes in the placenta that resulted in decreased heme oxygenase-1 (Hmox-1) expression and IUGR. These changes were caused by an increase of cytotoxic $CD8^+$ T cells producing inflammatory cytokines. This inflammatory surge was unopposed by $CD8^+CD122^+$ T regulatory cells. Notably, supplementation of progestogens mitigated the IUGR by restoring Hmox-1 expression as well as suppressing inflammation (68).

Intriguingly, stress-induced intrauterine inflammation takes place in an environment rich in glucocorticoids (6, 68). Glucocorticoids can affect placental gene expression and growth (6, 125), with consequences in the nutrition and gas exchange with the fetus. These effects together with potential fetal excessive glucocorticoid exposure are hypothesized to underlie intrauterine growth restriction i.e., in the case of maternal dietary protein restriction, or stress [reviewed in (8)].

Together these observations provide evidence that the functions of progesterone and glucocorticoids are not exchangeable and that a regulated balance is required in the uterus to promote fetal growth.

Prenatal Exposure to Excess Glucocorticoids: Fetal Programming of Postnatal Immunity

During late gestation, glucocorticoids are required to ensure structural and functional organ maturation in the fetus (126, 127). However, prenatal exposure to glucocorticoid surges is detrimental for fetal growth and may hold significant consequences for postnatal physiology (8). Fetal glucocorticoid excess can be induced e.g., by antenatal steroid treatments in the case of risk for preterm birth (128). Additionally, antenatal glucocorticoid exposure is proposed to underlie a number of conditions, such as maternal malnutrition (129), stress (6), and infection (130). In mice, prenatal stress and the consequent fetal glucocorticoid excess resulted in intrauterine growth restriction (IUGR) particularly in female offspring (6). These observations could be explained by sex specific stress responses at the placenta, which limits the transfer of maternal glucocorticoids to the fetus. Indeed, placentas from female offspring failed to upregulate placental protective mechanisms, such as 11β-HSD2 and ABC transporters in response to antenatal stress, whereas these protective mechanisms prevented glucocorticoid excess in male fetuses (6).

Growing evidence underscores a role of prenatal glucocorticoid exposure in offspring's immune ontogeny and impaired postnatal immunity (131, 132). These effects could be multifactorial, including indirect and direct effects in the immune system (8). For instance, prenatal stress or glucocorticoid excess can result in disarrangements in the HPA [reviewed e.g., in (133)]. Generally, it is widely accepted that postnatal HPA hypoactivity follows prenatal stress exposure (134). Metabolic disarranges in offspring exposed to prenatal stress or glucocorticoids have also been observed and include the programming of a thrifty metabolic phenotype (135). Both postnatal HPA and metabolism may affect postnatal immune responses. Remarkably, premature exposure to glucocorticoids may also affect the developing fetal immune system [reviewed in (8)]. For example, antenatal steroid treatment resulted in newborns with impaired immunity (136) e.g., due to poor neutrophil (137) and T cell (138) responses.

FINAL REMARKS

Recent data emerging from mice carrying cell specific gene deletions underscore that pathways downstream the GR in immune cells are critically involved in promoting immune tolerance during pregnancy (9, 10). As until recently this tolerance was considered to be primary modulated by signaling through the intracellular PR, these novel observations invite to reexamine aspects of endocrine immune regulation during

pregnancy. In early pregnancy such GR-mediated pathways are likely elicited by high levels of progesterone. However, glucocorticoids with high affinity for GR outpace progesterone levels in mid-late stages of gestation. Simultaneously the maternal inflammatory load intensifies due to the cumulative exposure to antigens derived from the conceptus (139). Whether this glucocorticoid predominance translates into a chief immunomodulatory role remains unknown and requires empirical validation. Taken together the here summarized data, it is tempting to anticipate the proximity of a paradigm shift with regards to immune-endocrine responses during pregnancy e.g., related to signaling pathways or potential therapies to promote immune tolerance during pregnancy.

Of note, glucocorticoids and progesterone appear to be present in a tight equilibrium during pregnancy. Even subtle disruptions of this equilibrium may have significant consequences for pregnancy progression and fetal development (8, 68) (**Figure 2**). However, detailed information on their modulation and potential associations to inflammatory mechanisms taking place in the context of pathological pregnancies remain largely elusive. This is at least partly due to the fact that progesterone and glucocorticoids are not routinely assessed during pregnancy. Such assessments could refine the identification of women that can benefit from endocrine therapies to achieve or support pregnancy and fetal growth.

Finally, the tight crosstalk between pathways downstream progesterone and glucocorticoids could have therapeutic implications. In clinical praxis, glucocorticoids are broadly employed to reduce inflammation in pathological settings. Still, due to the side effects of their long-term use, a great body of research has attempted to find active compounds that could replace corticosteroids particularly as a chronic therapy. It could be hypothesized that progesterone could be such an alternative. For example, the mitigation of the course of multiple sclerosis in pregnant women, with an intensification of the disease activity in the postpartum period (140), suggests an upstream immunomodulatory role of pregnancy-induced hormones (9, 141). However, a recent clinical trial failed to demonstrate an effect of progestogens in preventing post-partum relapses in women suffering from multiple sclerosis (141) implying a limited efficacy of the treatment applied in this trial. Hence, despite its high clinical relevance, the empirical evidence to support the use of progestogens as a replacement for glucocorticoids remains to date sparse and requires still thorough investigation.

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Both authors have made a substantial intellectual contribution to the work, and approved it for publication.

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