Synergy between interleukin-1β, interferon-γ and glucocorticoids to induce TLR2 expression involves NF-κB, STAT1 and GR

Akanksha Bansal¹, Cora Kooi², Keerthana Kalyanaraman¹, Sachman Gill¹, Andrew Thorne¹, Priyanka Chandramohan¹, Amandah Necker-Brown¹, Mahmoud M. Mostafa¹, Arya Milani¹, Richard Leigh², and Robert Newton¹*

Affiliations: ¹Department of Physiology & Pharmacology and ²Department of Medicine, Lung Health Research Group, Snyder Institute for Chronic Diseases, Cumming School of Medicine, University of Calgary, AB, Canada.

*Corresponding author
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Name of corresponding author: Dr. Robert Newton

Address: HRIC-4C56, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1

Telephone: 403.210.3938

Email: rnewton@ucalgary.ca

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ABBREVIATIONS: ALI, air-liquid interface; Bud, budesonide; COPD, chronic obstructive pulmonary disease; Dex, dexamethasone; GAS, γ-interferon-activated-sequence; GR, glucocorticoid receptor; GRE, glucocorticoid response element; ICS, inhaled corticosteroids; IFNG, interferon-γ; IFNG+Dex, IFNG plus dexamethasone; IKK, inhibitor of κB kinase; IL1B, interleukin-1β; IL1B+Dex, IL1B plus dexamethasone; IL1B+IFNG+Dex, IL1B plus IFNG plus dexamethasone; JAK, Janus kinase; MAPK, mitogen activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; STAT, signal transducer and activator of transcription; PAMPs, pathogen-associated molecular patterns; pHBECs, primary human bronchial epithelial cells; TLR, toll-like receptor.
Abstract

Glucocorticoids act via the glucocorticoid receptor (GR; NR3C1) to downregulate inflammatory gene expression and are effective treatments for mild-moderate asthma. However, in severe asthma and virus-induced exacerbations, glucocorticoid therapies are less efficacious, possibly due to reduced repressive ability and/or the increased expression of pro-inflammatory genes. In human A549 epithelial and primary human bronchial epithelial cells (pHBECs), toll-like receptor (TLR) 2 mRNA and protein were supra-additively induced by interleukin-1β (IL1B) plus dexamethasone (IL1B+Dex), interferon-γ (IFNG) plus dexamethasone (IFNG+Dex) and IL1B plus IFNG plus dexamethasone (IL1B+IFNG+Dex). Indeed, ~34-2100-fold increases were apparent at 24 h for IL1B+IFNG+Dex and this was greater than for any single or dual treatment. Using the A549 cell model, TLR2 induction by IL1B+IFNG+Dex was antagonized by Org34517, a competitive GR antagonist. Further, when combined with IL1B, IFNG or IL1B+IFNG, the enhancements by dexamethasone on TLR2 expression required GR. Likewise, inhibitor of κB kinase 2-inhibitors reduced IL1B+IFNG+Dex-induced TLR2 expression, and TLR2 expression induced by IL1B+Dex, with or without IFNG, required the nuclear factor (NF)-κB subunit, p65. Similarly, signal transducer and activator of transcription (STAT) 1 phosphorylation and γ-interferon-activated-sequence-dependent transcription were induced by IFNG. These, along with IL1B+IFNG+Dex-induced TLR2 expression, were inhibited by Janus kinase (JAK) inhibitors. As IL1B+IFNG+Dex-induced TLR2 expression also required STAT1, this study reveals cooperation between JAK-STAT1, NF-κB and GR to upregulate TLR2 expression. Since TLR2 agonism elicits inflammatory responses, we propose that synergies involving TLR2 may occur within the cytokine milieu present in the immunopathology of glucocorticoid-resistant disease, and this could promote glucocorticoid resistance.
Significance statement

This study highlights that in human pulmonary epithelial cells, glucocorticoids, when combined with the inflammatory cytokines, IL1B and IFNG, can synergistically induce the expression of inflammatory genes, such as TLR2. This effect involved positive combinatorial interactions between NF-κB/p65, GR and JAK-STAT1 signalling to synergistically upregulate TLR2 expression. Thus, synergies involving glucocorticoid-enhancement of TLR2 expression may occur in the immunopathology of glucocorticoid-resistant inflammatory diseases, including severe asthma.
Introduction

Synthetic glucocorticoids, administered as inhaled corticosteroids (ICS), are widely and successfully used to treat mild-to-moderate asthma (Rhen and Cidlowski, 2005). However, in patients with severe neutrophilic (aka T2 low) asthma, viral exacerbations, those who smoke or have chronic obstructive pulmonary disease (COPD), ICS medications exhibit reduced clinical efficacy (Barnes, 2013). Thus, partial, or complete, unresponsiveness to ICS therapy in such conditions is often referred to as glucocorticoid resistance or insensitivity. Furthermore, the elevation of ICS dose and/or the inclusion of oral glucocorticoids with the aim of achieving increased clinical effectiveness in such patients leads to systemic adverse effects that include reduced hypothalamus-pituitary-adrenal axis function, weight gain, osteoporosis, hypertension and hyperglycemia leading to diabetes (Liu et al., 2013).

Glucocorticoids act on the glucocorticoid receptor (GR; NR3C1) to suppress the expression of inflammatory mediators and thereby attenuate inflammation (Rhen and Cidlowski, 2005; Newton and Holden, 2007). These effects of glucocorticoid on airway epithelial cells are also thought to be essential in the resolution of lung inflammation caused by allergen inhalation (Klaßen et al., 2017). Glucocorticoids diffuse through the cell membrane and bind to cytoplasmic GR to drive its nuclear translocation. In the nucleus, ligand-bound GR acts as a transcription factor by binding to palindromic glucocorticoid response elements (GREs) which are located in the regulatory regions of target genes. These DNA elements are responsible for driving glucocorticoid-induced expression of many metabolic and anti-inflammatory genes (Jantzen et al., 1987; Imai et al., 1990; Newton and Holden, 2007). However, ligand-activated GR may also interact with inflammatory transcription factors, such as NF-κB and AP-1, and this effect is often suggested as directly causing transcriptional repression (De Bosscher and...
Haegeman, 2009). Despite this, an increasing body of evidence clearly shows that GR can positively interact with inflammatory transcription factors resulting in additive, or even synergistic transcriptional outcomes (Hofmann and Schmitz, 2002; Altonsy et al., 2014; Miyata et al., 2015; Newton et al., 2017; Oh et al., 2017). Thus, glucocorticoid-activated mechanisms variously increase or decrease the expression of inflammatory genes. As a consequence, glucocorticoids should more accurately be considered as being immunomodulatory, rather than simply being “anti-inflammatory” (Busillo and Cidlowski, 2013; Cruz-Topete and Cidlowski, 2015; Newton et al., 2017; Amrani et al., 2020).

Aside from being a primary target for therapy, the airway epithelium is first-in-line to encounter inhaled particulate matter and microbes that enter the airways (Tam et al., 2011; Lambrecht and Hammad, 2012). While these structural cells serve as a physical barrier, they are also fundamental in shaping inflammatory responses (Tam et al., 2011; Lambrecht and Hammad, 2012). In response to environmental insults and stimuli, airway epithelial cells release proinflammatory cytokines, chemokines, and inflammatory enzymes via activation of inflammatory signaling pathways (Lambrecht and Hammad, 2012). Cytokines, such as interleukin-1β (gene symbol: IL1B) and tumor-necrosis factor α (gene symbol: TNF) are central to inflammatory responses, to a large extent due to the activation of the IkB kinase (IKK)-nuclear factor (NF)-κB pathway (Pahl, 1999). Similarly, interferon-γ (gene symbol: IFNG) activates Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling and is a key regulator of immune responses related to viral infection and humoral immunity (Schroder et al., 2004). Interactions between IFNG- and TNF-mediated pathways are also implicated in promoting glucocorticoid resistance (Tliba et al., 2008; Britt et al., 2019). Furthermore, IL1B and IFNG play a variety of roles in airway inflammatory responses, including in severe asthma.
and both bacterial and viral exacerbations of asthma (Ngoc et al., 2005; Lambrecht et al., 2019). Since glucocorticoids are frequently employed as a treatment in these conditions, albeit with reduced efficacy, the co-regulation of inflammatory genes by glucocorticoids, IL1B and IFNG, is likely to be important to the mechanisms that underlie glucocorticoid insensitivity.

