Glucocorticoids prime the inflammatory response of human hippocampal cells through up-regulation of inflammatory pathways

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Abstract

Increased pro-inflammatory cytokines and an overactive hypothalamic-pituitary-adrenal (HPA) axis have both been implicated in the pathogenesis of depression. However, these explanations appear contradictory because glucocorticoids are well recognised for their anti-inflammatory effects. Two hypotheses exist to resolve this paradox: the mediating presence of glucocorticoid receptor resistance, or the possibility that glucocorticoids can be pro-inflammatory in some circumstances. We sought to investigate these hypotheses in a cell model with significant relevance to depression: human hippocampal progenitor cells. We demonstrated that dexamethasone in vitro given for 24 hours and followed by a 24-hour rest interval before an immune challenge produces pro-inflammatory effects in these neural cells, that is, potentiates the IL-6 protein secretion induced by stimulation with IL-1β (10ng/mL for 24 hours) by +49% ($P<0.05$) at a concentration of 100nM and by +70% ($P<0.01$) for 1μM. These effects are time- and dose-dependent and require activation of the glucocorticoid receptor. Gene expression microarray assays using Human Gene 2.1st Array Strips demonstrated that glucocorticoid treatment up-regulated several innate immune genes, including chemokines and Nod-like receptor, NLRP6; using transcription factor binding motifs we found limited evidence that glucocorticoid resistance was induced in the cells. Our data suggests a mechanism by which stress may prime the immune system for increased inflammation and suggests that stress and inflammation may be synergistic in the pathogenesis of depression.

1. Introduction

The neurobiology underlying depression is increasingly understood to involve interacting multi-system dysfunction (Dantzer, 2018; Frank et al., 2016; Miller and Raison, 2016). Two systems that have been most prominently implicated in this process are the hypothalamic-pituitary-adrenal axis (HPA) axis and the pro-inflammatory cytokine system (Dowlati et al., 2010; Holsboer, 2000; A. H. Miller et al., 2009; Pariante and Lightman, 2008).

The investigation of both systems individually has yielded evidence of their involvement in depression: raised cortisol levels and evidence of glucocorticoid resistance have been identified in depressed patients (Juruena et al., 2006; Pariante, 2017, 2009), including in meta-analysis (Stetler and Miller, 2011), as are increased levels of pro-inflammatory cytokines (Dantzer, 2018; Dowlati et al., 2010; Howren et al., 2009). However, neither of these individual systems have been able to provide a full explanation of the pathogenesis of depression, nor identify robust biomarkers. For example, while using mRNA gene expression we have been able to demonstrate both glucocorticoid resistance and inflammation in the same depressed patients (Cattaneo et al., 2013), a recent meta-analysis from our group has found only limited evidence that glucocorticoid resistance underpins inflammation in depression (Perrin et al., 2019). Given the well-known multi-level interactions between these two systems (Bellavance and Rivest, 2014; Zunszain et al., 2011) a more comprehensive understanding of depressive pathology may be derived from investigation of their interaction in depression.

Of particular interest in their interaction is the co-existence of increased levels of cortisol and pro-inflammatory cytokines in depression: this presents an apparent paradox, given the potent ability of cortisol to inhibit inflammation, including inhibition of the production and secretion of pro-inflammatory cytokines (Cain and Cidlowski, 2017; De Bosscher et al., 2003). This paradox has produced two competing hypotheses which seek to explain the interaction between these two systems in depression.

The first hypothesis proposes the presence of glucocorticoid resistance as a critical mediating factor: this entails reduced glucocorticoid signalling (even in the presence of increased levels of cortisol) (Cohen et al., 2012; Miller, 2008). This resistance is postulated to permit overactivity of the innate immune system (including raised level of pro-inflammatory cytokines) due to a relative lack of normal inhibitory glucocorticoid tone (Cohen et al., 2012; Miller, 2008). A second hypothesis focuses on recent evidence that cortisol possesses more complex immunomodulatory effects than have previously been ascribed to it: in particular, that under certain circumstances, glucocorticoids can be pro-inflammatory, in addition to its recognized anti-inflammatory effects (Cain and Cidlowski, 2017; Frank et al., 2013a; Sorrells et al., 2009).

The first hypothesis – glucocorticoid resistance underlies increases activity of the innate immune system – is supported by several lines of evidence. Stressed populations – such as brain cancer sufferers (Miller et al., 2008), people with low early life social class (G. E. Miller et al., 2009) and lonely people (Cole, 2008) - have peripheral blood cells that possess reduced transcripts bearing glucocorticoid receptor response elements, and increased transcripts with NF-κB response elements, along with increased plasma levels of inflammatory markers. This

has been interpreted as a stress-induced switch in balance from glucocorticoid signalling to immune signalling (Miller, 2008; Miller et al., 2008). It has also been found that glucocorticoid resistance and increased inflammation have been found in chronically stressed animals. For example, mice subjected to a social defeat stress paradigm demonstrate both glucocorticoid resistance and increased inflammation (Avitsur et al., 2009; Sheridan et al., 2000); specifically, immune cells from the spleens of these mice display glucocorticoid resistance, through a lack of sensitivity to corticosterone inhibition following lipopolysaccharide (LPS) simulation (Avitsur et al., 2009). Additionally, monocytes from these animals secrete higher levels of tumour necrosis factor-α (TNF-α) and IL-6 in response to LPS, linked with an increased likelihood of developing endotoxic shock (Avitsur et al., 2009). Lastly, this effect has been demonstrated in humans, whereby stressed individuals demonstrate increased glucocorticoid resistance, an increased likelihood to develop a cold after rhinovirus seeding, and increased levels of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) in nasal secretions in response to the virus (Cohen et al., 2012). A strong correlation between glucocorticoid resistance and levels of pro-inflammatory cytokines was detected (Cohen et al., 2012).

The second hypothesis – that glucocorticoids might possess novel pro-inflammatory properties – has found support in a body of evidence derived from animal experiments. There are several studies demonstrating that when rodents are exposed to a stressor before inflammatory stimulus, a potentiated inflammatory response is observed, as measured by levels of pro-inflammatory cytokines (De Pablos et al., 2014; Espinosa-Oliva et al., 2011; Munhoz et al., 2006; Nair and Bonneau, 2006). For example, exposure to inescapable tail-shock or to daily sessions of unpredictable chronic stress (Munhoz et al., 2006) potentiates the

increase in hippocampus and frontal cortex expression of pro-inflammatory mediators, like IL-1β, induced by a peripheral injection of LPS given 24 hours after the stressor regimen.

This phenomenon is glucocorticoid- and glucocorticoid receptor-dependent as it can be abolished by adrenalectomy or administration of the glucocorticoid receptor antagonist RU486 (De Pablos et al., 2014; Espinosa-Oliva et al., 2011; Munhoz et al., 2006; Nair and Bonneau, 2006). Interestingly, glucocorticoid treatment administered shortly before an inflammatory insult produces anti-inflammatory effects, emphasising the importance of timing, consistent with the proposed existence of a window of pro-inflammatory effects (Frank et al., 2010; Smyth et al., 2004). There is also preliminary evidence that this phenomenon occurs in humans (Yeager et al., 2011, 2016): one study in humans found that intermediate doses of hydrocortisone (corresponding to stress levels) infused 24 hours before LPS administration potentiated the rise of IL-6 levels compared with LPS alone, while high doses failed to (Yeager et al., 2011), with similar effects found in peripheral human monocyte/ macrophages (Yeager et al., 2018). Additionally, the mineralcorticoid receptor (MR) has also been implicated in the ability of glucocorticoids to potentiate inflammatory responses, suggesting the possibility of differential effects of glucocorticoid agonists which differentially affect the GR and MR (Duque and Munhoz, 2016).

We sought to mechanistically explain the interaction of glucocorticoid signalling and inflammatory cytokines in human hippocampal progenitor cells, a model with significant relevance to depression (Anacker et al., 2011; Sahay and Hen, 2007). This model has previously allowed us to analyse pathways involved in depression and the response to antidepressants, including identification of the role of IL-1β in the kynurenine pathway,

affecting hippocampal neurogenesis (Zunszain et al., 2012), the ability of antidepressant compounds to stimulate hippocampal neurogenesis (Anacker et al., 2011), the ability of antidepressants to modulate inflammatory responses (Horowitz et al., 2015), the role of SGK1 in hippocampal neurogenesis (Anacker et al., 2013) and the ability of omega-3 fatty acids and antidepressants to reverse the effects of an inflammatory insult on neurogenesis (Borsini et al., 2017).

We examined whether glucocorticoids could potentiate inflammatory processes in these cells, and the characteristics of this phenomenon, exploring a variety of treatment concentrations and duration regimes, using our previously described inflammation model of IL-6 protein secretion into the supernatant by the hippocampal cells stimulated by IL-1β (Horowitz et al., 2015). Furthermore, we sought to find out whether these effects were mediated by glucocorticoid resistance or up-regulation of inflammatory pathways, using both pharmacological agonists and antagonists of the glucocorticoid receptor and mRNA transcriptomics.

2. Methods

1. Cell culture

All experiments were performed with the multipotent human female hippocampal progenitor cell line HPC03A/07, generated by ReNeuron as described previously (Pollock et al., 2006). Cells were cultured as described previously (Johansson et al., 2008), with minor modifications. Cells were grown in reduced modified media (RMM) consisting of Dulbecco's

Modified Eagle's Media/F12 (Invitrogen) supplemented with 0.03% human albumin (Baxter Healthcare), 100 µg/mL human apo-transferrin, 16.2 µg/mL human putrescine DiHCl, 5µg/mL human recombinant insulin, 60ng/mL progesterone, 2mM L-glutamine, and 40ng/mL sodium selenite. To maintain proliferation, 10ng/mL human basic fibroblast growth factor (bFGF), 20ng/mL human epidermal growth factor (EGF), and 100nM 4-hydroxytamoxifen (4-OHT) were added. Cultures were grown in 75cm^2 filtered, cap-cell culture flasks (Nunclon) at 37°C in 5% CO2 and were regularly passaged at 60–80% confluence.

In the presence of growth factors (FGF2 and EGF) and 4-Hydroxy-Tamoxifen (4-OHT), progenitor cells will proliferate and remain undifferentiated. Removal of these growth factors induces differentiation of cells, on average, into 52% of TuJ1-positive cells (of which 35% are doublecortin-positive neuroblasts, 25% were MAP2-positive mature neurons, and 8% labelled positive for both, doublecortin and MAP2), 27% S100ß-positive astrocytes, 2% of O1-positive oligodendrocytes and 19% of GFAP-positive immature progenitor cells, following a protocol of 72 hours of proliferation, followed by 7 days of differentiation (Anacker et al., 2011). Cell fate is influenced by incubation with both glucocorticoids and IL-1β: we have previously shown that dexamethasone (1µM) decreased the number of MAP-2-positive neurons by 27% (with an attendant increase in astrocytes)(Anacker et al., 2011); and IL-1β reduced MAP-2 positive cells by 40% (Borsini et al., 2017). All experiments were conducted in proliferating hippocampal progenitor cells in this study, given their implication in the response to stress (Egeland et al., 2015).

2.2 Cell culture study design

In order to understand the interaction between the glucocorticoid signalling pathway and the inflammatory pathways the effect of co-incubation of human hippocampal progenitor cells with an inflammatory stimulus (IL-1β) and a potent glucocorticoid receptor agonist (dexamethasone) was contrasted with the pre-treatment of cells with dexamethasone before inflammatory stimulation. Pre-treatment with glucocorticoids has been shown in animals to produce a potentiation in response to a subsequent inflammatory stimulus (Frank et al., 2013a), as well as in rodent macrophages (Zhang and Daynes, 2007) and rodent hippocampal microglia (Frank et al., 2011) but this has never been investigated in human brain cells.

The timing and dosage of dexamethasone pre-treatment was varied to characterise the putative pro-inflammatory properties of glucocorticoids. Cortisol, acting principally on the MR, as well as the glucocorticoid receptor antagonist, RU486, were employed to further delineate the mechanism of the pro-inflammatory effect. Cells were plated in 6 well plates (Nunclon) at a density of 300 000 cells per well in 2mL of RMM and allowed to firmly attach for 24 hours. IL-1β was employed at a concentration of 10ng/mL, which had been previously shown to induce a robust inflammatory response (Horowitz et al., 2014).

Dexamethasone was employed in a wide range of concentrations (1nM – 10μM), as in studies seeking to determine glucocorticoid resistance in blood samples (Carvalho et al., 2008). All treatments used the same vehicle to exclude the possibility of any differences observed being the consequence of differing concentrations of solvents. Supernatants were collected and stored at -80°C for subsequent measurement.

