Preclinical Evidence for the Glucocorticoid-Sparing Potential of a Dual Toll-Like Receptor 7/8 Inhibitor in Autoimmune Diseases*

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ABBREVIATIONS: anti-dsDNA, anti-double-stranded DNA; anti-RiboP, anti-ribosomal P; anti-Ro/SSA, anti-Sjögren’s-syndrome-related antigen A; anti-SmRNP, anti-Smith ribonucleoprotein; BL, baseline; CD163/CD69/CD4/CD8, cluster of differentiation 163/69/4/8; Dex, dexamethasone; DMSO, dimethyl sulfoxide; DUSP1, dual specificity protein phosphatase 1; EDTA, ethylenediaminetetraacetic acid; FAS, Fas cell surface death receptor; GC, glucocorticoid; HC, healthy control; IC50, 50% inhibitory concentration; IFIT1, interferon induced protein with tetratricopeptide repeats 1; IFN, interferon; IFNA2/IFNA14, interferon alpha 2/14; IFNB1, interferon beta 1; IFN-α, interferon-alpha; IL-1β, anti-interleukin-1 beta; IL-6, interleukin-6; IRAK3, interleukin 1 receptor associated kinase 4; IRF, interferon-regulatory
factor; mDCs, myeloid dendritic cells; MX1, MX dynamin like GTPase 1; NF-κB, nuclear factor-kappa B; NR3C1, nuclear receptor subfamily 3 group C member 1; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; pDCs, plasmacytoid dendritic cells; scRNA-seq, single-cell RNA sequencing; SLE, systemic lupus erythematosus; TLR, toll-like receptor; TLR7/8i, toll-like receptor 7/8 inhibitor; TNFAIP3/A20, TNF alpha induced protein 3; TNF-α, tumor necrosis factor-alpha; TSC22D3/GILZ, TSC22 domain family member 3; VSIG4, V-Set and immunoglobulin domain containing 4

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ABSTRACT

Toll-like receptor 7 (TLR7) and TLR8 are single-stranded RNA-sensing endosomal pattern recognition receptors that evolved to defend against viral infections. However, aberrant TLR7/8 activation by endogenous ligands has been implicated in the pathogenesis of autoimmune diseases including systemic lupus erythematosus. TLR activation and Type-I interferon (IFN) were shown recently to impart resistance to glucocorticoids (GC), which are part of the standard of care for multiple autoimmune diseases. While GCs are effective, a plethora of undesirable effects limit their use. New treatment approaches that allow for the use of lower and safer doses of GCs would be highly beneficial. Herein, we report that a dual TLR7/8 inhibitor (TLR7/8i) increases the effectiveness of GCs in inflammatory settings. Human peripheral blood mononuclear cell studies revealed increased GC sensitivity in the presence of TLR7/8i for reducing inflammatory cytokine production, a synergistic effect that was most pronounced in myeloid cells, particularly monocytes. Gene expression analysis by NanoString and single-cell RNA sequencing revealed that myeloid cells were substantially impacted by combining low dose TLR7/8i and GC, as evidenced by the effects on nuclear factor-kappa B-regulated cytokines and GC-response genes, although IFNs were affected to a smaller degree. Low dose of TLR7/8i plus GC was more efficacious than either agent alone in the MRL/lpr mouse model of lupus, with improved proteinuria and survival. Overall, our findings indicate a GC-sparing potential for TLR7/8i compounds, suggesting TLR7/8i may offer a new strategy for the treatment of autoimmune diseases.
SIGNIFICANCE STATEMENT

Some features of autoimmune diseases may be resistant to glucocorticoids, mediated at least in part by toll-like receptor (TLR) activation, necessitating higher doses that are associated with considerable toxicities. We demonstrated that TLR7/8 inhibition and glucocorticoids work synergistically to reduce inflammation in a cell-type specific manner and suppress disease in a mouse model of lupus. TLR7/8 inhibition is a promising strategy for the treatment of autoimmune diseases and has glucocorticoid-sparing potential.
Introduction

Toll-like receptor 7 (TLR7) and TLR8 are endosomal pattern recognition receptors for single-stranded RNA that normally function in anti-viral defense (Mohammad Hosseini et al., 2015). However, there is a wealth of literature indicating that TLR7/8 may drive autoimmunity; therefore, TLR7/8 inhibition is a promising treatment approach for autoimmune diseases.

TLR7/8 are expressed in multiple immune cell types and their stimulation can drive cytokine production and cell activation, resulting in inflammation and autoimmunity. One of the most prominent effects of TLR7/8 activation is the stimulation of Type-I interferon (IFN) production which is seen in various dendritic cell (DC) subsets (Bender et al., 2020). Studies in mouse models of lupus showed that knockout of TLR7 may reduce disease development (Christensen et al., 2006; Fairhurst et al., 2008; Lee et al., 2008; Savarese et al., 2008) and that TLR7/8 inhibitors (TLR7/8i) have a therapeutic effect (Vlach et al., 2021). Additionally, TLR7/8 single nucleotide polymorphisms were identified in patients with systemic lupus erythematosus (SLE), which suggests that these TLRs may be a key driver of autoimmunity in SLE (Lee et al., 2016; Brown et al., 2022).