Toll-like receptor (TLR) 2 is located on the surface of airway epithelial cells and recognizes pathogen-associated molecular patterns (PAMPs) present in many bacteria, viruses and other microbes (Akira et al., 2001). In a prior study (Bansal et al., 2022), we investigated the synergistic induction of TLR2 expression in airway epithelial cells by glucocorticoids and pro-inflammatory cytokines (IL1B and TNF) which involved positive cooperativity between GR and NF-κB. However, while studies also suggest that glucocorticoids enhance TLR2 expression induced by combinations of TNF and IFNG (Homma et al., 2004; Winder et al., 2009), molecular mechanisms underlying such effects are not established. As many biological effects of IFNG are manifested via the transcription factor, STAT1 (Schroder et al., 2004), we interrogated possible roles and interactions between GR, NF-κB and STAT1 in the regulation of TLR2 expression.
**Materials and Methods**

**Stimuli, drugs and other inhibitors.** Recombinant human IL1B (R&D Systems) was dissolved in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich). Recombinant human IFNG (R&D Systems) was dissolved in de-ionized sterile water. Budesonide (gift from AstraZeneca, Sweden), Dexamethasone (Dex; Sigma-Aldrich), Organon 34517 (Org34517) (gift from Chiesi Farmaceutici, Parma, Italy), PS-1145 (N-(6-chloro-9h-pyrido[3,4-b]indol-8-yl)-3-pyridinecarboxamide) (Sigma-Aldrich), ML120B (N-(6-Chloro-7-methoxy-9H-pyrido[3,4-b]indol-8-yl)-2-methyl-3-pyridinecarboxamide dihydrochloride) (SML1174, Sigma-Aldrich), TPCA-1 (2-[(Aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide) (2559, Tocris), AZD1480 ((S)-5-chloro-N2-(1-(5-fluoropyrimidin-2-yl)ethyl)-N4-(5-methyl-1H-pyrazol-3-yl)pyrimidine-2,4-diamine) (5617, Tocris), Ruxolitinib (βR-cyclopentyl-4-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazole-1-propanenitrile) (7067, Tocris) and Barcitinib (3-Azetidineacetonitrile, 1-(ethylsulfonyl)-3-[4-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol-1-yl]-) (7222, Tocris) were dissolved in dimethyl sulfoxide (DMSO) as stocks of 10 mM. Final DMSO concentrations on cells were ≤ 0.1%. G418 (A1720, Sigma-Aldrich) was dissolved in sterile water as stocks of 100 mg/ml.

**Submersion culture of cells.** A549 human pulmonary type II epithelial cells (American Type Culture Collection; ATCC® CCL-185™; RRID: CVCL_0023) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (all Thermo Fisher Scientific). Non-transplanted normal human lungs obtained from the tissue retrieval service at the International Institute for the Advancement of Medicine (Edison, NJ) were used to isolate primary human bronchial epithelial cells (pHBECs), as previously described (Hudy et al., 2010). No personal identifying information was provided, and
local ethics approval was granted via the Conjoint Health Research Ethics Board of the University of Calgary (Study ID: REB15-0336). Cells were grown at 37°C in 5% CO2:95% air as submersion cultures using complete airway epithelial cell medium (PromoCell). To arrest growth and reducing activation of pathways, cells were incubated in serum- and additive-free basal media overnight prior to all experiments. A549 cells were periodically checked for possible mycoplasma contamination and tested negative. pHBECs were not tested for mycoplasma contamination.

**Air-liquid interface culture of pHBECs.** pHBECs were grown in ALI culture using transwell inserts (3408, Corning), as previously described (Warner *et al.*, 2019; Michi and Proud, 2021). Over 5 - 6 weeks this method produces a highly differentiated cell layer that is made up of mucus-producing goblet cells, ciliated columnar epithelial and basal cells that very closely resemble a normal airways epithelium (Michi and Proud, 2021). Details of our ALI methodology were recently published (Bansal *et al.*, 2022). Only highly differentiated ALI cultures were used. Prior to experiments, cells were fed basally with PneumaCult-ALI basal media (05002, StemCell), with no supplements for 18 h. The cells were then washed with PBS to remove excess mucus prior to both apical and basolateral application of drugs and/or stimuli in fresh medium. Apical treatments (200 µl/well) were diluted in 0.025M HEPES in F12 and removed after 6 h, while basolateral treatments (1 ml/well) were in PneumaCult-ALI basal medium.

**RNA extraction, cDNA synthesis and qPCR.** Total RNA was extracted and 500 ng was used for cDNA synthesis as described (Bansal *et al.*, 2022). cDNA was diluted 1:5 prior to using 2.5 µl for PCR (StepOnePlus™, Applied Biosciences or QuantStudio3, Thermo Fisher Scientific) with Fast SYBR Green Master Mix (4385618, Thermo Fisher Scientific) and primers specific for TLR2 (forward: GCT GCT CGG CGT TCT CTC and reverse: AAG CAG TGA
AAG AGC AAT GGG) and GAPDH (forward: TTC ACC ACC ATG GAG AAG GC and reverse: AGG AGG CAT TGC TGA TGA TCT), as described (Bansal et al., 2022). Relative cDNA concentrations were obtained using standard curves generated by serial dilution of a stimulated cDNA sample analyzed alongside experimental samples. Amplification conditions were: 95 °C for 20 s, then 40 cycles of 95 °C for 3 s, 60 °C for 30 s. Primer specificity was assessed by melt curve analysis using: 95 °C for 3 s, 60 °C for 30 s followed by ramping to 95 °C at 0.1 °C/s with continuous fluorescent measurement. A single peak in the change of fluorescence with temperature was indicative of primer specificity.

**Western blotting.** Western blotting was carried out using standard methods (King et al., 2009). Following cell lysis, proteins were size-fractionated in SDS-PAGE gels prior to transferring to nitrocellulose membranes. After blocking, membranes were probed with primary antibodies against TLR2 (12276, Cell Signaling; RRID: AB_2797867 and ab108998, Abcam; RRID: AB_10861644), IκBα (sc-371, SantaCruz; RRID: AB_10861644), Ser32/Ser36 phosphorylated-IκBα (9246, Cell Signaling; RRID: AB_2267145), p65 (sc-8008, SantaCruz RRID: AB_628017), Ser536 phosphorylated-p65 (3036, Cell Signaling; RRID: AB_331281), GR (PA1-511A, Thermo Fisher Scientific; RRID: AB_2236340), Tyr701 phosphorylated STAT1 (9167, Cell Signaling; RRID: AB_561284), Ser727 phosphorylated STAT1 (9177, Cell Signaling; RRID: AB_2198300), or GAPDH (MCA4739, Bio-Rad; RRID: AB_1720065). Following overnight incubation at 4 °C, the membranes were washed in Tris-buffered saline with 0.05% v/v Tween® 20 detergent (TBS-T) and incubated, as appropriate, with 1:5000 or 1:10,000 dilutions of rabbit or mouse horseradish peroxidase (HRP)-conjugated secondary immunoglobulin (Jackson ImmunoResearch) at room temperature. Immune complexes were detected by enhanced
chemiluminescence (Bio-Rad) and images were captured using a ChemiDoc Touch imaging system (Bio-Rad). Densitometric analysis was performed using ImageLab software (Bio-Rad). Note: TLR2 western blots shown in figure 1A were generated using ab108998 (Abcam). All other TLR2 blots were generated using 12276 (Cell Signaling) due to the discontinuation of ab108998.

**siRNA-mediated knockdown.** Pools of 4 non-targeting siRNAs (SI03650325, SI03650318, SI04380467, 1022064), GR siRNA (SI00003745, SI02654757, SI00003766, SI02654764), RELA siRNA (SI02663101, SI05146204, SI00301672, SI02663094), and STAT1 siRNA (SI03119025, SI04950960, SI02662884, SI05078556) (all from Qiagen) were mixed with 3 µl Lipofectamine® RNAiMax (13778150, Thermo Fisher Scientific) in 100 µl Opti-MEM (31985070, Thermo Fisher Scientific), before incubation at room temperature for 5 min. This mixture was then added to A549 cells, at approximately 70% confluency, in serum-containing growth medium and incubated for 24 - 48 h until the cells were confluent.

**Luciferase reporter constructs and assay.** A549 cells containing the NF-κB reporter, 6κBtk.luc, and the GRE reporter, 2×GRE.luc, are previously described (Newton et al., 1998; Chivers et al., 2004). To generate the 3×STAT1.luc reporter, oligonucleotides containing three tandem repeats of a consensus STAT1 motif (JASPAR database), also known as a γ-interferon-activated sequence (GAS) (Decker et al., 1997), were designed to generate flanking KpnI and XhoI sticky ends. These oligonucleotides were annealed by heating to 95°C for 5 min prior to cooling to room temperature over a 45 min period in 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA. Annealed STAT1 oligonucleotides were cloned into KpnI (R3142, New England Biolabs) and XhoI (R0146, New England Biolabs) digested pGL3.TATA.neo vector (Catley et al., 2004) to generate pGL3.3×STAT1.TATA.neo. Twelve µg of each reporter, or empty vector
(as control), was transfected into sub-confluent A549 cells in T-75 flasks using Lipofectamine 2000 (11668019, Thermo Fisher Scientific) 24 h prior to addition of 1 mg/ml G418 (Sigma-Aldrich). After 14-21 days, foci of G418-resistant cells were passaged in the presence of G418 (0.5 mg/ml) prior to cryo-storage. Luciferase assays were performed using the Firefly Luciferase Assay Kit 2.0 (30085, Biotium Inc.) and luminescence was measured in a 20/20n luminometer (Promega).

**MTT Assay.** Cell viability was measured using MTT Assay. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (#298-93-1, Sigma-Aldrich) was dissolved in Hank’s balanced salt solution (14170112, Thermo Fisher Scientific) to achieve a concentration of 1 mg/mL. Cells grown in 24-well plates were pre-treated with IKK2 inhibitors 90 minutes prior to being treated with IL1B (1 ng/mL). After 6 h, supernatants were removed and 100 μL of MTT solution was added to each well. After 10-20 minutes of incubation in 37°C in 5% CO2/95% air, MTT solution was removed and 200 μL of DMSO was added. The solution was transferred to a 96-well plate and absorbance was measured at 570 nm.