We compared four different treatment designs, in order to examine the ability of glucocorticoids to modulate inflammatory responses (see Figure 1). Specifically, we examined a condition involving inflammatory stimulus alone (IL-1β at a concentration of 10ng/mL) for 24 hours following 48 hours of vehicle treatment (Figure 1b) with a control condition of 72 hours of vehicle treatment (Figure 1a). In order to investigate the putative inhibitory effects of glucocorticoid co-treatment on inflammatory responses in the hippocampal cells we co-treated cells with an inflammation stimulus (IL-1β, 10ng/mL) and dexamethasone at a range of concentrations (1nM – 10μM), after 48 hours of vehicle treatment (Figure 1d). Finally, to investigate the hypothesised pro-inflammatory effects of glucocorticoid pre-treatment on subsequent inflammatory responses we treated cells with glucocorticoids (dexamethasone or cortisol, at a wide range of concentrations) for 24 hours, before a rest period of 24 hours, followed by treatment with an inflammatory stimulus (IL-1β at a concentration of 10ng/mL) for a further 24 hours (Figure 1c). Appropriate comparisons were made between control and treatment conditions.

2.3 Secreted Cytokine Quantification

IL-6 secreted into the supernatant was quantified using the human IL-6 Quantikine ELISA kit (R&D Systems). The procedure was performed according to the manufacturer's instructions. Absorbance was read at 450nm using a DTX 880 Multimode Detector (Beckman-Coulter). Cell supernatants were also run on the Human Cytokine Magnetic 25-Plex Panel (Invitrogen) according to the manufacturer's instructions. Data were analysed using a four-parameter logistic algorithm to derive concentrations of samples from known standards using SoftMax Pro (Molecular Devices). All concentrations were then normalised by cell number as

determined by crystal violet. Briefly, cell viability was determined by incubating fixed cells with 10% crystal violet, before absorbance was measured. Conditions causing greater than 15% variation in cell viability from control conditions were excluded from consideration. All conditions presented showed cell viability variation from control condition of less than 15%, indicating that the toxicity of these compounds was minimal at the doses employed in this study. Further details are provided in Horowitz et al. (2014). . Three to twelve independent experiments were conducted on independent cultures, and each sample was tested in duplicate. Data is presented as percentage change of IL-6 (or relevant chemokine or cytokine) levels detected in the supernatant compared to vehicle treatment or treatment with IL-1β.

2.4 Gene expression analysis

RNA was isolated using the RNeasy Micro Kit (Qiagen) following the manufacturer's instructions, and samples were kept frozen at -80°C until further use. RNA quantity and quality were assessed by evaluation of the A260/280 and A260/230 ratios using a Nanodrop spectrometer (NanoDrop Technologies). Superscript III enzyme (Invitrogen) was used to reverse-transcribe 1 μg total RNA, as previously described (Horowitz et al., 2014). Quantitative real-time PCR (qPCR) was performed using HOT FIREPol EvaGreen qPCR Mix (Solis BioDyne), according to the SYBR Green method and using a Chromo 4 DNA engine (BioRad). For each target primer set, a validation experiment was performed to demonstrate that PCR efficiencies were within the range of 90–100%. Relative expression of the target gene GR, was normalized to the arithmetic mean of expression levels of the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase and beta-actin and expressed as fold change compared with controls using the Pfaffl method (Pfaffl, 2001). At least three

independent experiments were conducted on independent cultures, and each sample was tested in duplicate.

2.5 Transcriptomic Analyses

RNA of human progenitor cells was isolated using RNeasy mini kit (Qiagen, Crawley, UK) and subsequently DNase treated (Ambion, Warrington, UK). Gene expression microarray assays were performed using Human Gene 2.1st Array Strips on GeneAtlas platform (Affymetrix) following the WT Expression Kit protocol described in the AffymetrixGeneChip Expression Analysis Technical Manual (http://media.affymetrix.com/support/downloads/manuals/geneatlas_WT Expression_expkit_manual.pdf). Briefly 250ng RNA were used to synthesize second strand cDNA with the Ambion Express Kit (Ambion, Life technologies) Subsequently 5.5μg of purified cDNA was then fragmented, labelled and hybridized onto HuGene2.1 Array strips. The reactions of hybridation, fluidics and imaging were performed on the Affymetrix Gene Atlas instrument according to the manufacturer's protocol.

2.6 Statistical and Bioinformatic Analyses

2.6.1 Transcriptomic Analyses

Affymetrix CEL files from the complete data set (total of twenty four samples; six biological replicates per sample for vehicle/vehicle/vehicle (VVV) (1), dexamethasone (1μM)/vehicle/IL-1β(10ng/mL) (DVI) (2), vehicle/vehicle/IL-1β (VVI) (3), vehicle/vehicle/IL-1β+dexamethasone (VVI+D) (4)) were imported into Partek Genomics Suite version 6.6

for data visualization and statistical testing. Quality control assessment and Principal-component analysis (PCA) was performed to identify outliers. All samples passed the criteria for hybridisation controls, labelling controls and 3'/5' Metrics. Background correction was conducted using Robust Multi-strip Average (RMA) (Irizarry et al., 2003) to remove noise from auto fluorescence. After background correction, normalisation was conducted using Quantiles normalisation (Bolstad et al., 2003) to normalise the distribution of probe intensities among different microarray chips. Subsequently, a summarisation step was conducted using a linear median polish algorithm (Tukey, 1977) to integrate probe intensities in order to compute the expression levels for each gene transcript.

To assess the effect of treatment, an ANOVA test was applied to assess for differences in gene expression between treatment conditions (3) and (1), (4) and (3), (2) and (3), (effect of inflammation, effect of dexamethasone co-treatment, effect of dexamethasone pre-treatment, respectively) (Figure 1). Differential gene expression across treatment was assessed by applying a p-value filter (for treatment) of $p<0.05$ to the ANOVA results, and a fold-change cut-off of 1.2. Genes differentially modulated across treatment have then used to run a pathway analyses by using Ingenuity Pathway Analyses Software.

2.6.2 Transcription factor-binding motifs analyses to test glucocorticoid resistance

To evaluate the glucocorticoid resistance hypothesis at the genomic level, we evaluated whether GR-target genes were down-regulated in the transcriptomic profile following dexamethasone treatment. The Transcription Element Listening System (TELiS) bioinformatics analysis (Cole et al., 2005) was used to quantify the prevalence of transcription

factor-binding motifs (TFBMs) in the promoters of differentially expressed genes in DVV vs VVV conditions. This analysis can detect if the target genes of a particular transcription factor (for example, the glucocorticoid receptor) are over-expressed in a set of differentially regulated genes.

3. Results

3.1 Transcriptomic analysis of the human hippocampal cells confirms that these cells are immunocompetent

These hippocampal cells constitutively express IL-1 receptors, as well as both components of the IL-6 receptor complex (IL-6 binding sub-unit (IL-6R, gp80) and the transmembrane protein gp130, required for signal transduction (Figure S1). We have previously demonstrated that the human hippocampal progenitor cells employed in the current experiments respond to inflammatory stimulation: the addition of IL-1β induces secretion of inflammatory molecules, including cytokines (IL-6, IL-8, IL-15, IFN-α IL-1RA) and chemokines (MCP-1, IP-10, RANTES) (Horowitz et al., 2014). In a separate study, we have also found that these cells respond to interferon-alpha by producing IL-6 (Borsini et al., 2018). As IL-6 has been closely related to the pathogenesis of depression, and it is robustly induced in our cell model, it was a primary focus in these experiments (Dowlati et al., 2010; Howren et al., 2009; Jansen et al., 2015)

Up-regulation of the gene expression of the majority of these cytokines and chemokines by IL-1β stimulation was confirmed by genome-wide gene expression arrays, and the 30 most up-regulated genes are shown in Table S1, with a heatmap of regulated genes (fold-change>5, $p<0.05$) shown in Figure S1. A number of other inflammatory proteins, including chemokines, interferon-induced proteins and genes from the tumour necrosis factor family were also induced (Table S1). Additionally, pathways known to be activated by inflammatory stimulation, such as the interferon pathway, IL-1 receptor signalling pathway and the inflammasome pathway were among the most significantly modulated pathways by IL-1β stimulation in the human hippocampal progenitor cells (Table 1).

3.2 Co-incubation with dexamethasone dose-dependently inhibits IL-6 in human hippocampal cells

Glucocorticoids are well known, and employed clinically, for their anti-inflammatory properties, including an inhibitory effect on the synthesis and release of pro-inflammatory cytokines (De Bosscher et al., 2003). In order to determine whether these properties were also evident in the human hippocampal progenitors these cells were treated with IL-1β (10ng/mL) alone and in combination with 1nM, 10nM, 100nM, 1μM and 10μM of dexamethasone for 24 hours.

At baseline, IL-6 protein levels in the supernatant were near to the lower detection limit of the ELISA assay used for measurement (0.7pg/mL). IL-1β treatment alone for 24 hours induced IL-6 strongly to an average of approximately 470pg/mL (+/-82.43, $p<0.01$) (represented as 100% in Figure 2b). Co-incubation with dexamethasone demonstrated dose-dependent

inhibition of IL-1β-induced IL-6 secretion into the supernatant, significant at dexamethasone concentrations of 100nM (-37% +/- 10%, $p<0.01$), 1μM (-40 %+/- 10%, $p<0.01$) and 10μM (-43% +/- 0.1%, $p<0.01$) (Figure 2), consistent with glucocorticoid's putative ability to inhibit inflammatory molecules.

3.3 Contrary to the effect of co-treatment, pre-treatment with glucocorticoids potentiates IL-6 release , in a time- and dose-dependent manner

As mentioned above, pre-treatment with glucocorticoids has been shown in animals to produce a potentiation in response to a subsequent inflammatory stimulus (Frank et al., 2013a). In previous studies, two characteristics of the treatment paradigm were thought to influence the potentiating effects of glucocorticoids on inflammation: the time between glucocorticoid treatment and inflammatory stimulation (Frank et al., 2013a) and the dose of the glucocorticoid pre-treatment (Yeager et al., 2011).

3.3.1 Time-dependent effects

In order to investigate the role of timing in determining this effect, human hippocampal cells were pre-treated with dexamethasone 1uM before stimulation with IL-1β (10ng/mL), thoroughly washed of dexamethasone, and left for variable periods (1hour, 12 hours, 24 hours and 48 hours) before stimulation with IL-1β (10ng/mL). Consistent with findings in animal brains (Johnson et al., 2002; Munhoz et al., 2006), priming with dexamethasone potentiated the response to immune stimulation as measured by IL-6 secreted into the supernatant (Figure 3). This effect was only present when there was a time interval of 24 hours between

glucocorticoid treatment and inflammatory stimulation (One-way ANOVA, $p<0.05$, $F(1,4)=2.953$, n=7-12; for 24 hour interval +37.6%, $p<0.05$, n=12) (Figure 3). This is consistent with the notion that the inflammation potentiating effects of glucocorticoids are influenced by their temporal relation to inflammatory stimulation, suggesting a window of pro-inflammatory effects (Frank et al., 2013a).

3.3.2 Dose-dependent effects

Some studies find that intermediate doses of glucocorticoids (equivalent to stress levels) are able to potentiate inflammatory release, as opposed to low (baseline) or high (clinically used) doses (Munhoz et al., 2010; Yeager et al., 2011). In order to investigate the influence of concentration, the time interval between dexamethasone treatment and inflammatory stimulation was held constant at 24 hours, and the concentration of dexamethasone was varied (1nM, 10nM, 100nM, 1μM, and 10μM).

All concentrations of dexamethasone potentiated subsequent IL-6 response, but the strongest effects were present at the intermediate concentrations of 100nM and 1μM, which achieved significance (One-way ANOVA, $p<0.05$, $F1,5=3.894$, n=5; DEX 100nM by +72.2%, $p<0.01$, n=5; DEX 1μM by 48.9%, $p<0.05$, n=5) (Figure 4), consistent with findings in animal (Munhoz et al., 2010) and human studies (Yeager et al., 2011).

Cortisol treatment 24 hours before immune stimulation was also able to produce potentiation effects on IL-6, reaching significance for 10nM, 100nM, and 1μM (One-way ANOVA, $p<0.0001$, $F1,5=9.089$, n=5; cortisol 10nM by +25.9%, $p<0.01$, n=5; cortisol 100nM by

+49.3%, $p<0.01$, n=5; cortisol 1μM by 30.2%, $p<0.01$, n=5), confirming the role of the physiological concentrations of glucocorticoids for these pro-inflammatory effects (Figure 5).

3.4 Differential effects of dexamethasone are generalised to other cytokines and chemokines

In order to establish whether the differential effect of dexamethasone during co-incubation and pre-treatment was generalised to other cytokines and chemokines beyond IL-6, the supernatant was measured using a multiplex immunoassay.