TLR7/8i development has been challenging; the receptors were difficult targets for drug development due to an inability to recombinantly express the membrane-bound receptors and, until recently, a lack of crystal structures. However, there are now compounds targeting TLR7/8 that are being tested in clinical trials, including enpatoran which is being evaluated in SLE, cutaneous lupus erythematosus and idiopathic inflammatory myositis (Phase 2 WILLOW [NCT05162586] and NEPTUNIA [NCT05650567] trials) (Bender et al., 2020; Port et al., 2021), afimetoran (Dudhgaonkar S, 2021) and E6742 (Yamakawa et al., 2022) in SLE, and MHV370 in primary Sjögren’s syndrome and mixed connective tissue disease (although the trials were
recently discontinued).

Glucocorticoids (GCs), such as dexamethasone (Dex), are one of the cornerstone treatments for autoimmune diseases such as SLE. GCs can regulate gene expression to reduce immune cell activity and inflammation very broadly, but also in a cell-type specific manner (Franco et al., 2019). Myeloid cells are often most strongly affected. GCs can effectively treat acute flares and reduce disease activity, but high doses are often required that have considerable systemic adverse effects (Porta et al., 2020). High GC use has detrimental effects not limited to musculoskeletal, endocrine, and cardiovascular systems, in addition to increasing the risk of infections (Ugarte et al., 2018). Cumulative systemic GC exposure has been linked to an increase in the risk of irreversible organ damage and all-cause mortality in SLE (Apostolopoulos et al., 2020; Bultink et al., 2021).

Recent evidence shows that patients may develop resistance to GCs mediated by IFN or TLR activation (Guiducci et al., 2010; Lepelletier et al., 2010; Northcott M, 2021; Dankers et al., 2022). This suggests that in addition to potentially reducing disease by blocking TLR7/8-mediated inflammation, TLR7/8i may also have a secondary effect of synergizing with and increasing sensitivity to GCs. Therefore, combination treatment with TLR7/8i and GCs may allow tapering of GC dose while preserving benefit and reducing undesirable GC effects. Gaining a better understanding of the potential GC-sparing effect of TLR7/8i and the mechanisms involved may support testing of this concept in clinical trials.

In the work presented herein, we tested whether combinatorial use of GCs and TLR7/8i can synergistically reduce inflammation and autoimmunity. We utilized a well characterized TLR7/8i (CMPD2), a close analog of the clinical candidate enpatoran. This compound is highly potent and selective (no inhibition of TLR3/4/9, kinases or other receptors in various profiling
assays) (Vlach et al., 2021) and can be used to better understand TLR7/8 biology. CMPD2 is a good tool molecule as it is not only selective and potent, but also has better bioavailability and pharmacokinetics in mice relative to many other TLR7/8 inhibitors. Following observation of the beneficial effects of GC and TLR7/8i co-treatment in primary human immune cells, we investigated the mechanism for synergy including the cell types, pathways and genes involved, and validated the hypothesis in a mouse model of SLE. Overall, this translational work indicates that TLR7/8 inhibition may work synergistically with GCs and suggests that the combination treatment approach may have beneficial effects in patients with autoimmune diseases.

**Materials and Methods**

**Cell Isolation and Treatments**

*Peripheral Blood Mononuclear Cell Experiments*

Blood was obtained from healthy donor leukopaks (New York Blood Center, New York, USA) and peripheral blood mononuclear cells (PBMCs) were isolated using ACCUPSin Tubes according to the manufacturer’s protocol (Sigma-Aldrich, Missouri, USA). Cell viability was assessed with Trypan Blue Stain (BioRad, California, USA). Cells were cultured in RPMI 1640 Medium (Gibco, ThermoFisher Scientific, Massachusetts, USA) with 10% fetal bovine serum (Corning, Arizona, USA) and 1x Penicillin-Streptomycin (Gibco).

PBMCs were pre-treated for 15 minutes with Dex (Sigma-Aldrich) starting at 10 µM with a 3-fold serial dilution and 1 µM CMPD2 (TLR7/8i synthesized in-house; structure published previously (Vlach et al., 2021)) with a 2-fold serial dilution. Cells were then stimulated with 3–5 µM of the TLR7/8 agonist R848 (InvivoGen, Toulouse, France), 3 µM of a TLR7 agonist (CL-087 synthesized in-house), or 1 µM of the TLR8 agonist motolimod (Selleckchem, Texas, USA).
Vehicle dimethyl sulfoxide was used at 0.01%. Plates were incubated overnight at 37°C and 5% CO2. Cell viability was assessed using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Wisconsin, USA).

Cytokine secretion in supernatants was detected using AlphaLISA Detection Kits for human interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and IFN-alpha (IFN-α) following the manufacturer’s protocol (Perkin Elmer, Ohio, USA). When pre-treatment with cytokines was required, PBMCs were exposed to 10 ng/mL IFN-α2a (Sigma-Aldrich) for 4–5 hours before being treated with the compounds and TLR7/8 agonists described above.

