Comparative Efficacy of Glucocorticoid Receptor Agonists on Th2 Cell Function and Attenuation by Progesterone

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BMC Immunology  BMC Series

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DOI:
10.21203/rs.2.14497/v1

SUBJECT AREAS
Immunology

KEYWORDS
asthma, Th2 cells, type 2 cytokine, IL-13, apoptosis, corticosteroid, sex hormone, progesterone
Abstract

Background: Corticosteroids (CS) suppress cytokine production and induce apoptosis of inflammatory cells. Prednisone and dexamethasone are oral CS prescribed for treating asthma exacerbations. While prednisone is more commonly prescribed, dexamethasone is long acting and a more potent glucocorticoid receptor (GR) agonist. It can be administered as a one or two dose regime, unlike the five to seven days required for prednisone, a feature that increases compliance. We compared the relative ability of these two oral CS to suppress type 2 inflammation. Since progesterone has affinity for the GR and women are more likely to relapse following an asthma exacerbation, we assessed its influence on CS action.

Results: Dexamethasone suppressed the level of IL-5 and IL-13 mRNA within Th2 cells with 10-fold less than prednisolone (the active form of prednisone). Dexamethasone induced a higher proportion of apoptotic and dying cells than prednisolone, at all concentrations examined. Addition of progesterone reduced their capacity to drive cell death, though dexamethasone maintained significantly more killing activity. Progesterone blunted dexamethasone-induction of FKBP5 mRNA, indicating the mechanism of action was by interference of the GC:GR complex.

Conclusions: Dexamethasone is both more potent and effective than prednisolone in suppressing type 2 cytokine levels and mediating apoptosis. Progesterone attenuated these anti-inflammatory effects, indicating its potential influence on corticosteroid responses in vivo. Collectively, our data suggest that when oral corticosteroid is required, dexamethasone may be better able to control type 2 inflammation, eliminate Th2 cells and ultimately lead to improved long-term outcomes. Further research in asthmatics is needed.

Background

Allergic asthma is typified by allergen-induced differentiation of T helper 2 (Th2) cells and their production of the type 2 cytokines IL-4, IL-5, and IL-13 [1]. Together these cytokines orchestrate much of the pathophysiology of asthma: IL-4 and IL-13 regulate B cell isotype switching to IgE [2] and upregulate endothelial expression of VCAM-1, mediating eosinophil infiltration [3]. IL-5 directs eosinophil differentiation and survival [4, 5] and IL-13 drives mucus production [6] and airway fibrosis.
More recently, group 2 innate lymphoid cells (ILC2) have also been shown to produce type 2 cytokines [10], though they are activated by TSLP, IL-33 or IL-25 released from the epithelium following exposure not only to allergens but also various microbes [11].

Corticosteroids (CS) are an effective treatment for most asthmatics [12] due in part to their ability to suppress type 2 cytokine expression, both in vitro and in vivo [13-17]. While corticosteroids also induce apoptosis of eosinophils [18] and peripheral blood mononuclear cells [19], within the T lymphocyte population there are differences in sensitivity to corticosteroids across the various subsets. For instance, Brinkmann et. al. showed that memory T cells were 100-fold less sensitive to steroid-mediated reduction in clonal expansion than naïve T cells [20]. While Banuelos et. al. observed that Th1 cells seem more sensitive to steroid-induced apoptosis than Th17 cells [21], the susceptibility of human Th2 cells has not been well studied. Since the persistence of symptoms in difficult-to-treat asthma is largely attributed to the presence and repeated activation of long-lived memory Th2 cells and subsequent inflammation [22, 23], therapies aimed at their elimination have the potential to mediate lasting effects.