Co-incubation of IL-1β (10ng/mL) with dexamethasone (1μM) showed numerical inhibitory effects for all detectable cytokines and chemokines compared with IL-1β stimulation alone (IL-8, IP-10, IL-1RA, RANTES and MCP-1, Figure 6, light grey bars vs. white bars), as already demonstrated for IL-6 (Figure 2), reaching statistical significance, after multiple comparisons correction, for RANTES (-45%, $p<0.001$) (Figure 6e).

Importantly, pre-treatment with dexamethasone (1μM) 24 hours before immune stimulation potentiated the increase of all detectable cytokines and chemokines IL-8 (+23%), IP-10 (+28%), IL-1RA (+82%), RANTES (+29%), MCP-1 (+10%) (Figure 6, black bars vs. white bar), compared with IL-1β stimulation alone, reaching statistical significance after multiple comparisons correction, for IL-1RA ($p<0.01$), and RANTES ($p<0.05$) (Figure 6d,e), consistent with changes detected for IL-6. Notably, the difference between co-treatment and pre-treatment with dexamethasone was significant for IL-8 ($p<0.05$), IL-1RA ($p<0.001$), RANTES ($p<0.001$) and MCP-1 ($p<0.05$).

Furthermore, the multiplex immunoassay revealed that pre-treatment with dexamethasone for only one hour before immune stimulation produced an effect that was intermediate between the effect of co-treatment and that of dexamethasone pre-treatment 24 hours before immune stimulation (Figure 6, dark grey bars), consistent with the time-dependent effects detected with IL-6 (Figure 3).

These experiments suggest that the differential effects of co-treatment or pre-treatment with glucocorticoids are generalised to the cytokines and chemokines involved in the inflammatory response.

3.5 Mechanism underlying differential effects of glucocorticoids on inflammatory responses

There have been two mechanisms proposed by which glucocorticoid pre-treatment may potentiate subsequent inflammatory effects: up-regulation of inflammatory pathways (Frank et al., 2013a) and induction of glucocorticoid resistance (Miller, 2008), necessitating activation of the glucocorticoid receptor. We examined for evidence of both proposed mechanisms.

3.5.1 Activation of the glucocorticoid receptor is necessary for the potentiating effects of dexamethasone pre-treatment on cytokines

Activation of the glucocorticoid receptor is required for dexamethasone to inhibit inflammation (De Bosscher et al., 2003), and it has been demonstrated that the pro-inflammatory effect of glucocorticoid pre-treatment can be blocked with the GR antagonist,

RU486, in animals (Munhoz et al., 2006). We therefore sought to establish whether activation of the glucocorticoid receptor is also necessary for both the anti- and the pro-inflammatory effect of dexamethasone in human hippocampal cells.

A dose-titration experiment established that a concentration of 1μM of the GR antagonist RU486 completely abrogated the anti-inflammatory, inhibitory effect of co-treatment with dexamethasone (1μM) on the IL-6 inflammatory response to IL-1β (10ng/mL) stimulation (Figure 7).

Therefore, to determine the role of the glucocorticoid receptor in the glucocorticoid priming phenomenon, cells were pre-treated with dexamethasone and RU486 during the pre-treatment and the rest interval (Figure 8). The presence of 1μM RU486 completely abrogated the potentiation effect of dexamethasone pre-treatment (Figure 8, 4th column vs 3rd column), consistent with the notion that GR activation is necessary for this effect.

The fact that activation of the GR is necessary both for the inhibitory effect of dexamethasone on IL-1β stimulation and the potentiation effect on IL-1β stimulation confirms the GR as a critical effector of differential dexamethasone action, and it is consistent with our previous findings showing that GR activation can both increase and decrease hippocampal neurogenesis in this cell line (Anacker et al., 2011).

We also demonstrated that the effect of dexamethasone pre-treatment is blocked by co-treatment with RU486 during the dexamethasone treatment period alone, that is, without treating during the rest period (fifth column, Figure 9), but not by treatment with RU486

during the ‘rest’ period alone (that is, not during pre-treatment) (sixth column, Figure 9). Given the importance of the ‘rest’ period on the pro-inflammatory effects of glucocorticoid pre-treatment established above (Section 3.3 and Figure 3) this suggests that molecular pathways induced during the treatment period (and requiring GR activation) produce the pro-inflammatory effects during the ‘rest’ period, and that during this ‘rest’ the GR is no longer involved in the process.

3.6 Up-regulation of inflammatory pathways

In order to investigate whether inflammatory pathways are up-regulated by dexamethasone pre-treatment we investigated genome-wide gene expression in different conditions (as in Figure 1) to identify differentially regulated inflammatory genes and gene pathways.

3.6.1 Dexamethasone co-incubation with IL-1β inhibits most inflammatory genes, but up-regulates a minority

As above (Section 3.1), inflammatory stimulation of the neural cells produced up-regulation of numerous immune genes. Analysis of genome-wide gene expression of co-treatment with dexamethasone and IL-1β (VVI+D) vs. IL-1β alone (VVI) demonstrated the inhibitory effect of dexamethasone on IL-1β-induced gene expression. Inflammatory genes were amongst the genes most strongly down-regulated, including the cytokine and chemokine genes TNF-α, IL-1α, IL-11, chemokine ligand 1 (CCL1), chemokine receptor 4 (CXCR4), chemokine (C-X-C motif) ligands 5, 9, 12, and 16, colony stimulating factor 2 (granulocyte-macrophage) (CSF2 or GM-CSF) and other immune genes (Figure 10, Table S2). This reflects the known

inhibitory influence of dexamethasone co-incubation on inflammatory processes (De Bosscher et al., 2003).

Of note, IL-6 was not found to be differentially regulated by dexamethasone, despite the pronounced inhibition observed at the level of secreted protein, perhaps due to a different time course of changes at the mRNA and protein levels (Koussounadis et al., 2015).

Additionally, the gene pathways most strongly regulated by dexamethasone co-treatment include many pathways known to be involved in inflammation, including inflammatory pathways such as TGF-β signalling, NF-κB signalling, toll-like receptor signalling and p38 MAPK signalling (Table 2).

Of note, it was also found that dexamethasone co-incubation *up-regulated* several immune genes, including chemokine (C-X-C motif) ligand 5 (CXCL5) ($p=0.019$, FC= 1.27), NOD-like receptor family, pyrin domain containing 6 (NLRP6), ($p=0.001$, FC=1.43), and the chemokine ligand 20 (CCL20) ($p<0.001$, FC=1.98). The up-regulation of NLRP6 is particularly notable as the up-regulation of NOD-like receptors have been strongly implicated as a mechanism by which glucocorticoids can potentiate inflammation (Busillo et al., 2011; Frank et al., 2013a) (Table S3).

3.6.2 Dexamethasone pre-treatment up-regulates some inflammatory pathways

We analysed genome-wide gene expressed of dexamethasone pre-treatment, comparing pre-treatment with dexamethasone followed by IL-1β (DVI) compared with IL-1β alone (VVI) to

further understand the mechanisms underlying the inflammatory potentiation effect. There were found to be 102 genes differentially regulated by dexamethasone pre-treatment (cut off: FC> 1.2, $p<0.05$), with 59 genes up-regulated and 43 genes down-regulated (Figure 11).

Interestingly, some immune genes were down-regulated by dexamethasone pre-treatment compared with no pre-treatment, including the interleukin 18 receptor 1($p=0.01$, FC=-1.26), TNF receptor-associated factor 1 (TRAF1)($p=0.04$, FC=-1.24), nuclear factor, interleukin 3 regulated ($p=0.04$, FC=-1.20) and colony stimulating factor 2 (CSF2 or GM-CSF) ($p=0.047$, FC=-1.23) (Table S4).

However, there were also a number of immune genes up-regulated by dexamethasone pre-treatment, including NLRP6 ($p=0.002$, FC=1.40), which, interestingly, as mentioned above, was significantly up-regulated also by co-treatment. In addition, T cell receptor genes, TRAV1, TRBV5-3, and TRAV9-1 were also significantly up-regulated, as were other immune system-related genes such as HLA-DQA2 and the immunoglobulin gene IGKV3D-11 (Table 3).

There was one gene pathway differentially regulated by dexamethasone pre-treatment - haematopoietic cell lineage ($p=0.009$). Interestingly, the haematopoietic cell lineage pathway was also regulated by dexamethasone co-incubation.

3.6.3 Dexamethasone regulated some genes in the same direction in both the pre-treatment and co-incubation conditions

Of the 102 genes regulated by dexamethasone pre-treatment (DVI vs VVI) and the 478 genes regulated by dexamethasone co-incubation (VVI+D vs VVI) there were 31 differentially regulated genes in common, assessed using a bioinformatics tool (Oliveros, 2015). Of these 31 genes, all were regulated in the same direction in both circumstances: that is, dexamethasone regulated 31 genes in the same direction whether dexamethasone treatment preceded or coincided with immune stimulation. For example, as mentioned above, NLRP6 was significantly up-regulated by both co- and pre-treatment, and nuclear factor, interleukin 3 regulated (NFIL3) and TNF-receptor associated factor 1 (TRAF1) were decreased in both conditions. However, most notably, there were also 71 genes that dexamethasone pre-treatment regulated uniquely compared with dexamethasone co-treatment, including the up-regulated immune-related genes outlined above, TRAV1, TRBV5-3, TRAV9-1, HLA-DQA2, IGKV3D-1; these may therefore be particularly relevant to the potentiation effects.

3.7 Testing the presence of glucocorticoid resistance

To explore the role of glucocorticoid resistance in this potentiation effect, we investigated whether glucocorticoid resistance was induced by dexamethasone pre-treatment, thus contributing to the potentiation of subsequent inflammatory responses.

3.7.1 Functional test of glucocorticoid resistance

We first used a functional test of glucocorticoid function – the ability of dexamethasone (1μM) to inhibit IL-1β-induced IL-6 secretion – using the same experimental design that

elicits the potentiation effects, that is, in the presence or absence of dexamethasone (1μM) pre-treatment, for 24 h, followed by 24 h rest period.

We found that dexamethasone pre-treatment only minimally affected the ability of the subsequent dexamethasone (1μM) to suppress IL-1β-induced IL-6 secretion (45.4% suppression with pre-treatment vs. 50.5% without), a change that was not significant and with pronounced variation in the dexamethasone pre-treatment group (Figure 12).

3.7.2 GR expression

We also investigated whether GR resistance may have been evident in changes to levels of GR gene expression. GR gene mRNA expression measured using qPCR, 48 hours following treatment with dexamethasone at concentrations of between 1nM and 10μM demonstrated no difference in levels of expression, compared with vehicle treatment (Figure 13).

3.7.3 GR-target genes using transcriptomic analysis

To evaluate the glucocorticoid resistance hypothesis at the genomic level, we evaluated whether GR-target genes were differentially expressed in genome-wide gene expression following dexamethasone treatment, which has been interpreted as evidence of glucocorticoid resistance (Miller et al., 2008), using the Transcription Element Listening System (TELiS) bioinformatics analysis (Cole et al., 2005) (see 2.7). To investigate for glucocorticoid resistance, we used the same treatment conditions that gave rise to the potentiation ofinflammatory effects we observed: namely, cells were treatment with dexamethasone for 24

hours at a concentration of 1μM, washed carefully, and then extracted the mRNA 48 hours later (to correspond to the time point at which potentiation of inflammatory effects were observed), compared with vehicle treatment for this time period (DVV vs VVV). Genome-wide gene expression changes were measured using the Affymetrix system described above. We wanted to test whether the differentially regulated genes that were down-regulated by dexamethasone pre-treatment showed an over-representation of response elements to the GR, as this would indicate glucocorticoid resistance, that is, reduced expression of these genes due to reduced functional activation of the GR. This was tested using the TELiS bioinformatics analysis which identifies whether genes containing transcription factor response elements are under- or over-represented in a gene group. Of the genes down-regulated by dexamethasone pre-treatment, 20 genes were recognised in the TELiS database.

Consistent with our hypothesis, there was a trend for response elements to the glucocorticoid receptor to be over-represented in the genes down-regulated by dexamethasone pre-treatment. Indeed, there was a 19.2-fold greater incidence of genes bearing a response element to the GR in the group of genes down-regulated by dexamethasone pre-treatment than would be expected by chance, although this trend was non-significant ($p=0.051$). This is suggestive of a down-regulation of GR-target genes following previous dexamethasone treatment, a finding consistent with glucocorticoid resistance (Miller et al., 2008). However, given the small number of genes analysed (20 genes recognised in the TELiS database), this finding should be cautiously interpreted.