**Data presentation and statistical analysis.** Data were assumed to be normally distributed and are generally presented as means ± S.D. with scatterplots to show individual data points. Multiple comparisons between groups of normally distributed data were made using one-way ANOVA, with the appropriate post-hoc test (Tukey’s or Dunnett’s), as indicated. Comparisons between two treatment groups were made using two-tailed, paired Student t-test. The number of independent experiments (N) is indicated in each graph and sample sizes (N) for experiments were not pre-specified. To test for greater than simple additivity, the sum of the effects (i.e., fold - 1) of each treatment (IL1B, IFNG or glucocorticoid alone) (sum) was compared to the effect of co-treatment (comb). GraphPad Prism software (version 6.01 and 10; Dotmatics, Boston, MA)
was used for statistical analyses and graph plotting. All analyses in the current study were exploratory and were not designed to test prespecified statistical null hypotheses. Consequently, all calculated $p$-values are descriptive and should not be viewed as hypothesis testing.
Results

Regulation of TLR2 protein expression by IL1B, IFNG and glucocorticoid in pulmonary epithelial cells. In previous studies, IL1B, at 1 ng/ml, produced maximal, or near maximal, NF-κB DNA-binding activity, as well as NF-κB-dependent reporter activity and cytokine release in A549 cells (Newton et al., 1996; Catley et al., 2004). Similarly, dexamethasone, at 1 μM, and budesonide, a clinically relevant glucocorticoid, at 300 nM, elicited maximal activation of simple GRE-dependent transcription and maximal expression of glucocorticoid-induced genes (Kelly et al., 2012; Rider et al., 2015). Since dexamethasone and budesonide produced near identical expression profiles in A549 cells (Mostafa et al., 2019), dexamethasone was used as the representative glucocorticoid in the A549 cells and pHBECS grown in submersion cultures, while budesonide was used in pHBECS grown at air-liquid interface (ALI).

In A549 cells, IL1B at 1 ng/ml and dexamethasone at 1 μM each very modestly induced TLR2 protein at 6 h with slightly increased effects observed at 24 h (Fig. 1A, upper panels). These effects were enhanced by IL1B plus dexamethasone (IL1B+Dex) such that the combination effect of IL1B+Dex (i.e., fold IL1B+Dex -1) (comb) was greater (p ≤ 0.01) than the summation (sum) of the effects of IL1B and dexamethasone (i.e., fold IL1B-1 + fold dexamethasone-1) (Fig. 1A, lower panels). Since these data are consistent with our prior report in which IL1B+Dex-induced TLR2 mRNA expression was maximal at 6 h and greater TLR2 protein expression was apparent at 24 h (Bansal et al., 2022), these times were also used to investigate the additional effect of IFNG on IL1B+Dex-induced TLR2. While IFNG (10 ng/ml), at a concentration that induces chemokine release in epithelial cells (Tudhope et al., 2007), produced very little effect at 6 h, co-treatment with IL1B produced a modest increase in TLR2 protein expression. However, co-treatment of IFNG plus dexamethasone (IFNG+Dex), increased
TLR2 protein expression, in a manner that was greater \( (p \leq 0.05) \) than simple additivity (Fig. 1A). Thus, by 24 h, IFNG+Dex increased TLR2 protein levels by 10.7-fold and again revealed \textit{supra}-additivity \( (p \leq 0.05) \). Upon combined stimulation with IL1B plus IFNG plus dexamethasone (IL1B+IFNG+Dex), TLR2 protein was increased \( (p \leq 0.05) \) to 11.3-fold at 6 h, and this was further increased to 33.6-fold \( (p \leq 0.05) \) by 24 h (Fig. 1A). In each case, this response was markedly \( (p \leq 0.05) \) more than each individual treatment and, at 24 h, TLR2 protein expression induced by IL1B+IFNG+Dex was significantly greater \( (p \leq 0.01) \) than that for each single treatment (Fig. 1A, upper panels;), as well as consistently, and significantly (all \( p \leq 0.05 \)) more than for each of the three dual treatments (Supplemental Fig. 1A). Moreover, comparison of the sum of the effects of each stimulus with the effect of the combined stimulation revealed obvious \( (p \leq 0.05 \& 0.01, \text{respectively}) \) \textit{supra}-additivity between IL1B, IFNG and dexamethasone at both 6 and 24 h (Fig. 1A, lower panels).

In pHBECs grown in submersion culture, IL1B, IFNG and dexamethasone alone produced low levels of TLR2 protein, whereas treatment with IL1B+Dex, IL1B+IFNG, or IFNG+Dex, each more clearly induced TLR2 protein at 24 h (Fig. 1B, upper graph). Summation of the effects for each of these pairs of stimuli \textit{(sum)} was less \( (p \leq 0.05) \) than the respective combination effect \textit{(comb)} and therefore \textit{supra}-additivity is revealed (Fig. 1B, lower graph). In the case of treatment with IL1B+IFNG+Dex, TLR2 protein was increased by \( \sim 2100 \)-fold \( (p \leq 0.05) \) and this was to a higher level than for each of IL1B, IFNG or dexamethasone alone (Fig. 1B, upper graph). Comparing the additive effects for each of IL1B, IFNG and dexamethasone \textit{(sum)} with the combination effect of IL1B+IFNG+Dex \textit{(comb)} showed marked \( (p \leq 0.05) \) \textit{supra}-additivity between these stimuli on TLR2 protein (Fig. 1B, lower graph).
In pHBECs grown at ALI, while each of IL1B, IFNG and budesonide produced little increase in TLR2 protein at both 6 and 24 h, induction (all \( p \leq 0.01 \)) was produced by IL1B plus budesonide (IL1B+Bud) and IL1B plus IFNG plus budesonide (IL1B+IFNG+Bud) (Supplemental Fig. 2A, upper panels). However, in ALI cultures of pHBECs, none of the combinations of IL1B, IFNG and budesonide revealed statistically significant supra-additivity on TLR2 protein at either 6 or 24 h (Supplemental Fig. 2A, lower panels). Nevertheless, in the case of IL1B+Bud and IL1B+IFNG+Bud, trends towards a greater effect for the combination treatment (comb) compared to the sum of the effects of each stimulus (sum) were apparent.

**Effect of IL1B, IFNG and glucocorticoid on TLR2 mRNA expression in pulmonary epithelial cells.** TLR2 mRNA was only marginally induced by IL1B, IFNG or dexamethasone alone at both 6 and 24 h in A549 cells (Fig. 2A, upper panels). However, the combination of IL1B+Dex increased TLR2 mRNA at 6 h in a manner that was greater (\( p \leq 0.01 \)) than simple additivity (Fig. 2A). Similarly, dexamethasone also enhanced IFNG-induced TLR2 mRNA at 6 and 24 h such that the effect of co-treatment with IFNG+Dex (comb) was higher (\( p \leq 0.05 \)) than the sum of the effects of IFNG and dexamethasone alone (sum) (Fig. 2A). These data indicate supra-additivity between IFNG and dexamethasone on TLR2 mRNA at both 6 and 24 h. Co-treating A549 cells with IL1B+IFNG also led to slight increases in TLR2 mRNA at 6 and 24 h, although no combinatorial effect of IL1B and IFNG on TLR2 mRNA was observed. However, combined stimulation of A549 cells with IL1B+IFNG+Dex profoundly induced TLR2 mRNA to 19 and 69-fold, at 6 and 24 h, respectively (\( p \leq 0.001 \) and 0.01, respectively) (Fig. 2A, upper panels). Such induction of TLR2 expression was greater (all \( p \leq 0.001 \)) than was evident for IL1B, IFNG or dexamethasone alone at both 6 and 24 h. Moreover, comparison of the sum of the effects of single treatments with that of the effect of the combined stimulus showed marked (\( p \leq

0.05 and 0.01, respectively) supra-additivity between the effects of IL1B, IFNG and dexamethasone at 6 and 24 h (Fig. 2A, lower panels). Furthermore, the addition of IL1B, dexamethasone or IFNG to each of the three dual treatments led to reproducible and statistically significant increases in TLR2 mRNA expression at both 6 and 24 h (Fig. 2A; Supplemental Fig 1B). Thus, clear positive combinatorial effects between IL1B, IFNG and dexamethasone on TLR2 mRNA expression were revealed in A549 cells.

Similarly, the effects of IL1B, IFNG and glucocorticoid on TLR2 mRNA were investigated in pHBECS. In submersion cultures of pHBECS, TLR2 mRNA was modestly induced by IL1B, IFNG and dexamethasone alone at both 6 and 24 h (Fig. 2B, upper panels). Treatment with IL1B+Dex induced ($p \leq 0.01$) TLR2 mRNA at 6 h, and IFNG+Dex induced ($p \leq 0.001$) TLR2 mRNA at 24 h (Fig. 2B, upper panels). While combined stimulation with IL1B+IFNG showed little increase in TLR2 mRNA at 6 and 24 h, dexamethasone enhanced ($p \leq 0.001$) the IL1B+IFNG-induced TLR2 mRNA at both times (Fig. 2B, upper panels). Moreover, IL1B+IFNG+Dex-induced TLR2 mRNA to 63 and 56-fold, at 6 and 24 respectively, and this was more (both $p \leq 0.01$) than each of IL1B, IFNG or dexamethasone alone. Comparison of the effects of the combination treatments (comb), IL1B+Dex, IFNG+Dex or IL1B+IFNG+Dex, to the sum of the effects of the respective individual treatments (sum) revealed obvious (all $p \leq 0.05$) supra-additivity at both 6 and 24 h (Fig 2B, lower panels).