In asthma, the need for systemic (e.g., oral, intravenous or intramuscular) corticosteroid agents arises when inhaled corticosteroids (ICS) fail to control symptoms or allergen/irritant exposure stimulates the inflammatory response and leads to an asthma exacerbation [24]. In severe asthma, patients can be dependent on oral CS agents with symptoms worsening upon withdrawal [12, 25]. Due to well-known and serious systemic side effects [26], however, long term use of oral CS is not advised and guidelines now recommend prioritizing anti-Th2 therapies for asthma control [12, 25]. Oral CS is recommended for acute conditions, such as exacerbations of asthma, and has been shown to significantly reduce the risk of hospitalization and relapse following discharge [27]. In adults, prednisone is the most commonly prescribed oral CS [28] though an alternative is dexamethasone, a more potent agonist for the glucocorticoid receptor (GR) [28, 29]. A number of studies have compared short burst oral prednisone and dexamethasone in treating exacerbations of asthma and shown they have similar safety profiles, especially in children [30-36]. Their relative ability to suppress type 2 inflammation, however, has not been closely examined.
Here, we performed a head-to-head comparison of the relative anti-inflammatory capacity of prednisolone (the active form of prednisone) and dexamethasone in vitro. Sex as a biological variable is an important consideration in asthma. Women are more likely than men to develop severe asthma [37] and to relapse after an asthma attack [38]. Fluctuation in female sex hormones during the menstrual cycle [39] and pregnancy [40] are associated with worsening of asthma symptoms [41]. Since progesterone has affinity for the GR [42], this hormone may influence how GCs influence type 2 immunity. As such, we also examined the ability of progesterone to interfere with the action of prednisolone and dexamethasone. Collectively, our study provides insight into the relative efficacy of these two CS therapies and suggests their effects may be dampened in women.

Results

Dexamethasone more potently reduces type 2 cytokine levels than prednisolone

To directly compare the ability of dexamethasone and prednisolone to suppress type 2 cytokine production, primary Th2 cells producing IL-5 and IL-13 [43] were treated (24 hours) with equimolar concentrations of either CS ($10^{-9}$–$10^{-7}$M) as in [15, 18]. Moreover, these concentrations are within the pharmacological range [44]. IL-13 mRNA was decreased following treatment with all concentrations of dexamethasone (Figure 1A). A 10-fold higher concentration of prednisolone was needed to significantly reduce IL-13, as the level of this cytokine was only lower in cells that had been cultured with concentrations of prednisolone $\geq 10^{-8}$ M (Figure 1A). The suppressive effect on IL-13 mRNA was significantly different between the two CS (Figure 1B). IL-5 mRNA level was also inhibited, though this cytokine required 10-fold more of either CS than IL-13 to exhibit a significant reduction (Figure 1C) and there was no difference between the concentration curves for either CS (Figure 1D). These results indicate that dexamethasone is more potent than prednisolone in suppressing the level of type 2 cytokines.

Dexamethasone has a higher maximal efficacy than prednisolone to induce apoptosis

To assess the efficacy of dexamethasone and prednisolone to induce apoptotic cell death we used a
CD4+ T lymphoblastoid cell line (CCRF-CEM). This line exhibits a Th2 phenotype, with enriched expression of the Th2 markers GATA3 and CRTh2 compared to Jurkat T cells (Figure Supplemental 1 (Figure S1)) and responds similarly to primary Th2 cells in terms of corticosteroid-induced apoptosis [45]. Flow cytometry was used to quantify Annexin V and 7-AAD staining following 48 hours of CS treatment. Early apoptotic cells were identified as those staining for Annexin V only, but not 7-AAD, and cells positive for 7-AAD alone were considered necrotic (Figure S2). Both prednisolone and dexamethasone induced necrosis (Figure 2A) and apoptosis (Figure 2B), though there were significantly more cells with each concentration of dexamethasone treatment. We assessed whether adding higher concentrations of prednisolone could overcome this difference, but found that even with 10-fold more prednisolone, dexamethasone still more effectively induced apoptosis (Figure 2C).

**Progesterone dampens corticosteroid-induced apoptosis**

Since progesterone has affinity for the GR [42], we investigated whether this sex hormone influences the efficacy of corticosteroid action. Th2 cells (CCRF-CEM) were incubated with dexamethasone for 48 hours in the presence or absence of progesterone and cell death and apoptosis assessed by flow cytometry. We found that progesterone, added with or 30 minutes prior, reduced dexamethasone-mediated cell death (Figure 3A) and apoptosis (Figure 3B). To assess the differential effect of progesterone on either corticosteroid, Th2 cells were cultured with prednisolone or dexamethasone in the presence or absence of progesterone. Head-to-head comparison revealed that while the proportion of prednisolone-induced (Figure 3C) and dexamethasone-induced apoptotic cells (Figure 3D) were similarly reduced by progesterone, due to its higher maximal efficacy dexamethasone maintained superior killing activity (Figure 3E).