Taken together with the data described above showing minimal reduction in the GR-mediated anti-inflammatory action and no changes in GR expression, these experiments indicate that

GR resistance, if indeed it is induced by dexamethasone pre-treatment, is small and unlikely to be the main mechanisms underpinning the potentiation of inflammatory effects.

4. Discussion

4.1 Overview of findings

We have demonstrated in this study that the glucocorticoids, dexamethasone and cortisol, can potentiateinflammatory effects in human hippocampal progenitor cells, a cell system with significant relevance to depression (Egeland et al., 2015; Sahay and Hen, 2007). Glucocorticoids demonstrated their well-recognised inhibitory effect on inflammatory processes when *co-incubated* with an inflammatory stimulus, but exerted a potentiating effect on inflammatory processes when their treatment *preceded* an inflammatory stimulus and a rest period was allowed, with increased expression of a wide variety of inflammatory molecules, including cytokines and chemokines implicated in the pathogenesis of depression (Dantzer, 2018). We found that this potentiation of inflammatory effect was time-dependent, only occurring when glucocorticoids preceded inflammatory stimulus by a rest period of 24 hours, but not of 1, 12 or 48 hours. The effect was also dose-dependent, only occurring for intermediate (stress-relevant) concentrations of glucocorticoid, not high (pharmacological) or low (basal) concentrations.

We found that the ability of glucocorticoids to potentiateinflammatory processes was dependent on activation of the glucocorticoid receptor, as it could be abrogated by co-treatment with RU486 (De Bosscher et al., 2003). Mechanistically, we found limited evidence

that this effect is dependent on the induction of glucocorticoid resistance, as gene expression of the glucocorticoid receptor was unchanged, and a test of functional glucocorticoid resistance showed minimal reduction; while the examination of glucocorticoid resistance at the genomic level found some support for a down-regulation of GR-target genes, consistent with glucocorticoid resistance, this finding was limited by the small number of genes included in the analysis, and of borderline significance.

On the other hand, we found support for the hypothesis that pre-treatment with glucocorticoids causes up-regulation of inflammatory pathways. In particular, glucocorticoid treatment led to an up-regulation of NLRP6, one of the NOD-like receptors, which have been strongly implicated in the pro-inflammatory effects of glucocorticoids in animals (Frank et al., 2015). Other innate and adaptive immune system genes, such as chemokines and T cell receptor genes, were also found to be up-regulated.

4.2. Glucocorticoids can be pro-inflammatory in human hippocampal progenitor cells

We found that glucocorticoids exhibited pro-inflammatory effects in human hippocampal cells. This is consistent with an accumulating body of work demonstrating that glucocorticoids can have pro-inflammatory effects in a variety of systems: animal brains (Frank et al., 2016), particularly the hippocampus (Johnson et al., 2003), as well as animal hippocampal microglia (Frank et al., 2007, 2011), and peripheral human monocyte/ macrophages (Yeager et al., 2018). This has led to a re-conception of glucocorticoids as more than anti-inflammatory hormones, possessing the ability to complexly modulate the immune system (Cain and Cidlowski, 2017). These findings have led some authors to suggest that

glucocorticoids function as a ‘neuroendocrine alarm signal’ (Frank et al., 2013b), whereby stress primes the immune system for subsequently heightened innate immune responses, as is characteristically present in depression (Miller and Raison, 2016). Our findings demonstrate that this phenomenon also occurs in human neural cells, with potential relevance to the pathogenesis of depression.

4.2 Time-, dose- and GR-dependent properties of the priming effect, consistent with animal and peripheral human cell studies

The present work demonstrates that the characteristics that typify the pro-inflammatory properties of glucocorticoids in animals and peripheral human cells are also evident in human neural cells (Cain and Cidlowski, 2017; Frank et al., 2016): in particular, the effects are dependent on activation of the glucocorticoid receptor, and are both time- and dose-dependent. We found that the pro-inflammatory effect was dependent on activation of the GR as the effect could be blocked by co-incubation of dexamethasone with RU486. This is consistent with findings in animal studies where RU486 administered during either psychological stress or glucocorticoid administration abrogates the potentiating effects of glucocorticoid treatment to microglia stimulated ex vivo (Frank et al., 2011). It is now thought that the glucocorticoid receptor transduces either anti- or pro-inflammatory effects through differential genomic, and non-genomic effects, and that these effects are dose- and time-dependent (Cain and Cidlowski, 2017).

Interestingly, we also observed that the effects of equi-molar doses of cortisol had greater potentiating effects than dexamethasone, perhaps explained by cortisol’s ability to activate

both the MR and GR, while dexamethasone only causes translocation of the GR(Duque and Munhoz, 2016). The MR has also been implicated in the pro-inflammatory properties of glucocorticoids, potentially because cytokine promoters contain elements responsive to mineralcorticoids (Duque and Munhoz, 2016). Further examination of this issue was beyond the scope of this paper, but might be pursued in future by examining the effect of aldosterone, a specific binder of the MR.

We found evidence that the pro-inflammatory properties of glucocorticoids were time-dependent in human neural cells. Co-incubation of glucocorticoids and an inflammatory stimulus in the human hippocampal cells produced well-recognised anti-inflammatory properties (De Bosscher et al., 2003), as have also been demonstrated in animal brains (Frank et al., 2010). In the present paper, incubation with dexamethasone for 24 hours produced potentiation of the inflammatory response to IL-1β stimulation only when the IL-1β stimulation was delayed by a further 24 hours, and not if it occurred 1 hour, 12 hours or 48 hours after dexamethasone incubation. This is consistent with findings in animals where 24 hours of delay is required after either stress or glucocorticoid treatment in order to produce pro-inflammatory effects (Frank et al., 2015); shorter periods produce anti-inflammatory effects (Frank et al., 2010). This is possibly the ideal time for the pro-inflammatory cascade activated by the GR during the dexamethasone pre-treatment to exert its full downstream action.

This specificity of timing has been attributed to the ability of glucocorticoids to prime cells, such as microglia, for subsequent inflammatory response by increasing their ability to sense danger signals, through up-regulation of receptor molecules such as toll-like receptors (TLRs)

and NLRP3, combined with an acute inhibitory effect on the release of cytokines (Frank et al., 2016). Such a model predicts that glucocorticoids are inhibitory to inflammatory processes in the acute phase, but prime subsequent inflammatory responses in the sub-acute phase (Cain and Cidlowski, 2017; Frank et al., 2013b). It has been found that this potentiated immune response can persist for 4-6 days after exposure to inescapable stress in rats (Johnson et al., 2002), which, based on animal to human time equivalencies, might approximate weeks in a human (Quinn, 2005). It is currently unclear how the time course of the potentiation in human hippocampal cells in vitro – for example, the finding that inflammatory potentiation was not demonstrated after a 48 hour delay - would correspond to the longer time-lines in vivo.

Consistent with findings in animals and human peripheral cells (Cain and Cidlowski, 2017), we also found that the pro-inflammatory effects were dose-dependent, occurring for intermediate concentrations of dexamethasone (and cortisol) but not for low or high concentrations, For example, it has been demonstrated that human subjects administered low and high concentrations of cortisol before an LPS challenge do not demonstrate a potentiation effect, but those administered intermediate concentrations (thought to be equivalent to plasma levels of cortisol in stress) do, with the effect maximal at 100nM of cortisol (Yeager et al., 2011), consistent with findings in our study. This has been interpreted as evidence that glucocorticoids display a biphasic dose-response curve (Cain and Cidlowski, 2017; Frank et al., 2013a), whereby stress levels of glucocorticoids may sensitise cells to harmful stimuli by up-regulating receptors for danger signals, whereas high concentrations of glucocorticoids overcome this sensitisation by restraining the immune response and blunting cytokine signals (Cain and Cidlowski, 2017; Frank et al., 2013a). It is not clear how time and dose might

interact in these effects, with further research required to elucidate the pleiotropic effects of glucocorticoids.

4.3. Glucocorticoid resistance vs. pro-inflammatory pathways as the mechanism underpinning the potentiation effects

Our study provides some insights into the mechanisms underlying this pro-inflammatory effect in human neural cells. Two hypotheses have been proposed to explain the co-existence of increased inflammation and increased cortisol in depression: the presence of glucocorticoid resistance (Miller, 2008) and the pro-inflammatory effects of glucocorticoids (Frank et al., 2016).

Glucocorticoid resistance has been demonstrated to associate with the increased inflammation seen in stressed populations (Cohen et al., 2012; Miller et al., 2008), although our recent meta-analysis on this topic (Perrin et al., 2019) finds that a surprisingly small number of studies measure glucocorticoid resistance and inflammation in the same depressed subjects, with only limited evidence supporting the notion that glucocorticoid resistance in depressed patients is the main mechanisms underpinning inflammation. Our present study is consistent with this clinical evidence, as we also found evidence for only minimal glucocorticoid resistance upon pre-treatment with dexamethasone, that is, the condition which produced pro-inflammatory effects. We found only minimal functional resistance (tested by the effective of dexamethasone on the IL-6 response), no changes in GR expression and only suggestive evidence of down-regulation of GR-target genes. It is possible that these effects were not pronounced because of the short time period for which glucocorticoids were exposed to the

cells. It is also possible that glucocorticoid resistance may have been more evident at lower concentrations of dexamethasone, as has been demonstrated in the peripheral cells of stressed subjects (Miller et al., 2002), where glucocorticoid resistance was more evident on exposure to 10nM of dexamethasone than 50nM or 250nM. We found similar findings in the peripheral cells of inflamed depressed patients with coronary heart diseases (Nikkheslat et al., 2015). One study has found that microglia from rodents exposed to repeated social defeat do not display glucocorticoid resistance and demonstrated that these microglia produce more IL-6, MCP-1 and TNF-α in response to LPS stimulation compared with microglia from control animals (Wohleb et al., 2011); this ex vivo finding is highly consistent with the present study. Altogether, our findings do not indicate glucocorticoid resistance as the sole or main mechanism underpinning the pro-inflammatory action of glucocorticoids.

Perhaps not surprisingly, we demonstrated that many cytokine and chemokine genes, for example, TNF-α, IL-1α, and CCL1, as well as their receptor targets, for example, chemokine receptor 4 (CXCR4), were down-regulated by dexamethasone co-treatment with IL-1β, consistent with the classic anti-inflammatory action, and many studies that find that dexamethasone down-regulates pro-inflammatory immune genes (Webster et al., 2002).

However, the most important genomic finding in our study is that both co- and pre-treatment with glucocorticoids up-regulate multiple pro-inflammatory genes and pathways, consistent with findings in other cell types exposed to glucocorticoids (Cain and Cidlowski, 2017). For example, co-treatment upregulates the chemokines, chemokine ligand 5 (CXCL5), chemokine ligand 20 (CCL20), and Nod-like receptor family, pyrin domain containing 6, (NLRP6), while pre-treatment upregulate T cell receptor genes, TRAV1, TRBV5-3, TRAV9-1, and most

notably, again, NLRP6, indicating that it remained elevated for 48 hours following dexamethasone treatment, potentially indicating that one of the molecular mechanisms underpinning the potentiation effects is the persistent activation of pro-inflammatory pathways (see below). These findings are consistent with a genome-wide expression study of glucocorticoid-treated blood mononuclear cells, which found up-regulation of genes of the innate immune system, including chemokines, cytokines and Toll-like receptors (Galon et al., 2002). Another study found that glucocorticoids increase the expression of danger signal receptors, such as TLR2 and TLR4, as well as NLRP3, a NOD-like receptor, in cultured and primary macrophages (Busillo et al., 2011). Although the exact genes upregulated by glucocorticoids in our and these other studies are different, the overall pattern of a pro-inflammatory signal persistently activated (while the main inflammation phenotype is inhibited in the short term) is consistent across all of these studies (Busillo et al., 2011; Galon et al., 2002), with specific differences in the gene profiles probably arising from the difference in concentration and duration of glucocorticoid treatment, cell type examined and the time elapsed before cells were harvested for analysis.

Notably, CCL20 and CXCL5, chemokines up-regulated in our study by dexamethasone were also found to be potentiated by pre-treatment with glucocorticoids in human macrophages subsequently stimulated with LPS (van de Garde et al., 2014). These genes are regulated by the MyD88 pathway, which regulates the expression of IL-1β and CCL20 (van de Garde et al., 2014), and thus our findings provide evidence that the MyD88 pathways may be involved in the ability of glucocorticoids to potentiate inflammation in human hippocampal cells. Notably, the MyD88 pathways is also involved in the pathway that activated NF-κB, and transcribes pro-IL-1β and NLRP3 (Kaufmann et al., 2017).