In ALI cultures of pHBECS, TLR2 mRNA was modestly induced by IL1B and budesonide alone, and clear combinatorial effects, with supra-additivity ($p \leq 0.05$), between IL1B and budesonide were observed at both 6 and 24 h (Supplemental Fig. 2B). While IFNG barely induced TLR2 expression in the ALIs, co-treatment with IL1B led to increased ($p \leq 0.05$) TLR2 mRNA at both 6 and 24 h (Supplemental Fig. 2B, upper panels). Similarly, IFNG+Bud co-
treatment increased \( p \leq 0.001 \) TLR2 mRNA above baseline. Moreover, budesonide enhanced \( p \leq 0.01 \) IL1B+IFNG-induced TLR2 mRNA to 11.6 and 7.8-fold at 6 and 24 h, respectively. Thus, at both times, TLR2 mRNA induced by the combined stimulation with IL1B+IFNG+Bud was higher \( all \ p \leq 0.01 \) than by IL1B, IFNG or budesonide alone (Supplemental Fig. 2B, upper panels). Indeed, comparison of the sum of the effects of IL1B, IFNG, and budesonide alone (\textit{sum}) with the effect of their combined stimulation \( (comb) \) revealed \textit{supra}-additivity between IL1B+IFNG+Bud at 24 h \( p \leq 0.05 \), but not at 6 h (Supplemental Fig. 2B, lower panels). However, this synergistic effect was no more than for IL1B+Bud combination, i.e., IFNG had no additional effect over IL1B+Bud combination.

As GAPDH expression may vary with some treatments, \( C_T \) values to show GAPDH expression for all treatments are shown (Supplemental Fig 3A-C). In each case, this was unaffected by IL1B, IFNG or dexamethasone treatments in A549, pHBECS and ALI cultures.

**IKK involvement in IL1B+IFNG+Dex-induced TLR2 expression.** Prior work showed a role for NF-κB in the induction of TLR2 by IL1B plus glucocorticoid (Bansal \textit{et al.}, 2022). Here, a role for the canonical IKK pathway, involving IKK2, in the IL1B+IFNG+Dex-induced TLR2 expression was tested in A549 cells using the IKK2-selective inhibitors, PS-1145, ML120B and TPCA-1 (Gamble \textit{et al.}, 2012). For each compound, concentrations of 30 \( \mu \)M produced maximal inhibition of IL1B-induced NF-κB luciferase reporter activity (Fig. 3A; Supplemental Fig. 4A).

TLR2 mRNA and protein expression were modestly induced by IFNG+Dex, at 6 and 24 h, and these effects were significantly enhanced \( p \leq 0.001 \) and 0.01, respectively) by IL1B (Fig. 3B-C). In cells pre-treated with 30 \( \mu \)M PS-1145, ML120B or TPCA-1, the enhancement of IFNG+Dex-induced TLR2 mRNA and protein by IL1B were reduced \( all \ p \leq 0.05 \) by each compound (Fig. 3B-C). In each case, PS-1145 and ML120B produced partial inhibition, whereas
TPCA-1 resulted in more complete inhibition of TLR2 mRNA and protein expression. Neither of these compounds affected the cell viability in the experimental conditions employed (Supplemental Fig. 4B). These data support a role, albeit possibly only partial, for IKK2 in the induction of TLR2 expression by IL1B+IFNG+Dex.

Effects of IFNG on the possible activation of NF-κB were also examined. Treatment of A549 cells with IFNG did not elicit serine 32 and 36 phosphorylation of IκBα (Supplemental Fig. 4C). Likewise, IFNG alone did not induce NF-κB reporter activity and showed no effect on IL1B-induced NF-κB reporter activity, whether in the absence or presence of dexamethasone (Supplemental Fig. 4D, top panel). Thus, while IKK2 is implicated in the induction of TLR2 expression by IL1B+IFNG+Dex, there was no effect of IFNG on activation of the NF-κB pathway or the induction of NF-κB transcriptional activity.

**Involvement of GR in IL1B+IFNG+Dex-induced TLR2 expression.** A role for GR in the synergistic induction of TLR2 expression by IL1B plus glucocorticoid was previously documented (Bansal et al., 2022). In the current study, the role of GR in the induction of TLR2 expression by IL1B+IFNG+Dex was tested using the competitive GR antagonist, Org34517 (Peeters et al., 2004; Joshi et al., 2015a). TLR2 mRNA and protein were both modestly induced by IL1B+IFNG at 6 and 24 h, respectively (Fig. 4A-B; Supplemental Fig. 5A-B). Increasing the concentration of dexamethasone markedly enhanced IL1B+IFNG-induced TLR2 mRNA at 6 h and protein at 24 h. In each case, these enhancements by dexamethasone were competitive with Org34517, at 1 μM, as shown by rightward shifts in the response curve to dexamethasone (Fig. 4A-B). Schild analysis resulted in pA2 values of 8.07±0.16 and 7.85±0.29 for TLR2 mRNA and protein, respectively, which is consistent with prior reports of competitive antagonism at GR. IL1B+IFNG-induced TLR2 mRNA and protein were unaffected by Org34517.
Possible effects of IL1B and IFNG on GRE-dependent transcriptional activity were investigated using A549 cells harbouring a 2×GRE.luc reporter. GRE reporter activity was not induced by either IL1B or IFNG and following induction by dexamethasone at 6 h, reporter activity also remained largely unaffected by IL1B and IFNG treatments (Supplemental Fig. 4D, middle panel). Thus, while the antagonism by Org34517 supports a role for GR in the induction of TLR2 by IL1B+IFNG+Dex, GRE reporter activity was unaffected by IL1B and/or IFNG suggesting that an enhancement of simple GRE-dependent transcription does not directly contribute to the observed synergy in respect of TLR2 expression.

Activation of JAK-STAT signaling by IFNG. STAT1 is the major transcription factor involved in IFNG-dependent transcriptional responses that occur via conventional JAK-STAT signaling (Schroder et al., 2004). To explore possible activation of STAT1, IFNG-induced phosphorylation of STAT1 at Y701 and S727, which are suggested to be involved in nuclear accumulation and transcriptional activation, respectively, were examined (Sadzak et al., 2008). Treatment of A549 cells with IFNG at 10 ng/ml induced rapid phosphorylation of STAT1 at Y701 with statistical significance observed from 15 min onwards (Fig. 5A; Supplemental Fig. 6A). This peaked at 1 h before declining slightly by 6 and 24 h. Phosphorylation of STAT1 at S727 was detectible in unstimulated cells and this was increased ($p \leq 0.05$) by 1 h post IFNG treatment (Fig. 5B; Supplemental Fig. 6B). This phosphorylation declined slightly by 6 h but was further elevated at 24 h. However, while total STAT1 levels were unaffected by IFNG treatment at times until 6 h, there was a ~2-fold ($p \leq 0.05$) increase in total STAT1 at 24 h (Fig. 5B; Supplemental Fig. 6B). This effect largely accounts for the increase in S727 phosphorylation observed at 24 h. Overall, these data indicate activation of STAT1 by IFNG.
The effects of IL1B, IFNG and dexamethasone were tested on phosphorylation of STAT1 at Y701 and S727 at 1 h. STAT1 phosphorylation at Y701 was not induced by IL1B, either in the presence or absence of dexamethasone (Fig. 5C, upper panels). IFNG strongly \( (p \leq 0.05) \) induced STAT1 phosphorylation at Y701, and this was unaffected by the addition of IL1B and/or dexamethasone (Fig. 5C, upper panels). While dexamethasone did not induce STAT1 S727 phosphorylation, IL1B alone induced \( (p \leq 0.01) \) levels of S727 phosphorylated STAT1 that remained high with IL1B+Dex cotreatment (Fig. 5C, lower panels). IFNG alone also induced \( (p \leq 0.01) \) STAT1 S727 phosphorylation, and this was unchanged with IFNG+Dex cotreatment (Fig. 5C, lower panels). Combination of IL1B+IFNG increased S727 STAT1 phosphorylation to a higher level than either IL1B or IFNG alone, both \( p \leq 0.01 \), and this was unaffected by dexamethasone (Fig. 5C, lower panels). Total STAT1 expression was not affected by IL1B, IFNG or dexamethasone, whether alone or in combination (Supplemental Fig. 7A). Overall, these data show increased STAT1 phosphorylation at Y701 and S727 following IFNG treatment. S727 phosphorylation was also increased by IL1B, and this appeared to be additive with the effect of IFNG. In each case, dexamethasone, alone or in combination with IL1B and/or IFNG did not affect STAT1 phosphorylation at Y701 and S727.