**Progesterone inhibits GR signaling**

To investigate the mechanism by which progesterone reduced the corticosteroid effect, we examined expression of the nuclear progesterone receptor (PGR). We found that in the Th2 cell line (CCRF-CEM) PGR mRNA was undetectable by qRT-PCR, though present in a breast cancer cell line (MCF-7) (Figure 4A). We also found no induction of progesterone immunomodulatory binding factor 1 (PIBF1), a factor
known to be induced by progesterone [46, 47] (Figure 4B). These results suggested that the progesterone effect on CS-induced apoptosis was not through progesterone receptor signaling. Since progesterone has also been shown to have affinity for the GR [42], we next assessed whether it could influence the dexamethasone mediated induction of FKBP5 mRNA, a gene highly induced by CS [45, 48, 49]. Indeed, the level of FKBP5 mRNA following exposure to dexamethasone was significantly blunted when progesterone was added to the culture (Figure 4C), indicating this sex hormone interferes with GC:GR signaling.

Discussion
Prednisone and dexamethasone are both oral corticosteroid therapies recommended for treating patients with moderate to severe asthma exacerbations. While prednisone is more commonly used [28], studies in children and adults have shown that dexamethasone has a similar safety profile and efficacy in terms of reducing relapse rates, hospitalizations, symptoms and time to return to normal activity [30, 31, 34–36]. Moreover, its long half-life and shorter treatment duration have proven popular among patients, caregivers and clinicians, especially in children with acute asthma. Dexamethasone is a more potent GR agonist than prednisone [29]; however, whether it is more effective in reducing inflammation is less well understood. Here, we examined the anti-inflammatory effects of these two CS agents in vitro and found that dexamethasone was more potent and effective than prednisolone. As such, our data suggest that treating exacerbating asthmatics with dexamethasone may result in better control of symptoms and/or severity of disease, aspects of asthma mediated by persistent inflammation.

Studies comparing the effectiveness of standard dose regimes of dexamethasone (12 –16 mg/day, 1–2 days) to prednisone (60 mg/day, 5 days) found no difference in asthma relapse rates within two weeks [34–36]. These studies, however, failed to examine longer time points or differential CS effects on cellular or cytokine responses. Our study shows that dexamethasone significantly decreased IL–13 mRNA at a 10-fold lower concentration (10−9 µM) than prednisolone. IL–5 appeared to be generally less CS sensitive, with significant suppression observed only at 10−8 µM dexamethasone. However, at the highest concentration examined (10−7 µM), still physiologically relevant [44], both CS agents
almost completely suppressed IL-13 and IL-5 mRNA levels. This result indicates that in vivo type 2 cytokines are likely sufficiently controlled with an optimal dose of either prednisolone or dexamethasone.

Type 2 cytokines mediate many features of asthma and therapies that block their action are effective and now recommended as frontline controllers in severe asthma [25]. Attention, however, is now turning toward the importance of therapies that reduce the proportion and/or development of the type 2 cytokine producing cells with the long term hope of actually modifying or curing asthma [50]. Circulating Th2 cells are an important aspect of immune memory and their level controls a patient’s susceptibility to respond to allergens and the development of persistent symptoms. For this reason, we examined differences in the ability of these two CS agents to induce Th2 cell apoptosis. We found that dexamethasone was superior to prednisolone in driving apoptosis (~10-fold more effective) - no matter the concentration added, prednisolone was never able to induce apoptosis to a similar degree as dexamethasone. This differed from the effect on cytokines, where $10^{-7}$ M of either prednisolone or dexamethasone completely inhibited type 2 cytokine mRNA levels. This may be due to a different mechanism(s) of action. Corticosteroid suppression of IL-5 and IL-13 has been shown to be due to GR interfering with transcription factors binding to the promoter of these type 2 cytokine genes [14], while GC-induced apoptosis has been associated with induction of pro-apoptotic genes such as BIM (BCL-2-interacting mediator of cell death) [51]. Clearly, studies involving patients with asthma are needed to assess whether oral dexamethasone therapy more effectively reduces Th2 cells in vivo.