NOD-like receptors have been particularly implicated in the pro-inflammatory effects of glucocorticoids (Cain and Cidlowski, 2017; Frank et al., 2016). NOD-like receptors form inflammasomes that are critical to the neuroinflammatory cascade, through activation of the cytokine precursor pro-IL-1β. The NLRP3 inflammasome requires both a priming and an activation step (Frank et al., 2016). It has been found that glucocorticoids up-regulate NLRP3 in macrophages, and that these cells subsequently exhibit potentiated inflammatory responses (Busillo et al., 2011), suggesting that glucocorticoids are able to prime these cells. Consistent with this, exposure to stress increases NLRP3 in hippocampal microglia that subsequently show potentiated inflammatory responses (Weber et al., 2015). Furthermore, NLRP3 inhibition during chronic mild stress prevents pro-inflammatory cytokine increases (and depressive behaviour) in rats (Liu et al., 2015), and NLRP3 is activated in the peripheral blood mononuclear cells of depressed patients (Kaufmann et al., 2017), suggesting that this activation process may be relevant in depression. Interestingly, activation of NLRP3 can induce glucocorticoid resistance (Paugh et al., 2015), suggesting that up-regulation of inflammatory pathways and induction of glucocorticoid resistance may not be mutually exclusive pathways – a proposition for which we find some evidence in our study. We extend the known involvement of members of the NOD-like receptor family in the ability of glucocorticoids to be pro-inflammatory to human hippocampal cells, where NLRP6, another NOD-like receptor, may be particularly important.

4.4 Potential role for NLRP6 in pro-inflammatory effects of glucocorticoids

NLRP6, a key gene in our paper that is up-regulated by both pre- and co-treatment with dexamethasone regulates inflammation and host defence against microorganisms, like other members of the NOD-like receptor family (Levy et al., 2017). NLRP6 participates in inflammasome formation, NF-κB and MAPK signalling regulation (Levy et al., 2017). These inflammasome complexes function as innate sensors of endogenous or exogenous stress or damage associate molecular patterns (Wlodarska et al., 2014). NLRP6 has previously been investigated most closely in the intestine (Levy et al., 2017), but the NLRP6 inflammasome has also been demonstrated to play an important role in response to injury in the brain (Wang et al., 2017). NLRP6 has been found to be present in astrocytes and neurons, but not in microglia (Wang et al., 2017), whereas NLRP3 has been found to be highly expressed in microglia, but not reported to be expressed in neurons or astrocytes (De Rivero Vaccari et al., 2014). This suggests that the increase in NLRP6 in our neural cells may be a mechanism by which glucocorticoids potentiate inflammation in neurons, mirroring the role for NLRP3 in pro-inflammatory effects identified in microglia (Frank et al., 2016) and macrophages (Busillo et al., 2011).

4.5 Limitations

One potential limitation was the use of progesterone in the culture media, as in previous protocols with these cells (Anacker et al., 2013; Johansson et al., 2008; Pollock et al., 2006). As progesterone activates steroid receptors this may influence some of the pathways examined in this paper, including those activated by glucocorticoids and RU486. However, if progesterone does have a relevant effect at the doses used in this study, its effects should be

subtracted out by its use in both control and intervention conditions; however, this does not rule out the possibility of interaction effects.

All experiments in this study were conducted in hippocampal progenitor cells, given their importance in regulating stress responses (Egeland et al., 2015), however, it would also be interesting to explore the effects of glucocorticoid exposure to subsequent inflammatory stimulation in the mature cell phenotypes of neurons, astrocytes and oligodendrocytes that develop from these cells, particularly given the recent finding that pre-natal exposure to stress can affect vulnerability to stress exposure later in life, which might include inflammatory responses (Provençal et al., 2019). It would be particularly interesting to explore the effect of treating the progenitor cell stage with glucocorticoids to determine the effect of inflammatory responsiveness on the subsequently developed mature phenotypes. Future studies could also re-capitulate the paradigms employed here in differentiated cells, exploring the differential sensitivity of different cell types to glucocorticoid exposure and their differential response to inflammatory stimulation.

4.6 Conclusions

Our findings suggest a mechanism by which glucocorticoids, thought to contribute to the pathogenesis of depression (Stetler and Miller, 2011), may synergise with inflammation to contribute to the pathogenesis of depression, rather than being solely counter-regulatory to inflammation (Cain and Cidlowski, 2017). This fits with increasing recognition that glucocorticoids have pro-inflammatory properties in a wide variety of circumstances: in particular functioning as a priming signal before an inflammatory insult (Cain and Cidlowski,

2017). The present study extends this finding to human hippocampal progenitor cells, which have been particularly implicated in the pathogenesis of depression (Egeland et al., 2015; Sahay and Hen, 2007). It is consistent with the body of evidence that suggests early stressful experiences, especially during childhood, increase levels of inflammation in adults at baseline (Baumeister et al., 2015), and when subject to laboratory stressors (Pace et al., 2006), and that this is associated with an increased risk of developing depression (Aschbacher et al., 2012). This research also provides further evidence of the mechanism thought to underlie this pro-inflammatory state: the ability of glucocorticoid surges to prime the innate immune system of the human brain (Frank et al., 2013b).

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Figures

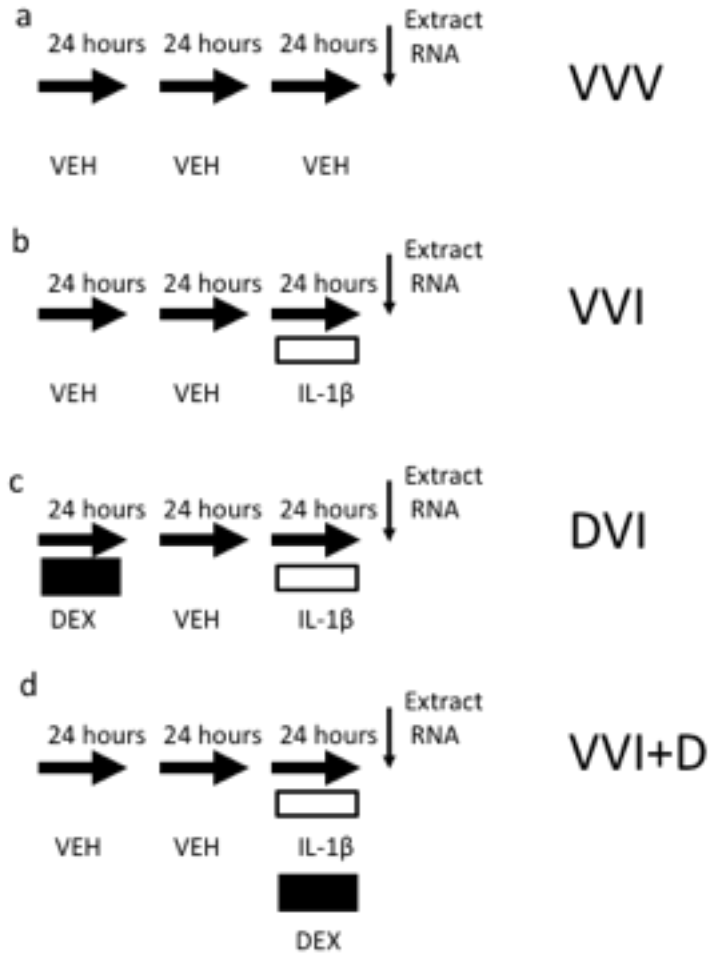


Fig. 1. Conditions analysed for genome-wide gene expression. There were four conditions examined: (a) vehicle treatment; (b) inflammatory stimulation alone (IL-1β at a concentration of 10ng/mL); (c) pre-treatment with dexamethasone (1μM), followed by a rest period, then stimulation with IL-1β; and (d) co-treatment with dexamethasone (1μM) and inflammatory stimulation (IL-1β at a concentration of 10ng/mL).

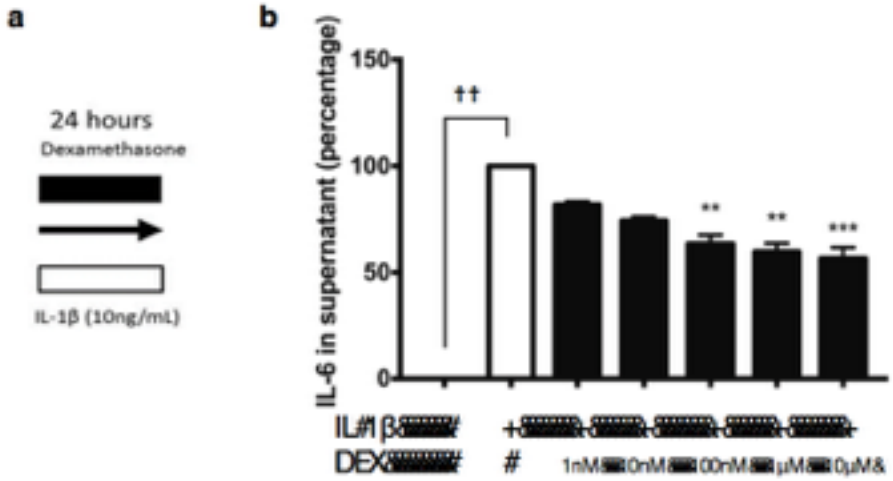


Fig. 2. Dexamethasone inhibits release of IL-6 dose-dependently upon co-incubation.

(A) Cells were co-incubated with dexamethasone and IL-1β (10ng/mL) for a period of 24 hours. (B) Dexamethasone reduced the large increase in IL-6 detected in the supernatant upon IL-1β treatment alone. The inhibitory effect of dexamethasone was dose-dependent. Maximal effect was achieved with DEX 10μM, reducing IL-6 by 43% ($p<0.01$). Results are presented as a fraction of IL-6 detected in the supernatant upon IL-1β treatment alone ± SEM**$p<0.01$, ***$p<0.001$ compared to IL-1β treated condition. ++ $p<0.01$ compared to vehicle treatment. n=3-11.

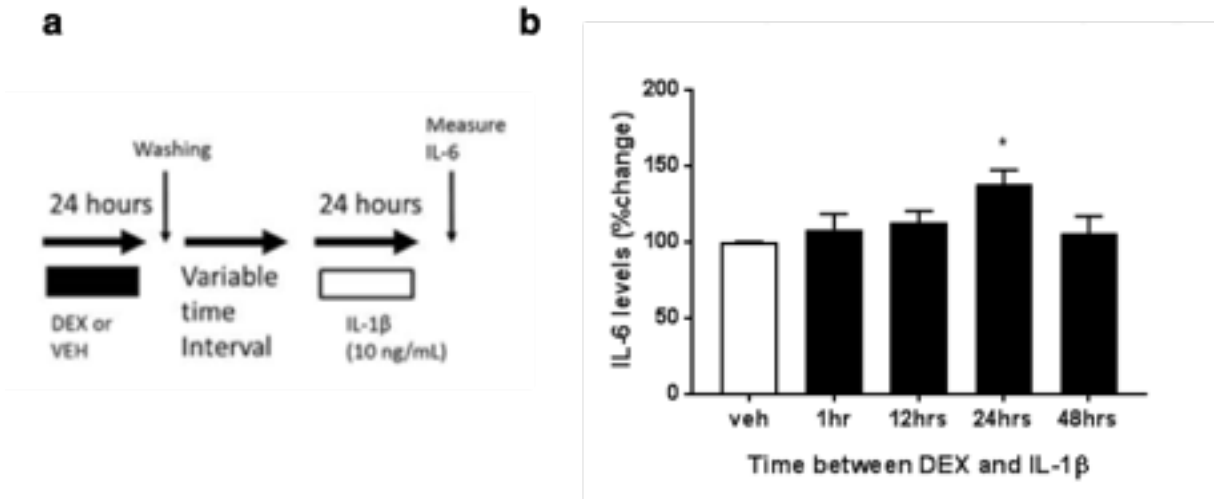


Fig. 3. Effect of time interval between DEX pre-treatment and IL-1β stimulation. (A) Hippocampal progenitor cells were treated with vehicle or DEX (1μM) for 24 hours, before an interval of no treatment. This interval was varied from 1 hour to 48 hours before subsequent stimulation with IL-1β (10ng/mL). (B) Only a time interval of 24 hours significantly potentiated levels of IL-6. A time interval of 1 hour, 12 hours and 48hours did not significantly potentiate subsequent inflammatory responses. Data is presented as percentage change of IL-6 detected in the supernatant from vehicle pre-treatment before IL-1β stimulation +/-SEM. *$p<0.05$ compared to vehicle pre-treatment before inflammatory stimulation. n=7-12