To test for possible JAK involvement, the JAK inhibitors, AZD1480, baricitinib, and ruxolitinib, were tested on the ability of IFNG to induce STAT1 phosphorylation. IFNG-induced phosphorylation of STAT1 at Y701 and S727 was prevented by each JAK inhibitor in a concentration-dependent manner (Fig. 6A-C; Table 1). Inserts in panel C provide the log EC\(_{50}\) values obtained for each pair of curves describing the effect of each compound on S727 and Y701 phosphorylated STAT1 in each individual experiment (Fig. 6C). Both AZD1480 and baricitinib revealed greater \( (p \leq 0.01 \) and 0.05, respectively) sensitivity of inhibition in respect of
S727 compared to Y701 phosphorylation of STAT1. With ruxolitinib, this effect was much reduced and while reaching $p = 0.049$, it should be noted that one set of data was excluded (See: Supplemental Fig. 8). The apparent difference in log EC$_{50}$ values should therefore be treated with caution. Total STAT1 expression remained unaffected by the various concentrations of JAK inhibitors in the presence of IFNG (data not shown). Furthermore, unstimulated A549 cells showed lower levels of STAT1 phosphorylation at S727 and these were unaffected by 1 μM of each of the JAK inhibitors (Fig. 6D, left panel). IL1B treatment increased STAT1 S727 phosphorylation compared to baseline which also remained unaffected by the JAK inhibitors (Fig. 6D, left panel). Treatment with IFNG increased STAT1 phosphorylation at S727, and this was completely abrogated to baseline by the JAK inhibitors (Fig. 6D, right panel). Treatment with IL1B+IFNG induced S727 phosphorylation to a greater level than IFNG alone, and each JAK inhibitor partially, but significantly (all $p \leq 0.05$), reduced this response (Fig. 6D, right panel). As stated above, STAT1 phosphorylation at Y701 was not observed in unstimulated or IL1B-treated A549 cells and this was not changed by the JAK inhibitors (data not shown). IFNG and IL1B+IFNG treatments strongly induced similar levels of STAT1 Y701 phosphorylation and this induction was completely prevented by all three JAK inhibitors (Fig. 6E). Total STAT1 expression was unaffected by IL1B, IFNG or IL1B+IFNG treatments, either in the presence or absence of JAK inhibitors (Supplemental Fig. 7B). While these data support the involvement of JAKs in the activation of STAT1 by IFNG, whether in the presence or absence of IL1B, the apparent differences in selectivity on phosphorylation of STAT1 at Y701 versus S727 raise the possibility of mechanistic differences in each process.

**Activation of STAT1-driven transcription by IFNG.** To explore the transcriptional activation of STAT1 by IFNG, A549 cells harbouring a luciferase reporter containing three
consensus STAT1 motifs, corresponding to a conventional GAS, upstream of a luciferase gene driven by a basal promoter (3×STAT1.luc), were stimulated with various concentrations of IFNG (Fig. 7A; Supplemental Fig. 9A). Reporter activity, measured at 6 h, was induced by IFNG in a concentration-dependent manner (EC$_{50}$ = 0.28 ng/ml) with maximal activity occurring at ~10 ng/ml. This correlated with the concentration of IFNG that showed maximal synergy with IL1B and glucocorticoid on the induction of TLR2 expression. To confirm a role for the consensus STAT1 motifs in the induction of reporter activity, A549 cells were stably transfected with a reporter construct where the 3×STAT1 motifs were replaced with mutated sites (Fig. 7B, top panel). As above, IFNG, at 10 ng/ml, induced ($p \leq 0.001$) 3×STAT1.luc reporter activity, whereas cells containing the mutated STAT1 reporter showed no enhancement of reporter activity (Fig. 7B, bottom panel). These data indicate that the consensus STAT1 motif conferred IFNG-inducibility. To confirm a role for STAT1 in IFNG-induced reporter activity, A549 cells were transfected with various concentrations of four different STAT1-targeting siRNAs, or a pool of STAT1 siRNAs, alongside a pool of non-targeting siRNAs as a control. While the non-targeting control siRNAs had no effect, each individual STAT1-targeting siRNA dramatically reduced STAT1 protein expression, with maximal effects apparent at 1 and 10 nM (Fig. 7C; Supplemental Fig. 10). A pool of these four STAT1 targeting siRNAs also reduced ($p \leq 0.05$) STAT1 protein expression by ~90% at both 1 and 10 nM (Fig. 7C). Furthermore, IFNG-induced STAT1 reporter was unaffected by various concentrations of the control siRNA-pool, whereas the STAT1 siRNA-pool markedly diminished ($p \leq 0.001$) reporter activity at all siRNA concentrations (Fig. 7D). This confirms the involvement of STAT1 in the activation of this reporter by IFNG. Moreover, in A549.3×STAT1.luc cells pretreated with various concentrations of the JAK inhibitors, AZD1480, barcitinib, and ruxolitinib, IFNG-induced STAT1
transcriptional activity was abrogated in a concentration-dependent manner (pEC$_{50}$ = 7.05±0.22, 6.86±0.21, and 6.98±0.19 for AZD1480, barcitinib, and ruxolitinib, respectively) (Fig. 7E; Supplemental Fig. 9B). These data confirm that IFNG activates a JAK pathway and leads to STAT1-dependent transcription via consensus STAT1 motifs.

**Involvement of JAK signaling in IL1B+IFNG+Dex-induced TLR2 expression.** To investigate a possible role for JAKs in IL1B+IFNG+Dex-induced TLR2 expression, A549 cells were pretreated for 1 h with AZD14580, barcitinib, and ruxolitinib (each at 1 μM) prior to stimulation with IL1B, IFNG and dexamethasone. TLR2 mRNA and protein were modestly induced by IL1B+Dex at 6 and 24 h, respectively, and this was markedly enhanced (both p ≤ 0.01) by IFNG (Fig. 8). AZD14580, barcitinib, and ruxolitinib each reduced the enhancement of IL1B+Dex-induced TLR2 mRNA by IFNG back to the level achieved by IL1B+Dex (Fig. 8A). As similar data were also obtained for TLR2 protein (Fig. 8B), these data support involvement of JAK signaling in the enhancement of IL1B+Dex-induced TLR2 expression by IFNG.

The effect of IL1B and dexamethasone on IFNG-induced STAT1 transcriptional activity was also investigated using the A549.3×STAT1.luc cells. IL1B and dexamethasone did not induce STAT1 reporter activity. IFNG treatment induced strong activation of STAT1 reporter that was modestly, but significantly (p ≤ 0.05), repressed by IL1B, and largely unaffected by dexamethasone (Supplemental Fig. 4D, lower panel). Since IFNG-induced STAT1 reporter activity was either not affected or reduced by IL1B and/or dexamethasone, simple effects on STAT1-dependent transcription by combined stimulation of IL1B+IFNG+Dex is unlikely to explain the synergy observed between IL1B, IFNG and dexamethasone at inducing TLR2 expression.
Roles of p65, GR and STAT1 on TLR2 expression induced by IL1B, IFNG and dexamethasone. As shown in supplemental figure 4D, IL1B-induced NF-κB reporter activity was unaltered by IFNG and/or dexamethasone. Similarly, as seen above, dexamethasone-induced GRE reporter activity was not affected by IL1B and/or IFNG. Likewise, IFNG-induced STAT1 reporter activity was also unaffected by IL1B and/or dexamethasone. Therefore, the effects of IL1B on GR and STAT1 signaling, or of dexamethasone on NF-κB and JAK-STAT pathway, or of IFNG on the NF-κB or GR signaling, do not explain the synergy between IL1B, IFNG and dexamethasone at the level of TLR2 expression. Nevertheless, the involvement of p65, GR and STAT1 transcription factors, as the primary factors activated by each pathway, seems highly plausible in the synergy in TLR2 expression by combined stimulation with IL1B, IFNG, and dexamethasone. This was tested by knocking down the expression of each factor. To confirm the knockdown p65, A549 cells were transfected with various concentrations of four different p65-targeting siRNAs, or a pool of p65 siRNAs, alongside a pool of non-targeting siRNAs as a control. While the non-targeting control siRNAs had no effect on p65 protein expression, each individual p65-targeting siRNA, as well the p65 siRNA pool, dramatically reduced p65 protein expression at 1 and 10 nM (Supplemental Fig. 11A). NF-κB reporter activity was also tested to assess the effectiveness of the p65 knockdown. Thus, increasing concentrations of each of the p65 siRNAs, along with the p65 siRNA pool, reduced the IL1B-induced NF-κB reporter activity, where the greatest effect was observed at 1 and 10 nM and the non-targeting control siRNAs had no effect (Supplemental Fig. 11B). To achieve knockdown of GR, siRNAs used in a prior study were employed (Thorne et al., 2023). Further, the role of each of p65, GR and STAT1 was tested on TLR2 expression. Therefore, A549 cells were transfected with siRNA pools targeting each of p65, GR and/or STAT1, followed by treatment with various combinations of IL1B, IFNG and
dexamethasone for 24 h. With IL1B+Dex, the control and STAT1-targeting siRNAs had no marked effect on TLR2 protein, whereas marked reductions ($p \leq 0.01$ and 0.05, respectively) in TLR2 protein were observed following knockdown of either p65 and/or GR (Fig. 9A-B). This confirms roles for both p65 and GR in the induction of TLR2 expression by IL1B+Dex. Likewise, in cells treated with IFNG+Dex, the knockdown of STAT1 and GR reduced (both $p \leq 0.01$) TLR2 protein levels, while control and p65 siRNA pools had no effect (Fig. 9, A and C). Therefore, STAT1 and GR appear to be involved in IFNG+Dex-induced TLR2 expression. Similarly, in cells treated with the combination of IL1B+IFNG+Dex, while non-targeting control siRNAs had no significant effect, the p65 and GR siRNA pools almost completely abolished (both $p \leq 0.001$) the IL1B+IFNG+Dex-induced TLR2 protein (Fig. 9D). The STAT1 siRNA pool also significantly ($p \leq 0.05$), but only partially, reduced TLR2 expression induced by IL1B+IFNG+Dex (Fig. 9 D). These data confirm roles for p65, GR and STAT1 in the induction of TLR2 expression by IL1B, IFNG and dexamethasone in combination. Finally, in the case of IL1B+IFNG-treated A549 cells, only the p65 siRNA pool reduced ($p \leq 0.05$) TLR2 protein, while the controls, GR and STAT1 targeting siRNAs had no effect on TLR2 expression (Fig. 9, A and E). This suggests that p65, but not STAT1, appears to be involved in the TLR2 expression induced by IL1B+IFNG.
Discussion