Since corticosteroid withdrawal studies have shown that suppression of type 2 inflammation requires continuous exposure to steroid [45, 52, 53], a therapeutic approach aimed at eliminating Th2 cells may provide a more sustained repression of allergic responses. Th2 cells are highly differentiated with a strong ability to survive, mediated through expression of the anti-apoptotic factor BCL-2 [54, 55]. The BCL-2 inhibitor ABT-199, in clinical trials for leukemia [56], was also shown to reduce the level of airway eosinophils and Th2 cells in a mouse model of asthma [57]. Huang et. al. showed that elimination of Th2 cells (using an antibody against CRTh2) resulted in significantly fewer eosinophils and lower levels of type 2 cytokines and chemokines in the blood, lymph nodes and lung in a mouse...
model of asthma [58]. Indeed, a number of therapies directly targeting Th2 cells, such as blockade of CRTh2 and TSLP, have shown promising results in clinical trials for asthma [59, 60].

Women and men respond differently to acute asthma. For example, women are more likely to relapse following an asthma exacerbation than men [38]. Furthermore, the female sex hormone progesterone has affinity for the GR [42]. As such, we examined whether progesterone could dampen the ability of CS to induce Th2 cell apoptosis and its mechanism of action. We found that progesterone (1μM, considered to be a physiologic level during pregnancy [42]), interfered with both prednisolone and dexamethasone and that the effect was identical if hormone was added in combination or as a pre-treatment. The Th2 cell line (CCRF-CEM) had no PGR mRNA and no production of PIBF1 mRNA in response to progesterone, indicating the effect was not due to activation of nuclear progesterone receptors [46]. We did, however, find that progesterone reduced the level of FKBP5 mRNA, a gene known to be induced by GC exposure and GR binding to its promoter [48, 49]. As such, our data suggest that progesterone antagonizes GC:GR signaling, rather than acting through its own receptor. This finding is in line with Guo et al., who showed that treating murine NK cells with the progesterone analog P4, in the presence of PGR blockade, antagonized GR signaling and reduced IFNγ and CD69 expression following CpG/IL–12 stimulation [61]. Progesterone was also shown to antagonize dexamethasone-induced apoptosis of murine thymocytes [62]. Collectively these studies support our hypothesis that circulating progesterone levels may influence the efficacy of CS action in vivo, which could explain why some women experience worse symptoms at times when progesterone levels are elevated [39, 40] and are more likely to be diagnosed with severe asthma [37].

Dexamethasone is a long-acting corticosteroid (36–72 hour half-life) with 30 times more GR activity than hydrocortisone. Due to these factors, long term dexamethasone treatment is associated with serious side effects such as suppression of the hypothalamic-pituitary-adrenal (HPA) axis and so is generally reserved for acute therapy, such as exacerbations of asthma [28]. Prednisolone is less potent (4 times the activity of hydrocortisone) and is shorter acting (12–36 hour half-life) [28] and so historically has been used as a controller medication. In 2012, due to the known side effects of long term oral steroid use, the GINA guidelines downgraded the use of oral CS in chronic asthma and
recommend them only after other controllers, such as anti-IgE and anti-IL-5 [63]. Nevertheless, a recent study of severe asthmatics reported a third of their study population was using oral prednisone, with an average duration of 4 years and mean dose 17.5 mg/day [64]. The ranges of oral prednisone prescribed vary greatly from very low (1 - 7.5 mg/day) to low doses (10 - 40 mg/day) [63]. Though less than recommended for exacerbation (60 mg/day) [35, 36], these doses equate to in vitro concentrations of ~ 0.3 - 0.8 µM (7.5 - 17.5 mg/day). In light of our data, the dose range of prednisolone for chronic use may be sufficient to suppress type 2 cytokine levels, but relatively inefficient as an inducer of apoptosis, particularly in women. These data indicate that inhaled CS formulations of higher affinity GR agonists, such as fluticasone furoate [65], may also be effective in eliminating Th2 cells and suggest this should be examined in future studies.

In conclusion, our study provides an in vitro examination of the anti-inflammatory capacity of two clinically relevant corticosteroids, prednisolone and dexamethasone. We found that dexamethasone more potently and effectively reduced type 2 cytokine expression and diminished Th2 cell numbers than prednisolone. In the presence of progesterone, dexamethasone maintained a superior ability to drive Th2 cell death over prednisolone. Though further study is needed to assess these effects in patients with asthma, our results do suggest that amongst oral CSs dexamethasone may be the better therapeutic option for treating exacerbations of asthma, particularly in women.