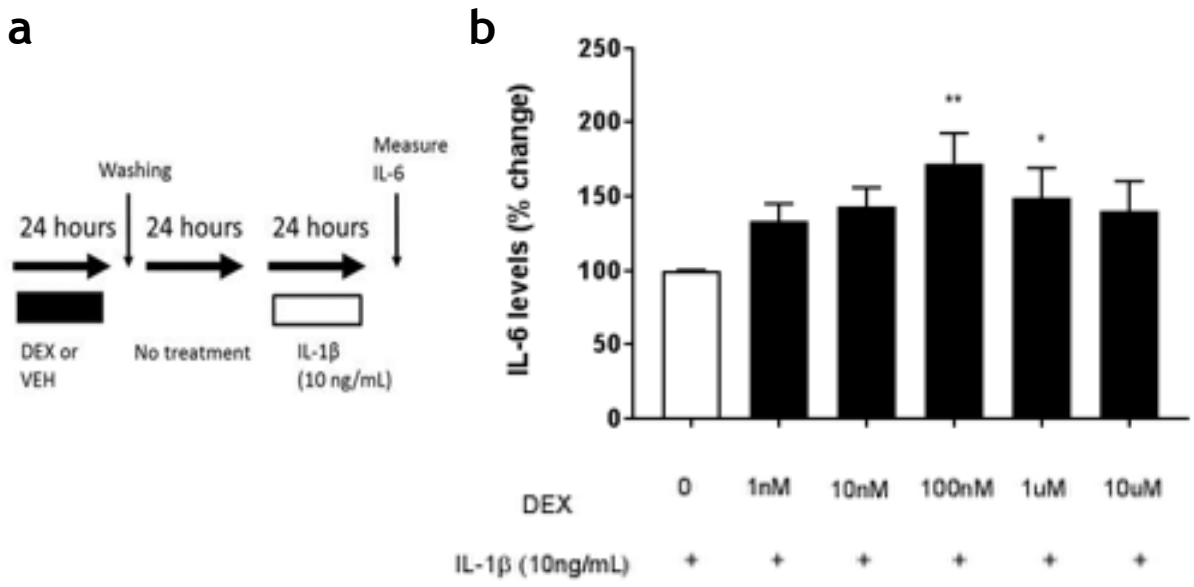


Fig. 4. Dose-dependent effects of dexamethasone priming. (A) Hippocampal progenitor cells were treated with concentrations of dexamethasone varying from 1nM to 10μM for 24 hours, before thorough washing and 24 hours of no treatment. Cells were then treated with IL-1β (10ng/mL) for 24 hours and the amount of IL-6 produced in the supernatant measured. (B) All tis of dexamethasone used for pre-treatment increased the IL-6 secreted into the supernatant, with significance reached for concentrations of 100nM and 1μM. Data is presented as percentage change from levels of IL-6 detected in the vehicle pre-treatment condition +/- SEM. *$p<0.05$, **$p<0.01$ compared with vehicle pre-treatment condition.

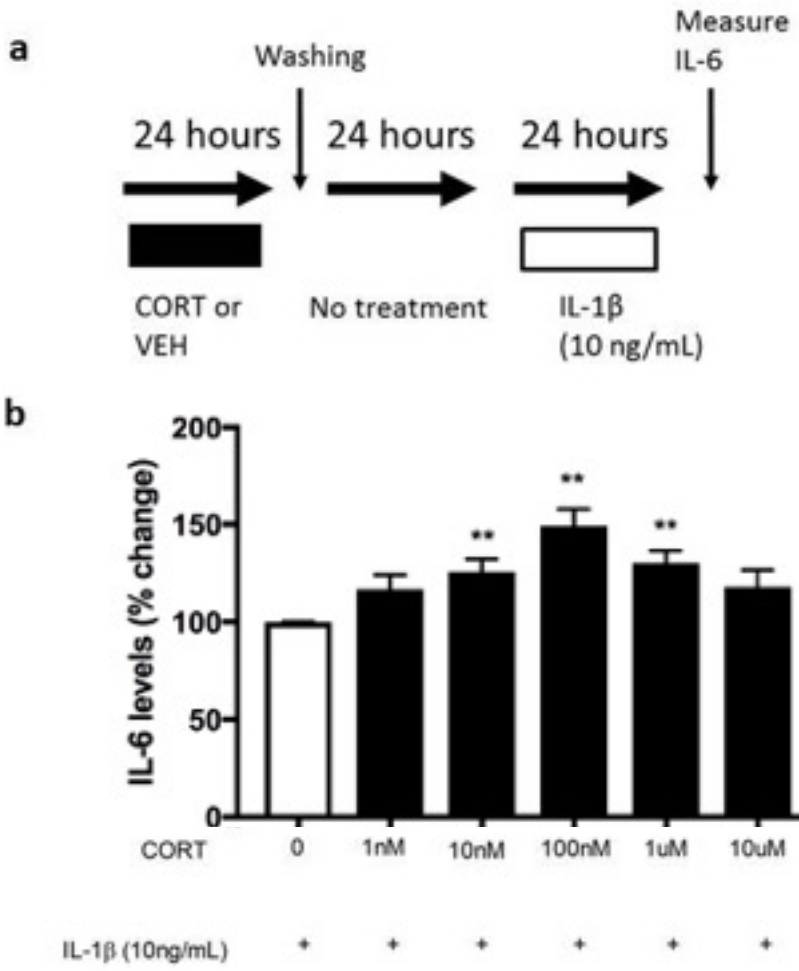


Figure r cells were treated before thorou 1β (10ng/mL) fo d. (b)All concen nto the supernatant, with significance reached for concentrations of 10nM, 100nM, and 1μM. Data is presented as percentage change from levels of IL-6 detected in the vehicle pre-treatment condition. * $p<0.05$, ** $p<0.01$ compared with vehicle pre-treatment condition.

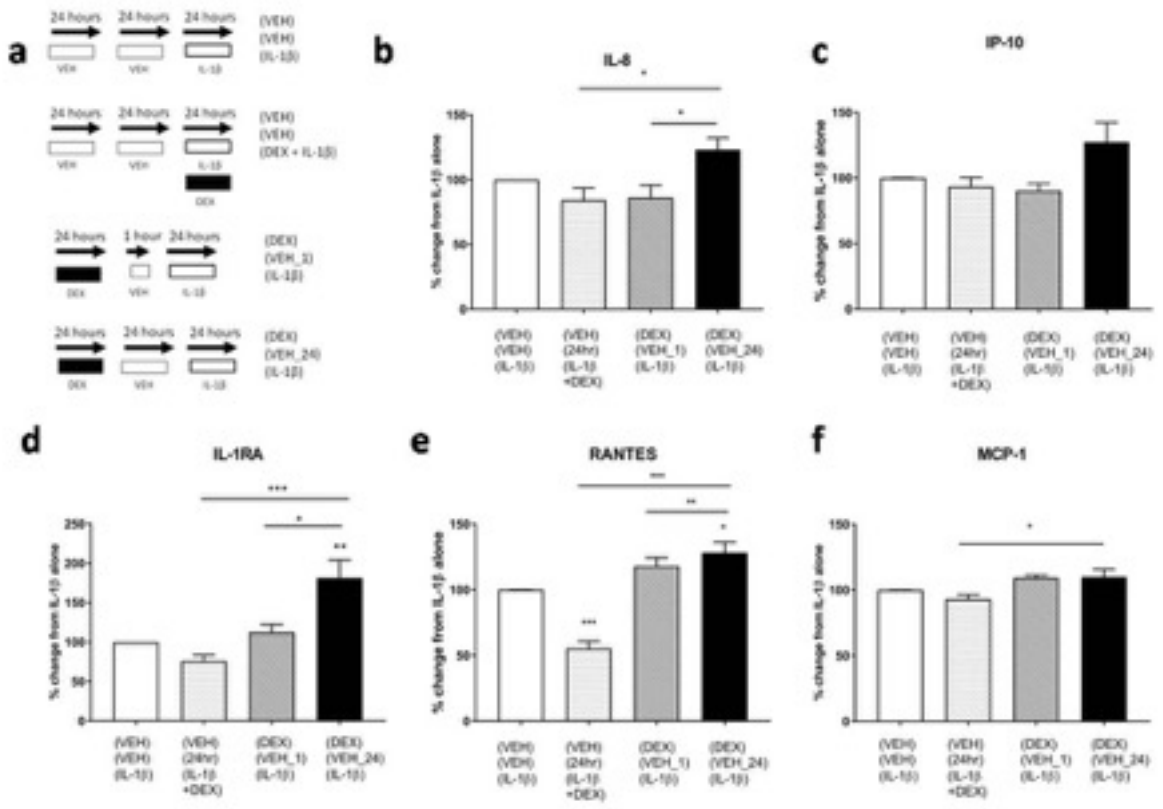
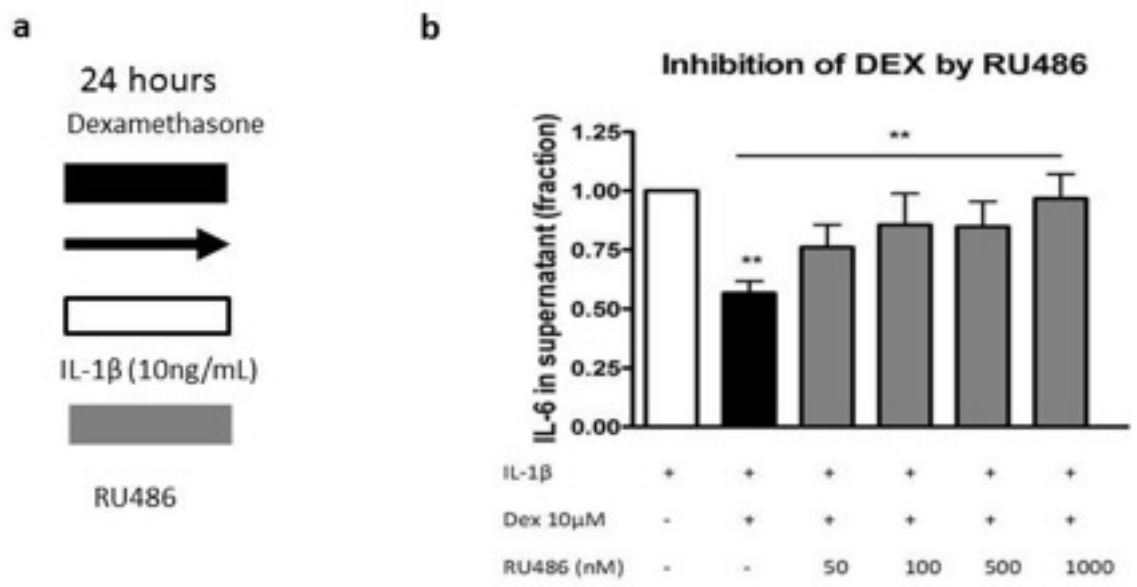


Fig. 6. Time-dependent effects of glucocorticoid treatment on multiple chemokines and cytokines. (A) Four different conditions as in Figure 22. (B)-(F) The effect of timing on five different immunoproteins. Data is presented as percentage change from levels of immunoprotein detected in the IL-1β alone treatment condition. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to IL-1β treatment alone, or the indicated condition. n= 6-8.

Fig. 7. Dose-dependent inhibition of dexamethasone effects by GR antagonist RU486. (A) Human neural progenitor cells were co-incubated with IL-1β (10ng/mL), DEX 10μM and concentrations of RU486 from 50nM to 1μM for 24 hours before measurement of IL-6 in the



supernatant. (B) DEX 10μM (black column) inhibits secretion of IL-6 into the supernatant upon co-incubation with IL-1β, compared with IL-1β treatment alone (white column). RU486, a glucocorticoid receptor antagonist, dose-dependently inhibits this effect of DEX on IL-1β-induced IL-6 secretion (grey columns). The effect of DEX was completely abrogated by a concentration of 1μM of RU486 (right-most grey column).