Glucocorticoids have complex impacts on inflammation and variously downregulate or enhance gene expression (Busillo and Cidlowski, 2013; Cruz-Topete and Cidlowski, 2015; Amrani et al., 2020). Aside from metabolic effects, many glucocorticoid-enhanced genes, by controlling signaling and gene expression, are regulatory, whereas others are pro-inflammatory (Busillo et al., 2011; Newton et al., 2017). Mechanisms and relevance for these different effects remain poorly understood, but are required to build a more comprehensive understanding of glucocorticoid action that considers both anti-inflammatory effects with potential enhancements of inflammatory responses. The current study focused on synergy between glucocorticoid and cytokines to induce TLR2 expression in human pulmonary epithelial cells. Consistent with prior reports, dexamethasone when combined with each of IL1B or IFNG supra-additively enhanced TLR2 expression in A549 cells (Shuto et al., 2002; Hermoso et al., 2004; Homma et al., 2004; Sakai et al., 2004; Hoppstädter et al., 2019; Bansal et al., 2022). Building from this, we report that in A549 cells and pHBECs, a clear three-way supra-additivity occurred between the glucocorticoid, IL1B and IFNG as a triple combination. At the mRNA level, the effect of this triple combination surpassed the responses to the individual and dual treatments. Indeed, occurring at maximally effective concentrations of each stimulus, this represents synergy between the three pathways that was readily apparent and replicated at both the mRNA and protein levels of TLR2 expression. Since all the current analyses were at 24 h, or less, further synergy could be observed at longer times, but this would require additional testing. Furthermore, replication of these effects in pHBECs grown in submersion culture supports A549 cells as a model for mechanistic investigations. Nevertheless, while pHBECs grown in ALI culture showed supra-additivity between IL1B and glucocorticoid on TLR2 mRNA, effects of
IFNG were not apparent. This discrepancy could be due to several reasons. For example, IL1B plus glucocorticoid may induce maximal TLR2 expression such that combinatorial effects with IFNG require lower levels of IL1B plus glucocorticoid stimulation. Moreover, signaling and the accessibility of regulatory elements responsible for TLR2 expression induced by IL1B, IFNG and glucocorticoid may differ between undifferentiated pHBECS in submersion culture and highly differentiated ALI cultures. Equally, disparities in the expression of necessary factors between undifferentiated and differentiated pHBECS could differentially regulate TLR2, while enhancer usage certainly can change with differentiation state (Natoli, 2010). In addition, differential contributions by the various differentiated ALI cell types may confound the overall effect. Although not explored, this differential responsiveness could be relevant to heighten host responses in damaged pseudostratified epithelia where cells undergo dedifferentiation as a necessary part of healing and restoration of barrier function.

To better understand combinatorial effects between glucocorticoid, IL1B and IFNG, we identified key factors involved with each stimulus. The regulation of gene expression by IL1B and IFNG commonly involves NF-κB and STAT1, respectively (Pahl, 1999; Schroder et al., 2004), and can produce profound transcriptional synergy (Hiroi and Ohmori, 2005). However, such synergy was not readily observed for TLR2. Rather, TLR2 synergies required the presence of glucocorticoid, prompting us to explore roles for NF-κB, STAT1 and GR. The two glucocorticoids, dexamethasone and budesonide, used in the current study are both GR-selective and produce synonymous effects on gene expression (Hellal-Levy et al., 1999; Millan et al., 2011; Mostafa et al., 2019). By employing Org34517, a competitive GR antagonist, that unlike RU496 (mifepristone) (Jewell et al., 1995; Chivers et al., 2004; Rider et al., 2018), may not lead to GR translocation (Peeters et al., 2008), and which shows a much-reduced level of partial
agonism (Joshi et al., 2015a,b), we indicate a role for GR. Given possible off-target effects of antagonists on other nuclear hormone receptors, we also used silencing of GR to provide compelling evidence for GR involvement in the synergy between IL1B, IFNG and glucocorticoid. This aligns with glucocorticoid-induced GR binding to GRE-containing DNA regions upstream of human TLR2 to activate transcription (Bansal et al., 2022).

The NF-κB pathway was explored using maximally effective concentrations of the IKK2-selective inhibitors, PS-1145, ML120B and TPCA-1. Partial, but significant, reductions in IL1B+IFNG+Dex-induced TLR2 expression by PS-1145 and ML120B, as well as the near complete inhibition by TPCA-1, support roles for the IKK2-NF-κB pathway. However, the partial effects of PS-1145 and ML120B, combined with their greater selectivity for IKK2 compared to TPCA-1 (Gamble et al., 2012), also raise the possible involvement of non-IKK2 pathways. Conversely, the greater inhibition of NF-κB-dependent transcription and TLR2 expression observed for TPCA-1 compared to PS-1145 and ML120B implies off-target effects for this compound. Indeed, reports that TPCA-1 prevents IFNG-induced responses, in the absence of NF-κB activation, support this concern (Tudhope et al., 2007). Nevertheless, following p65 silencing, the resultant loss of TLR2 expression when induced by IL1B+IFNG+Dex, but not by IFNG+Dex, indicates a key role for NF-κB. Given p65 binding to the human TLR2 promoter (Bansal et al., 2022), the current data confirms p65/NF-κB in synergy between IL1B, IFNG and glucocorticoid.

IFNG treatment of A549 cells induced STAT1 phosphorylation at S727 and Y701 with Y701 phosphorylation markedly preceding that for S727. While consistent with STAT1 activation (Wen et al., 1995; Sadzak et al., 2008), the delay in S727 phosphorylation compared to the rapid increase in Y701 phosphorylation suggests mechanistic differences. It is likely that IFNG
receptor activation produces rapid JAK-dependent phosphorylation of Y701, facilitating STAT1 nuclear localization (Sadzak et al., 2008). Conversely, delayed S727 phosphorylation may involve an independent pathway or be consequent on prior Y701 phosphorylation. Notably, S727 phosphorylation was induced by IL1B, and additive summation with the effect of IFNG suggests additional, likely non-JAK, pathways to phosphorylate S727. Indeed, MAPKs, which are activated by IL1B in these cells (Shah et al., 2014), can phosphorylate S727 (Kovarik et al., 1999; Ramsauer et al., 2002; Zhang, 2004). However, in considering effects of IL1B on S727 phosphorylation, it is salient that IFNG-induced STAT1/GAS reporter activity was reduced upon co-treatment with IL1B and therefore questions a positive role for this phosphorylation event.

While the JAK inhibitors, AZD1480, baricitinib, and ruxolitinib were highly efficacious and similarly potent on IFNG-induced STAT1 reporter activity, their effects on STAT1 phosphorylation suggest site-specific differences. Specifically, S727 phosphorylation, compared to Y701 phosphorylation, appeared more sensitive to inhibition by AZD1480 and baricitinib relative to ruxolitinib. Various possibilities may explain this. Given the time lag between Y701 and S727 phosphorylation, it is possible that high levels of JAK activity are required for subsequent S727 phosphorylation. Therefore, modest inhibition of the relevant JAK could reduce S727 phosphorylation while having a reduced, or no effect, on Y701 phosphorylation. However, this hypothesis predicts parallel inhibitory effects of each inhibitor on each phosphorylation. This was likely not observed for ruxolitinib and this raises the possibility that the differential selectivity of the inhibitors for JAK1, JAK2, JAK3, and Tyk2 could explain the data. Indeed, AZD1480 and baricitinib are relatively selective for JAK1 and JAK2, whereas ruxolitinib shows a less selective inhibition profile against the four JAKs (Fridman et al., 2010; Quintás-Cardama et al., 2010; Ioannidis et al., 2011). Therefore, different JAKs may plausibly produce these two
phosphorylation events. This will need to be resolved by additional studies. Further, all three compounds revealed similar, but lower, potency on the STAT1 reporter. Thus, while STAT1 phosphorylation was relatively sensitive to JAK inhibition, the reporter system may show a “reserve” requiring higher levels of kinase inhibition to reduce reporter activity. This is consistent with the silencing of STAT1 where >90% loss of STAT1 protein produced just over 50% inhibition of reporter activity. Similar effects on other pathways involving kinase activation of transcriptional responses are observed, which, anecdotally, aligns with signal amplification and signaling reserves towards the distal parts of pathways.