Materials And Methods
This study was approved by the Western University Health Sciences Research Ethics Board (Approval number 106770). All subjects gave informed consent.

Th2 cells
Primary Th2 cells were derived from healthy donor peripheral blood mononuclear cells (PBMCs) and cultured as described in [66]. The immortalized cell lines CCRF-CEM (ATCC® CCL-119™) and Jurkat (ATCC® E6-1, TIB-152™) were purchased from American Type Culture Collection (VA). They are both CD4+ T cell lines derived from acute human T cell leukemia. Cell lines were grown in RPMI-1640 media (Sigma-Aldrich) supplemented with 10% FBS (Hyclone Scientific, Fisher Scientific, ON, Canada) and 1X penicillin-streptomycin-glutamine (Gibco, Invitrogen, Thermo Fisher Scientific). Cells were
incubated at 37°C, in 85% humidity and 5% CO₂ and maintained at 0.2 - 0.3 x 10^6 cells/mL with re-seeding every 2 days.

**Cell Culture Conditions**

Dexamethasone, prednisolone and progesterone (Sigma Aldrich, ON, Canada) were prepared in 100% ethanol and serially diluted in culture medium prior to treatment of primary Th2 cells (1.3 x 10^6 cells/mL) and CCRF CEM cells (0.2 x 10^6 cells/mL). While prednisone is most commonly prescribed to patients, it needs to be metabolized in the liver to prednisolone, the active drug. Therefore, we used prednisolone for these *in vitro* experiments. Cells were treated with 10^{-9}—10^{-7} M of each CS agent as in [15, 18]. These concentrations were based on the relative anti-inflammatory activity of dexamethasone being 6.25-fold more potent than prednisolone (https://clincalc.com). Moreover, they are within the pharmacological range - oral administration of dexamethasone (12 mg, ~ 0.26 µM) and prednisone (60 mg, ~ 2.76µM) [44]. For experiments with progesterone, cells were either incubated with progesterone for 30 minutes prior to dexamethasone or added simultaneously with dexamethasone. Gene expression was assessed after 24 hours and staining for flow cytometry conducted after 48 hours.

**Gene Expression**

RNA (400ng) was isolated with RNeasy Plus Mini/QIAshredder (Qiagen, Hilden, Germany) and reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, California). Real time polymerase chain reaction (RT-PCR) was conducted using TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, California). Assays for IL-13 (Hs00174379_m1), IL-5 (Hs01548712_g1), PGR (Hs01556702_m1), PIBF1 (Hs00197131_m1), FKBP5 (Hs01561006_m1), CRTh2 (Hs00173717_m1), GATA3 (Hs00231122_m1), IFN (Hs00989291_m1) and the housekeeping gene GAPDH (Hs02786624_g1) were used (ThermoFisher). Thermal cycling was according to manufacturer’s instruction. Fold increase relative to the control condition was assessed for experimental treatments using the 2^{-ΔΔCT} method.

**Flow Cytometry**
Following 48 hours CS treatment, cells (0.5x10^6 cells/ml) were washed with FACS buffer (0.5% bovine serum albumin, 0.1% sodium azide, 3% FBS), pelleted at 4°C, re-suspended in Annexin V binding buffer (100µL/condition; 10mM HEPES pH 7.4, 140mM NaCl, 2.5mM CaCl_2) and stained with Alexa Fluor 647 Annexin V (1µL) and 7-AAD Viability Staining Solution (5µL) (BioLegend, CA, USA) for 15 minutes. Cells were diluted with additional Annexin V binding buffer (400µL) and data acquired using an LSR II (BD Biosciences). Flow cytometry analysis was conducted using FlowJo (Version 10, Ashland, OR, USA) and reported as the proportion of total cell population.

**Statistical Analysis**

Two- and one-way repeated measures analysis of variance (ANOVA) were used to determine statistical significance at p < 0.05 (SigmaPlot, version 12.5).

**Declarations**

Ethics approval and consent to participate: Informed consent to participate in the study was obtained from all participants.

Consent for publication: Not applicable

Availability of data and material: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Funding: Operating grants from the Canadian Institute of Health Research (CIHR), Grant-in-Aid from AllerGen/Canadian Allergy, Asthma and Immunology Foundation, bridge funding from Western University and summer student funding from the Schulich School of Medicine and Dentistry’s Dean’s Undergraduate Research Opportunities Program (DUROP).