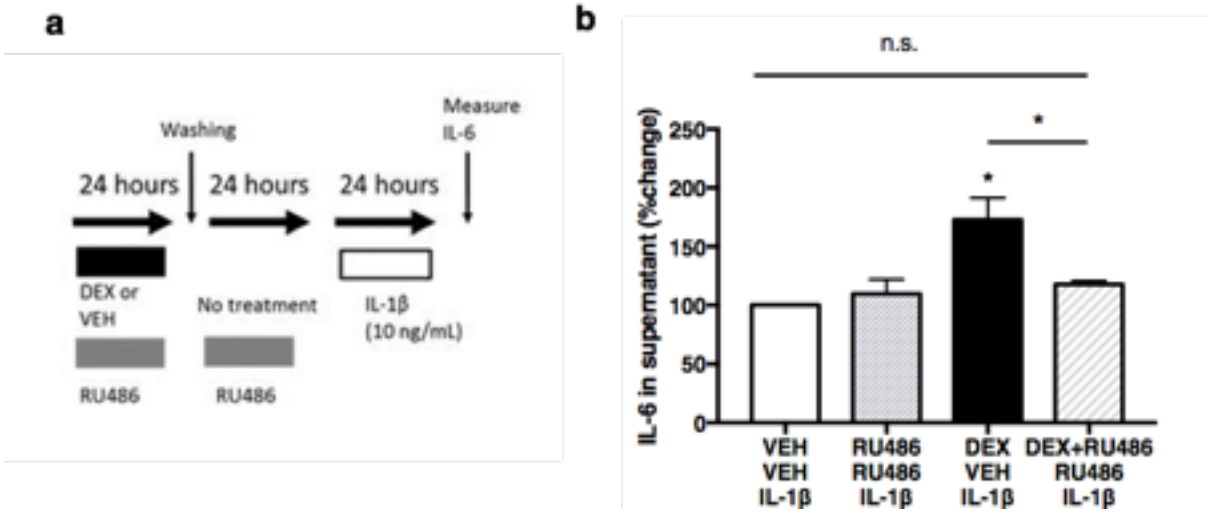


Fig. 8. The pro-inflammatory effects of dexamethasone are GR-dependent. (A) Cells were co-treated with RU486 (1μM) during DEX (1μM) pre-treatment and the period of no treatment

before inflammatory stimulation. (B) RU486 co-treatment (fourth column) abolishes the pro-inflammatory effect of DEX pre-treatment (third column). Treatment with RU486 alone during the first 48 hours of the paradigm has no significant effect (second column). *$p<0.05$ compared with vehicle pre-treatment or DEX pre-treatment, where indicated. V- vehicle treatment; R – RU486 (1μM), D - DEX 1μM; I-IL-1β (10ng/mL).

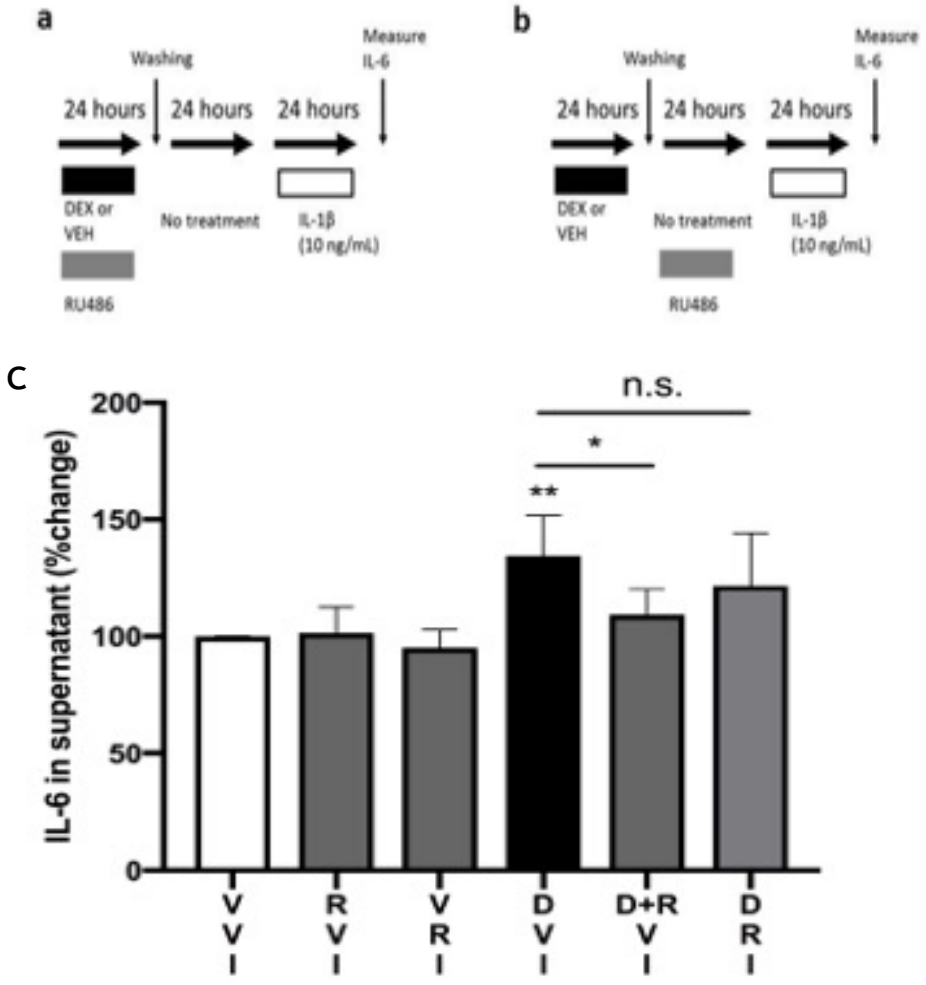


Fig. 9. The pro-inflammatory effects of dexamethasone are induced during pre-treatment. (A) Cells were either co-incubated with RU486 during DEX pre-treatment or (B) during the

interval between DEX pre-treatment and inflammatory stimulation. (C) Co-incubation of RU486 (500nM) during DEX (1μM) pre-treatment (second column from right) significantly reduced the pro-inflammatory effect of DEX pre-treatment (black column), while treatment with RU486 during the interval between DEX pre-treatment and inflammatory stimulation (right-most column) showed a non-significant reduction. RU486 alone in either period (left-most grey columns) showed no significant effect upon inflammatory response.* $p<0.05$, ** $p<0.01$ compared with vehicle pre-treatment. V- vehicle treatment, R – RU486 (500nM), D - DEX 1μM; I- IL-1β (10ng/mL).

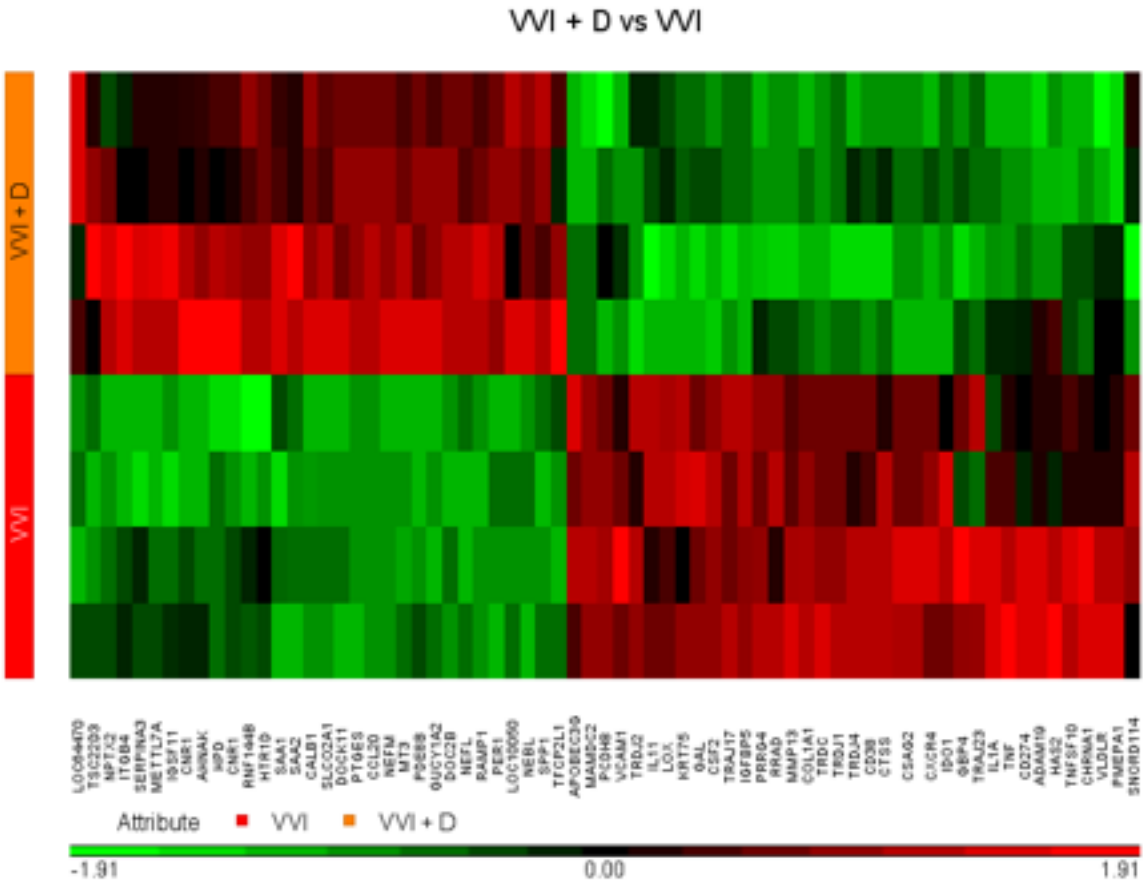


Figure 10 Heatmap of genes differentially regulated by dexamethasone co-treatment with IL-1β compared to IL-1β treatment alone. The genes represented here are those with a fold-

change of greater than 1.5, with significant $p<0.05$ after correction for multiple comparisons. A list of the genes most strongly up-regulated by this treatment are shown in Table S2.

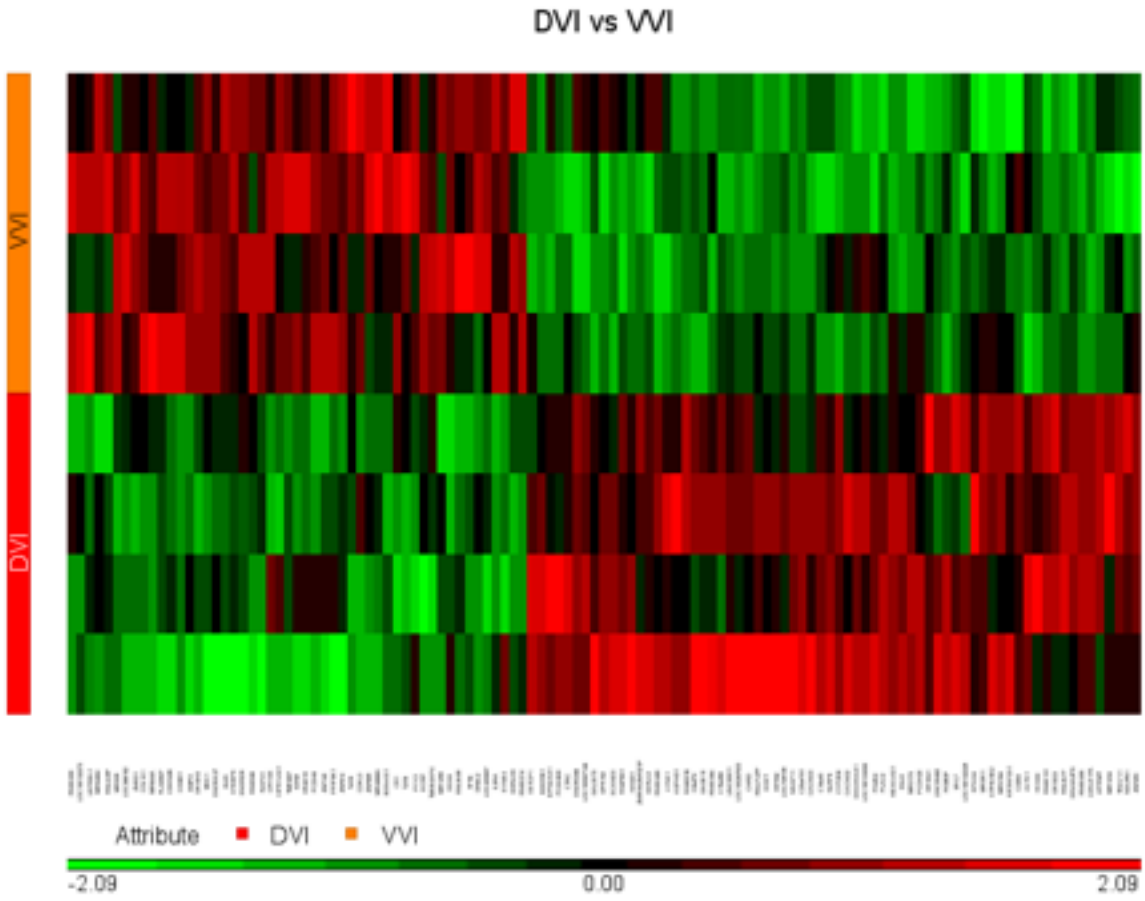


Figure 11 Heatmap of genes differentially regulated by dexamethasone treatment preceding IL-1β compared to IL-1β treatment alone. The genes represented here are those with a fold-change of greater than 1.2, with significant $p<0.05$ after correction for multiple comparisons. A list of the genes most strongly up-regulated by this treatment are shown in Table S2.