Regarding synergies produced by IL1B, IFNG and glucocorticoid on TLR2 expression, we provide strong evidence for roles of pathways leading to NF-κB, STAT1 and GR. However, co-activation of these pathways had little effect on transcriptional activation produced by each factor acting via its respective consensus motif. This necessitates gene-specific explanations for the observed synergy. In prior studies, GR and p65 binding to upstream regions of the TLR2 locus was unaffected by co-treatments and suggested that the presence of both factors at the TLR2 promoter was sufficient for combinatorial effects (Bansal et al., 2022). However, these same promoter regions were unresponsive to IFNG (data not shown) indicating involvement of STAT1 at distinct genomic regions. Further studies are therefore required to delineate any such regions.

The current findings advance understanding of the mechanisms leading to synergy but do not address the functional significance of TLR2 synergy. Prior investigators have debated pro- and anti-inflammatory effects for TLR2 (Imasato et al., 2002; Homma et al., 2004; Winder et al., 2009; Hoppstädter et al., 2019). However, TLR2 agonism in cells showing IL1B+Dex-induced TLR2 stimulated NF-κB reporter activity and induced CXCL8 and CCL5 expression (Bansal et
This is consistent with other TLR and IL1 family receptors, which activate NF-κB, MAPKs and inflammatory responses (O’Neill and Bowie, 2007). Likewise, in pHBECS treated with TNF or IFNG plus dexamethasone, TLR2 agonists induce expression of inflammatory genes that include IL6, CXCL8 and human β-defensin-2 (Hertz et al., 2003; Homma et al., 2004; Winder et al., 2009). Moreover, as TLR2 agonism reduced responsiveness to β2-agonists and glucocorticoids (Alkhouri et al., 2014; Rahman et al., 2016), its expression may be unhelpful in chronic inflammatory diseases. Indeed, TLR2 expression is widely documented as being elevated in severe asthma (Simpson et al., 2007; Singhania et al., 2018; Zhang et al., 2021), a condition in which patients are subjected to high-intensity therapies that will high feature β2-adrenoceptor agonists and glucocorticoids. Conversely, enhanced TLR2 expression and activation of inflammatory responses may facilitate host defence responses while glucocorticoids simultaneously suppress general inflammation and promote healing (Busillo and Cidlowski, 2013; Cruz-Topete and Cidlowski, 2015; Amrani et al., 2020). Thus, the co-operation between GR, NF-κB and STAT1 to induce TLR2 expression may be important for normal host defence, but could be undesirable in the (glucocorticoid) treatment of chronic inflammatory disease, including severe asthma, where the expression of cytokines such as IL1B and IFNG is expected. Indeed, while priming of the innate immune response is reported for TLR2 agonists and may be enhanced in the presence of glucocorticoid (Alshaghdali et al., 2021; Deliyannis et al., 2021; Girkin et al., 2021), the antagonism of TLR2, or targeting of TLRs, has also been shown to reduce expression of inflammatory mediators (Su and Weindl, 2018; Thapa et al., 2023).
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Authorship Contributions

Participated in research design: Newton, R., Bansal, A.


Contributed new reagents or analytical tools: Newton, R., Leigh, R.


Wrote or contributed to the writing of the manuscript: Newton, R., Bansal, A., Leigh, R.
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Singhania A, Wallington JC, Smith CG, Horowitz D, Staples KJ, Howarth PH, Gadola SD,


Footnotes

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Person receiving reprint requests:

Robert Newton

HRIC-4C56, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1
Figure Legends

Fig. 1. Combinatorial effect of IL1B, IFNG and glucocorticoid on TLR2 protein expression in pulmonary epithelial cells. (A) A549 cells, or (B) pHBEcs grown in submersion culture, were either not treated, or treated with IL1B (1 ng/ml), IFNG (10 ng/ml) and/or dexamethasone (Dex; 1 µM), as indicated. Cells were harvested at 6 or 24 h for western blot analysis of TLR2 and GAPDH. Representative blots from N independent experiments are shown. ns = non-specific band. (Upper graphs) Densitometric data were normalized to GAPDH, expressed as fold of untreated and mean ± SD were plotted as bar graphs with overlaid scatter plots. Significance was tested using ANOVA with a Tukey post-hoc test. Asterisks (*, **, or *** ) represent significance relative to untreated. Hash (#, ##, or ###) represent significance relative to IL1B + IFNG + Dex. (Lower graphs). The sum of each effect (i.e., fold - 1) within a group of treatments (sum) and the effect (fold - 1) for each combination treatment (comb) is expressed as mean ± SD and plotted as bar graphs overlaid with scatter plots. Significance was tested by paired t-test. *, # p ≤ 0.05, **, ## p ≤ 0.01, ### p ≤ 0.001.

Fig. 2. Effect of IL1B, IFNG and dexamethasone on TLR2 mRNA in human pulmonary epithelial cells. (A) A549 cells, or (B) pHBEcs grown in submersion culture, were either not treated, or treated with IL1B (1 ng/ml), IFNG (10 ng/ml) and/or dexamethasone (Dex; 1 µM), as indicated. Cells were harvested at 6 or 24 h for qPCR analysis of TLR2 and GAPDH. (Upper graphs) TLR2 data from N independent experiments were normalized to GAPDH, expressed as fold of untreated and mean ± SD were plotted as bar graphs with overlaid scatter plots. Significance was tested using ANOVA with a Tukey post-hoc test. Asterisks (*, **, or *** ) represent significance relative to untreated, or as indicated. Hash (#, ##, or ###) represent significance relative to IL1B + IFNG + Dex. (Lower graphs). The sum of each effect (i.e., fold -
1) within a group of treatments (sum) and the effect (fold - 1) for each combination treatment (comb) is expressed as mean ± SD and plotted as bar graphs overlaid with scatter plots. Significance was tested using paired t-test. *, # p ≤ 0.05, **, ## p ≤ 0.01, ### p ≤ 0.001.

**Fig. 3. Effect of IKK inhibitors on the enhancement of IFNG plus dexamethasone-induced TLR2 expression by IL1B.** (A) A549 cells containing the NF-κB reporter, 6κBtk.luc, were either not treated, or pre-treated with 1% DMSO or indicated concentrations of PS-1145, ML120B or TPCA-1 for 90 min, prior to stimulation with IL1B (1 ng/ml). Cells were harvested for luciferase assay. Data from N independent experiments was expressed as fold (bars) and % IL1B (lines) and plotted as mean ± SD. Scatterplots showing individual data points appear as Supplemental Fig. 4A. (B) A549 cells were pretreated with PS-1145, ML120B or TPCA-1, each at 30 µM, for 1.5 h prior to stimulation with IFNG (10 ng/ml) plus dexamethasone (Dex; 1 µM) and IL1B (1 ng/ml) as indicated. Cells were harvested at 6 h for RNA extraction and qPCR analysis of TLR2 and GAPDH mRNA. (C) Cells were treated as in B and harvested at 24 h for western blot analysis of TLR2 and GAPDH protein. (B-C) TLR2 data, from N independent experiments, were normalized to GAPDH and are expressed as mean ± SD and plotted as bar graphs overlaid with scatter plots. Significance was assessed by ANOVA with a Tukey multiple comparison test. Differences from untreated: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, and from IL1B + IFNG + Dex: # p ≤ 0.05, ## p ≤ 0.01, ### p ≤ 0.001.

**Fig. 4. Effect of Org 34517 on the enhancement of IL1B plus IFNG-induced TLR2 expression by dexamethasone.** A549 cells were pretreated with Org 34517 (Org; 1 µM) for 1 h, prior to stimulation with IL1B (1 ng/ml) plus IFNG (10 ng/ml) in absence or presence of various concentrations of dexamethasone (Dex). (A) After 6 h, cells were harvested for RNA extraction and qPCR analysis of TLR2 and GAPDH mRNA. (B) At 24 h, cells were harvested for western
blot analysis of TLR2 and GAPDH. In each case, TLR2 data, from N independent experiments, were normalized to GAPDH and are plotted as means ± SD. Significance between treatments without and with Org was assessed by paired t test (two-tailed). Note: unpaired t test was used at 0.1 µM Dex due one sample failure in the western blotting. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. For illustration, curves were fitted following Schild analysis that assumed a common $E_{max}$ and a Schild slope factor of 1 with the lower asymptote fixed as the response to IL1B + IFNG. This produced $pA_2$ values of 8.07 (±0.16) and 7.85 (±0.29) for TLR2 mRNA and protein, respectively. Scatterplots for all data in A & B are shown as Supplemental Fig. 5A and B.