Authors’ contributions: AL conducted cell culture and apoptosis experiments with CCRF-CEM cells and flow cytometry staining. LAS conducted culture of primary Th2 cells, collected and analyzed all flow cytometry data and prepared the manuscript. TK established staining protocols, drug dosing and assisted with flow cytometry. BHR highlighted the need for this investigation and contributed clinical insight for the study design, data interpretation and manuscript revision. LC conceived of the study, oversaw all experiments and wrote the final manuscript.

Acknowledgements The authors wish to acknowledge Meerah Vijeyakumaran for assistance with primary Th2 cell culture and flow cytometry and Liliane Cabral-Fernandes assisted with quantifying cytokine gene expression. Dr. B. H. Rowe is supported by CIHR as the Scientific Director of the Institute of Circulatory and Respiratory Health (ICRH) in Ottawa, ON.

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Supplemental Figure Legends

Figure files S1 & S2 are only available for download from the Supplementary Files section.

Supplemental Figure S1: The CCRF-CEM cell line exhibits a Th2 phenotype The CD4⁺ T lymphoblastic leukemia cell line CCRF-CEM are enriched in mRNA levels of CRTh2 and GATA3, markers of the Th2 cell phenotype, compared to Jurkat, another CD4⁺ T cell line (n = 3, A). Type 2 (IL-13) and type 1 (IFN[]) cytokine levels in CCRF-CEM and Jurkat cells following stimulation (24 hours) with PMA (20ng/ml) and ionomycin (1M) (B). Data represent mean and standard error. *p < 0.05 determined by one-way ANOVA

Supplemental Figure S2. Gating strategy used to determine % Annexin V⁺ and % 7-AAD⁻ positivity of CCRF-CEM cells

Figures
A comparison of prednisolone- and dexamethasone-mediated reduction of type-2 cytokines.
mRNA levels in primary Th2 cells. Fold differences in mRNA level from corticosteroid (CS) treated cells compared to vehicle are provided (n=3). Quantification of IL-13 mRNA following steroid treatment (A). Comparison of CS ability to suppress IL-13 (B). Quantification of IL-5 mRNA following CS treatment (C). Comparison of the ability of either CS to suppress IL-5 (D). Data represent mean and standard error. Pred, prednisolone; Dex, dexamethasone.*p < 0.05 determined by one-way (A&C) or two-way (B &D) RM ANOVA
A comparison of prednisolone- and dexamethasone-induced apoptosis in a Th2 cell line (CCRF-CEM). Data are expressed as percentage of 7AAD+ cells (A) and Annexin V+ 7AAD− cells (B) identifying dead cells and apoptotic cells, respectively (n=11). Comparison of corticosteroid-induced apoptosis as fold-difference over vehicle (C). Data represent mean and standard error. Pred, prednisolone; Dex, dexamethasone. *$p < 0.05$ determined by two-way (A&B) or one-way (C) RM ANOVA.
Corticosteroid-induced cell death is dampened by the female sex hormone progesterone.

Dexamethasone-induced cell death (A, % 7AAD+) and apoptosis (B, % Annexin V+ 7AAD−)
of Th2 cells (CCRF-CEM) in the presence or absence of progesterone (2 μM, n=5). Head-to-head comparison of apoptosis following treatment with prednisolone (C) or dexamethasone (D) with or without progesterone (n=4). In the presence of progesterone, dexamethasone showed significantly more ability to induce apoptosis (% Annexin V+ 7AAD-) than prednisolone (E, n=4). Data represent mean and standard error. Pred, prednisolone; Dex, dexamethasone; Prog, progesterone; Pre, pretreatment. *p < 0.05 determined by one-way (A-D) or two-way (E) RM ANOVA
Figure 4

Expression of the nuclear progesterone receptor (PGR) is undetectable in a Th2 cell line (CCRF-CEM) but present in a breast adenocarcinoma cell line (MCF-7) used as a positive control (n=3; A). Effect of progesterone with or without dexamethasone on expression of PIBF1 (n=3, B). Progesterone reduced the dexamethasone-mediated increase in FKB5 mRNA level, but had no effect when applied alone (n=5, C). Data represent mean and standard error. *p < 0.05 determined by one-way ANOVA

Supplementary Files
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Fig S1.png