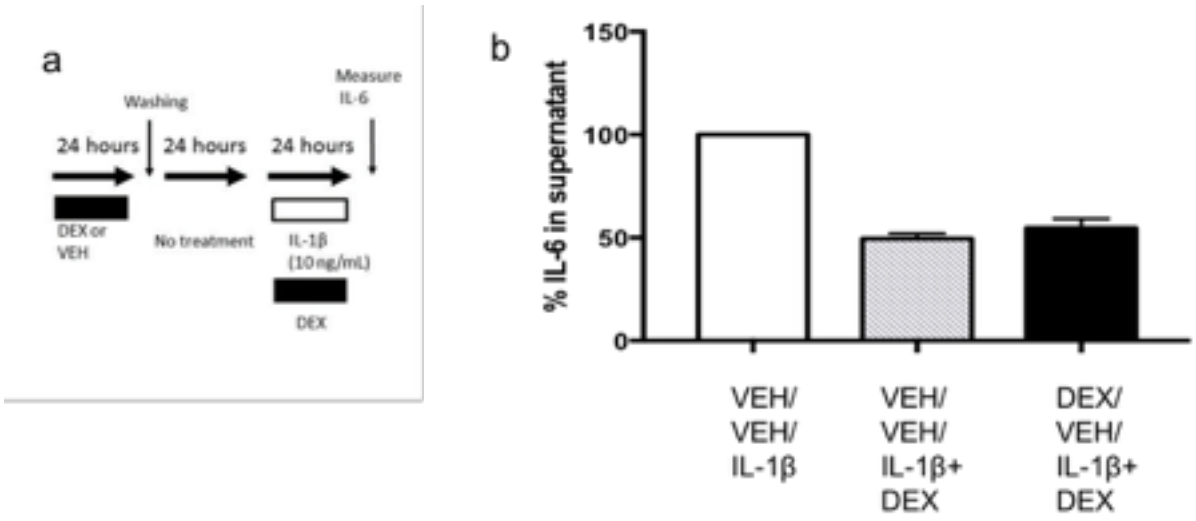


Fig. 12. Glucocorticoid resistance with and without glucocorticoid pre-treatment. (A) Glucocorticoid sensitivity was measured by determining the percentage inhibition of IL-1β (10μ/mL)-induced IL-6 secretion by DEX 1μM. The VEH condition entailed no pre-treatment, and the DEX condition involved DEX 1μM pre-treatment, 24 hours before co-treatment, analogous to the paradigm employed above. (B) DEX inhibited IL-6 secretion by 50.5% (compared with IL-1β stimulation alone) in the VEH pre-treatment condition, and 45.4% in the DEX pre-treatment condition, though there was no difference in significance. Data is presented as percentage change from IL-1β stimulation alone ± SEM. n=6.

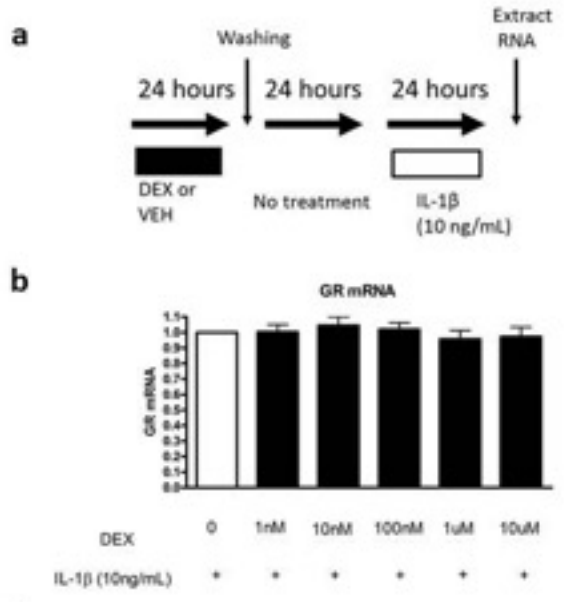


Fig. 13. No change in gene expression of GR or NF-κB in glucocorticoid potentiation (a)mRNA was extracted from experiments conducted as previously (24 hours DEX, 24 hours no treatment, 24 hours IL-1β) with the concentration of DEX varied from 1nM to 10μM. (b) No significant difference in GR mRNA expression was found between DEX pre-treatment at any concentration and vehicle pre-treatment. Data are shown as fractional change from vehicle pre-treatment ± SEM. n=4

Tables

Pathway	P-Value
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.04E-09
Molecular Mechanisms of Cancer	1.02E-08
Interferon Signalling	2.34E-07
Endocannabinoid Cancer Inhibition Pathway	9.77E-07
Role of NFAT in Cardiac Hypertrophy	1.62E-06
Colorectal Cancer Metastasis Signalling	1.66E-06
Axonal Guidance Signalling	6.46E-06
Amyotrophic Lateral Sclerosis Signalling	8.32E-06
TWEAK Signalling	8.91E-06
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	1.62E-05
Wnt/β-catenin Signalling	1.70E-05
Cell Cycle: G1/S Checkpoint Regulation	2.00E-05
IL-1 Signalling	2.69E-05
Wnt/Ca+ pathway	2.75E-05
Apoptosis Signalling	3.31E-05
IL-8 Signalling	3.47E-05
TNFR2 Signalling	4.57E-05
Hepatic Fibrosis / Hepatic Stellate Cell Activation	4.90E-05
Death Receptor Signalling	5.01E-05
Adipogenesis pathway	5.25E-05
Ephrin B Signalling	5.89E-05
Neuroinflammation Signalling Pathway	6.46E-05
Type I Diabetes Mellitus Signalling	6.92E-05
Ephrin Receptor Signalling	8.51E-05

Role of PKR in Interferon Induction and Antiviral Response	9.12E-05
Cyclins and Cell Cycle Regulation	1.05E-04
Protein Kinase A Signalling	1.15E-04
Glioblastoma Multiforme Signalling	1.35E-04
Aryl Hydrocarbon Receptor Signalling	1.58E-04
Choline Biosynthesis III	1.78E-04
Synaptogenesis Signalling Pathway	1.95E-04
Acute Myeloid Leukemia Signalling	2.24E-04
P2Y Purigenic Receptor Signalling Pathway	2.24E-04
G Beta Gamma Signalling	2.34E-04
Relaxin Signalling	2.63E-04
α-Adrenergic Signalling	2.69E-04
CREB Signalling in Neurons	2.82E-04
Pancreatic Adenocarcinoma Signalling	2.88E-04
Cardiac Hypertrophy Signalling	3.02E-04
Cardiac Hypertrophy Signalling (Enhanced)	3.31E-04
PI3K Signalling in B Lymphocytes	3.47E-04
Endocannabinoid Developing Neuron Pathway	3.80E-04
Androgen Signalling	4.27E-04
Chronic Myeloid Leukemia Signalling	4.47E-04
Induction of Apoptosis by HIV1	4.57E-04
Inflammasome pathway	4.57E-04
Cardiac β-adrenergic Signalling	4.79E-04
IL-6 Signaling	4.79E-04
TNFR1 Signaling	4.79E-04
Osteoarthritis Pathway	5.13E-04

Table 1 50 ingenuity pathways most significantly modulated by IL-1β treatment.

Gene pathway	p-value

Hepatic Fibrosis / Hepatic Stellate Cell Activation	4.47E-06
Osteoarthritis Pathway	4.68E-06
Granulocyte Adhesion and Diapedesis	6.76E-06
Agranulocyte Adhesion and Diapedesis	1.41E-05
Amyotrophic Lateral Sclerosis Signalling	1.66E-05
TGF-β Signalling	2.40E-05
VDR/RXR Activation	2.40E-05
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	7.41E-05
TREM1 Signalling	8.13E-05
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	8.13E-05
STAT3 Pathway	9.33E-05
p38 MAPK Signalling	1.41E-04
Cardiac Hypertrophy Signalling (Enhanced)	1.55E-04
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	3.89E-04
NF-κB Signalling	4.27E-04
IGF-1 Signalling	5.75E-04
Toll-like Receptor Signalling	7.76E-04
Atherosclerosis Signalling	9.77E-04
Ephrin Receptor Signalling	1.00E-03
Neuroinflammation Signalling Pathway	1.32E-03
Role of IL-17F in Allergic Inflammatory Airway Diseases	1.35E-03
Endocannabinoid Cancer Inhibition Pathway	1.41E-03
Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	1.45E-03
Role of IL-17A in Psoriasis	2.00E-03
IL-7 Signalling Pathway	2.00E-03
eNOS Signalling	2.14E-03

FAT10 Cancer Signalling Pathway	2.24E-03
TNFR2 Signalling	2.57E-03

Table2. Gene pathways significantly regulated by dexamethasone co-incubation.

Gene	Gene name	p-value	Fold-Change
CALB1	calbindin 1	0.005205	1.52
NLRP6	NLR family, pyrin domain containing 6	0.001942	1.40
CASQ1	calsequestrin 1	0.008988	1.39
ZNF493	zinc finger protein 493	0.034524	1.33
OR2A14	olfactory receptor, family 2, subfamily A, member 14	0.018139	1.31
SCGB1D2	secretoglobin, family 1D, member 2	0.027948	1.29
FAM66B	family with sequence similarity 66, member B	0.044288	1.29
CGB8	chorionic gonadotropin, beta polypeptide 8	0.00313	1.29
RN5S407	RNA, 5S ribosomal 407	0.04602	1.28
LRRC32	leucine rich repeat containing 32	0.044109	1.28
F11R	F11 receptor	0.019957	1.28
CACNG3	calcium channel, voltage-dependent, gamma subunit 3	0.031778	1.27
DOCK9-AS1	DOCK9 antisense RNA 1	0.020203	1.27

RN5S401	RNA, 5S ribosomal 401	0.006838	1.26
RPS18	ribosomal protein S18	0.042952	1.26
RN5S131	RNA, 5S ribosomal 131	0.024136	1.26
LYZL2	lysozyme-like 2	0.028925	1.25
C20orf181	chromosome 20 open reading frame 181	0.04731	1.25
PDZD2	PDZ domain containing 2	0.015709	1.25
RNU6-77	RNA, U6 small nuclear 77	0.001105	1.25
CDSN	corneodesmosin	0.007558	1.25
PRCD	progressive rod-cone degeneration	0.013879	1.25
RAB3IL1	RAB3A interacting protein (rabin3)-like 1	0.016996	1.25
WAS	Wiskott-Aldrich syndrome (eczema-thrombocytopenia)	0.010956	1.24
TSTD3	thiosulfate sulfurtransferase (rhodanese)-like domain containing 3	0.044267	1.23
IGKV3D-11	immunoglobulin kappa variable 3D-11	0.043524	1.23
LINC00511	long intergenic non-protein coding RNA 511	0.032733	1.23
BHLHA15	basic helix-loop-helix family, member a15	0.046192	1.22
TRAV1-1	T cell receptor alpha variable 1-1	0.015794	1.22
TRBV5-3	T cell receptor beta variable 5-3 (non-functional)	0.014098	1.22
TRAV9-1	T cell receptor alpha variable 9-1	0.041023	1.22
RAMP3	receptor (G protein-coupled) activity modifying protein 3	0.033979	1.22

RGS7	regulator of G-protein signalling 7	0.006123	1.22
PLAC2	placenta-specific 2 (non-protein coding)	0.04911	1.22
GRHL1	grainyhead-like 1 (Drosophila)	0.013868	1.22
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma deriv	0.038929	1.22
AGPHD1	aminoglycoside phosphotransferase domain containing 1	0.004394	1.22
EREG	epiregulin	0.046585	1.22
C8orf74	chromosome 8 open reading frame 74	0.041721	1.22
CYP4A11	cytochrome P450, family 4, subfamily A, polypeptide 11	0.047322	1.22
PLCXD3	phosphatidylinositol-specific phospholipase C, X domain containing	0.021273	1.21
UTY	ubiquitously transcribed tetratricopeptide repeat gene, Y-linked	0.009369	1.21
FAM221B	family with sequence similarity 221, member B	0.005735	1.21
OR8S1	olfactory receptor, family 8, subfamily S, member 1	0.038976	1.21
FAM171B	family with sequence similarity 171, member B	0.012797	1.21
KRT40	keratin 40	0.036118	1.21
RAMP1	receptor (G protein-coupled) activity modifying protein 1	0.03359	1.21

MSRA	methionine sulfoxide reductase A	0.038493	1.20
HLA-DQA2	major histocompatibility complex, class II, DQ alpha 2	0.016277	1.20

Table 3. All genes up-regulated by dexamethasone pre-treatment, arranged in order of fold-change.