**Fig 5. Effect of IL1B, glucocorticoid and IFNG on STAT1 phosphorylation.** A549 cells were either untreated, or treated with IFNG (10 ng/ml) prior to harvesting for western blot analysis at the indicated times. Representative blots of (A) phospho-STAT1 (P-STAT1 Y701) and GAPDH, and (B) P-STAT1 (S727), total STAT1, and GAPDH from N independent experiments are shown. In each case, densitometric data normalized to GAPDH are plotted as means ± SD (error bars). Significance relative to untreated at ¼ h was assessed by ANOVA with a Dunnett’s multiple comparison test. (C) A549 cells were either not treated, or treated with IL1B (1 ng/ml), IFNG (10 ng/ml) and/or dexamethasone (Dex; 1 µM), as indicated. Cells were harvested at 1 h for western blot analysis of P-STAT1 (Y701 and S727) and GAPDH. Representative blots of P-STAT1 (Y701 & S727) and GAPDH from N independent experiments are shown. Densitometric data for P-STAT1 (Y701) (upper graph) and P-STAT1 (S727) (lower graph) were normalized to GAPDH, expressed as means ± SD and plotted as bar graphs overlaid with scatter plots. Significance relative to untreated (*) or IL1B + IFNG (#) was tested using ANOVA with a Tukey post-hoc test. */# $p \leq 0.05$, **/## $p \leq 0.01$, ***/### $p \leq 0.001$. Scatterplots for all data in A & B are shown as Supplemental Fig. 6.
Fig. 6. Effect of JAK inhibitors on STAT1 phosphorylation. (A) A549 cells were pre-treated with AZD1480, barcitinib, and ruxolitinib at the indicated concentrations for 30 min, followed by stimulation with IFNG (10 ng/ml) for 1 h. Cells were harvested for western blot analysis of phospho-STAT1 (P-STAT1) and GAPDH. Representative blots for P-STAT1 (Y701), P-STAT1 (S727), and GAPDH from 5 independent experiments are shown. (B) Densitometric data for Y701 P-STAT1 and S727 P-STAT1 from western blots shown in A were normalized to GAPDH and plotted mean ±SD. (C) Data were expressed as %IFNG effect for each of AZD1480, barcitinib, and ruxolitinib, and plotted as means ± SD. Log EC50 of each compound for the inhibition of P-STAT1 (Y701) and P-STAT1 (S727) is expressed as mean ± SD and shown as scatter plots in the inserts within each graph of panel C. Note: one dataset was excluded for ruxolitinib and the full data are shown as Supplemental Fig. 8. (D & E) A549 cells were either not treated or pre-treated with AZD1480, barcitinib, and ruxolitinib, each at 1 µM, for 1 h prior to stimulation with IL1B (1 ng/ml) and IFNG (10 ng/ml) as indicated. Cells were harvested at 1 h for western blot analysis of P-STAT1 and GAPDH. Representative blots for P-STAT1 (Y701 & S727) and GAPDH from N independent experiments are shown. Densitometric data for (D) P-STAT (S727) and (E) P-STAT1 (Y701) were normalized to GAPDH, expressed as means ± SD and plotted as bar graphs overlaid with scatter plots. Significance relative to untreated (*), IFNG (#) or IL1B + IFNG ($) was tested using ANOVA with a Tukey post-hoc test. */#/$/ $ p ≤ 0.05, **#/##/$$ p ≤ 0.01, ***#/###/$$$$ p ≤ 0.001

Fig. 7. Characterization of a STAT1-driven luciferase reporter. (A) A549 cells stably transfected with a luciferase reporter containing three copies of a consensus STAT1-binding motif (shown in B) were either not stimulated (NS) or stimulated with the indicated concentrations of IFNG prior to harvesting for luciferase assay after 6 h. Luciferase activity from
$N$ independent experiments was expressed as fold of NS and plotted as mean ± SD. (B) *Upper panel*, reporter construct showing three copies of the consensus/wild type (*WT*, *bold blue*) or mutant (*Mut*, *bold blue+ red*) STAT1-binding motif is shown. *KpnI* and *XhoI* restriction sites used to clone the construct in pGL3.TATA.neo vector are underlined, with cut sites shown as black arrow-heads. *Lower panel*, A549 cells were stably transfected with the WT or Mut STAT1 reporter constructs and either not stimulated (*NS*) or treated with IFNG (10 ng/ml) for 6 h followed by harvesting for luciferase assay. Luciferase activity from $N$ independent experiments was expressed as fold of NS and mean ± SD was plotted as bar graphs overlaid with scatter plots. Significance relative to untreated cells was determined using paired *t*-test. (C) A549 cells were either not stimulated (*NS*), or incubated with lipid control (*lip*) alone or with the indicated concentrations of either control siRNA-pool (*C si-pool*), or STAT1 siRNA-pool (*STAT1 si-pool*) for 48 h. Cells were then incubated overnight with serum-free media prior to harvesting for western blot analysis. Representative blots of STAT1 and GAPDH from $N$ independent experiments are shown. Data obtained from densitometry analysis were normalized to GAPDH and mean ± SD was plotted as bar graphs overlaid with scatter plots. Significance relative to *C si-pool* at each siRNA concentration was tested by paired *t*-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (D) A549 cells stably transfected with the WT STAT1 reporter construct were treated with lipid or siRNAs as described in C, prior to stimulation with IFNG (10 ng/ml) for 6 h. Cells were harvested for luciferase assay and raw luciferase units (RLU) from $N$ independent experiments were expressed as mean ± SD and plotted as bar graphs overlaid with scatter plots. Significance relative to *C si-pool* at each siRNA concentration was tested by paired *t*-test. (E) A549 cells stably transfected with the WT STAT1 reporter construct were either not stimulated (*NS*) or pre-treated with the indicated concentrations of AZD1480, barcitinib, or ruxolitinib for
30 mins followed by stimulation with IFNG (10 ng/ml). Cells were harvested for luciferase assay after 6 h. Luciferase activity from \( N \) independent experiments was expressed as fold of NS and plotted as mean ± SD. Scatterplots for all data in A & E are shown as Supplemental Fig. 9.

**Fig. 8. Effect of JAK inhibitors on the enhancement of IL1B plus dexamethasone-induced TLR2 expression by IFNG.** A549 cells were either not stimulated or pretreated with AZD1480, barcitinib or ruxolitinib, each at 1 µM, for 1 h prior to stimulation with IL1B (1 ng/ml) plus dexamethasone (\( Dex \); 1 µM), and IFNG (10 ng/ml) as indicated. (A) Cells were harvested at 6 h for RNA extraction and qPCR analysis of TLR2 and GAPDH mRNA. (B) Cells were harvested at 24 h for western blot analysis of TLR2 and GAPDH protein. In each case, TLR2 data, from \( N \) independent experiments, were normalized to GAPDH and mean ± SD were plotted as bar graphs overlaid with scatter plots. Significance was assessed by ANOVA with a Tukey multiple comparison test. Differences from untreated: ** \( p \leq 0.01 \), *** \( p \leq 0.001 \), and from IL1B + IFNG + Dex: ## \( p \leq 0.01 \), ### \( p \leq 0.001 \).

**Fig. 9. Effect of p65, GR and STAT1 knockdown on IL1B, IFNG and dexamethasone-induced TLR2 expression in A549 cells.** (A) A549 cells were incubated with pools of either control siRNAs (C si-pool), p65 siRNAs (p65 si-pool), GR siRNAs (GR si-pool) or STAT1 siRNAs (STAT1 si-pool) (1 nM each) for 48 h. Cells were then incubated overnight with serum-free media prior to harvesting for western blot analysis. Representative blots of p65, GR, STAT1 and GAPDH from 5 independent experiments are shown. (B - E) A549 cells were incubated with siRNA-pools as in A, followed by treatment with IL1B (1 ng/ml), IFNG (10 ng/ml) and/or dexamethasone (\( Dex \); 1 µM), as indicated, for 24 h. Cells were harvested for western blot analysis. Representative blots of TLR2 and GAPDH from \( N \) independent experiments are shown. Densitometric data were normalized to GAPDH, expressed as fold of untreated and mean ± SD.
were plotted as bar graphs overlaid with scatter plots. Significance relative to C si-pool in each case was tested using ANOVA with a Dunnett’s post-hoc test. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.
Tables

Table 1. Concentration-response analysis of JAK inhibitors on IFNG-induced STAT1 phosphorylation and STAT1 reporter activity. pEC$_{50}$ values of the three JAK inhibitors – AZD1480, baricitinib and ruxolitinib on inhibition of P-Y701 STAT1, P-S727 STAT1, and STAT1 reporter activity in IFNG-treated A549 cells, are shown (related to Fig. 6A-C and 7E). Data from 4-5 independent experiments are shown.

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<td>6.9±0.2</td>
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Figure 3

A

B

C

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Figure 6

A

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<tr>
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<td>+</td>
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</table>

B

C

D

E

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Figure 8

A

Ruxolitinib
Barcitinib
AZD1480
IFNG
IL1B + Dex

6

TLR2/GAPDH mRNA

2.0
1.5
1.0
0.5
0.0

**
## ## ##

N = 6

B

Ruxolitinib
Barcitinib
AZD1480
IFNG
IL1B + Dex

24

TLR2/GAPDH protein

2.0
1.5
1.0
0.5
0.0

**
## ## ##

N = 6
Figure 9

(A) 

- Time (h): 0, 24, 48
- Samples: STAT1 si-pool, GR si-pool, p65 si-pool, C si-pool
- Proteins: GAPDH, TLR2

(B) 

- Time (h): 24
- Samples: STAT1 si-pool, GR si-pool, p65 si-pool, C si-pool, IL1B+Dex
- Proteins: GAPDH, TLR2

(C) 

- Time (h): 24
- Samples: STAT1 si-pool, GR si-pool, p65 si-pool, C si-pool, IFNG+Dex
- Proteins: GAPDH, TLR2

(D) 

- Time (h): 24
- Samples: STAT1 si-pool, GR si-pool, p65 si-pool, C si-pool, IL1B+IFNG+Dex
- Proteins: GAPDH, TLR2

(E) 

- Time (h): 24
- Samples: STAT1 si-pool, GR si-pool, p65 si-pool, C si-pool, IL1B+IFNG
- Proteins: GAPDH, TLR2

N = 5

Statistical significance:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001

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