**Monocyte, Plasmacytoid Dendritic Cell and B Cell Experiments**

Monocytes, plasmacytoid dendritic cells (pDCs) and B cells were isolated from PBMCs using the Pan Monocyte Isolation Kit (human), Plasmacytoid Dendritic Cell Isolation Kit II (human), or B Cell Isolation Kit II (human) following the manufacturer’s protocol and using an autoMACS Pro Separator (all from Miltenyi Biotec, California, USA). The reagents used, treatment conditions, cell viability assessment and detection of cytokines were similar to that described for PBMCs.

**Flow Cytometry**

Isolated PBMCs were treated with 200 nM Dex and/or 5 nM CMPD2 and stimulated with 3 µM R848. To detect cytokines, cells were treated with 1x Brefeldin-A (BD Biosciences, California, USA) 30 minutes after R848 stimulation to block secretion and then incubated for 4–5 hours at 37°C and 5% CO2. To detect cell surface Dex-regulated markers, stimulated cells were cultured overnight, washed with phosphate-buffered saline (PBS; Gibco) and stained with a live/dead discrimination dye (Zombie Red Fixable Viability Kit [BioLegend, California, USA] or Fixable Viability Stain 575V [BD Biosciences]) for 10 minutes in the dark at room temperature.
Following incubation, cells were washed twice with FACS Buffer (BD Biosciences). All subsequent steps were performed on ice.

T cells, B cells, monocytes, pDCs and myeloid dendritic cells (mDCs) were detected by staining in Brilliant Stain Buffer (BD Horizon, New Jersey, USA) in the presence of 1x Fc Block (BD Biosciences) and with the following surface markers (see Supplemental Table 1 for details): anti-CD3, anti-CD19, anti-IgD, anti-CD14, anti-CD16, anti-HLA-DR, anti-CD123, anti-CD303 and anti-CD1c. Cells were incubated in the dark for 30 minutes at 4°C and subsequently washed twice with FACS Buffer.

For detection of GC-induced markers and cell activation, cells were co-stained with antibodies against CD163, VSIG4, CD80 and CD69 (Supplemental Table 1). Staining was performed for 30 minutes at 4°C and cells were then washed and resuspended in FACs Buffer.

For cytokine detection, cells were fixed in 1x fixation buffer (Fixation/Permeabilization Solution Kit, BD Biosciences) for 20 minutes or overnight at 4°C, washed twice with FACS Buffer, and subsequently permeabilized (Fixation/Permeabilization Solution Kit, BD Biosciences) before intracellular staining with anti-IFNα, anti-IL-6, anti-TNF-α and anti-interleukin-1 beta (IL-1β) (Supplemental Table 1) for 20 minutes at 4°C in the dark. Following this incubation, cells were washed with 1x permeabilization buffer, spun down, resuspended in FACs Buffer and run on a LSRFortessa X-20 Cell Analyzer (BD Biosciences) with FlowJo v10.8 Software (BD Biosciences).

**NanoString Analysis**

NanoString analysis was performed using a custom-designed panel containing housekeeping genes and a number of genes known to be regulated by TLR7/8 activation (Bender et al., 2020).
and GCs (Hu et al., 2018) (Supplemental Table 2). After 1.5 hours of treatment, 1x 10^6 PBMCs were resuspended in 50 mL of RLT lysis buffer (proprietary component of RNeasy kits containing high concentration of guanidine isothiocyanate) containing 2-Mercaptoethanol and were passed through a QIAshredder to facilitate cell lysis (all from Qiagen, Maryland, USA). The lysates (1.5 μL) were then analyzed by NanoString (NanoString, Washington, USA) according to the manufacturer’s instructions with the nCounter Pro Analysis System. Gene expression levels were normalized using housekeeping genes. Log2 fold change calculated relative to untreated control cells and counts are presented.

**Single-cell RNA Sequencing**

Single-cell RNA sequencing (scRNA-seq) was performed using the 10x Genomics platform (California, USA). The Chromium Next GEM Single Cell 3’ Kit v3.1 was used for sample processing and library construction. The cells were loaded into the Chromium Controller with a target of 10,000 cells collected per sample and were captured and processed according to the manufacturer’s instructions. Library QC was performed using the Agilent Bioanalyzer and High Sensitivity DNA kit (California, USA) and DNA concentrations were measured using a Qubit fluorometer (ThermoFisher Scientific). Sequencing was performed using the Illumina NextSeq 2000 (California, USA). Libraries were loaded at a concentration of 650 pM and were sequenced using a P3 200 cycle sequencing kit. FASTQ files were generated locally using the DRAGEN processor.

For data analysis, raw FASTQ files were used as input to the scRNA-seq workflow of the Python-based snakePipes pipeline (v2.6.1, (Bhardwaj et al., 2019)). STARsolo methods were used for read mapping and quantification. The Single Cell 3’ v3 whitelist (10x Genomics) was
provided for barcode identification. The GENCODE Human Release 38 (GRCh38.p13) was used as a reference (Frankish et al., 2019). Read counts were used as input to the Seurat 4.0 package (Hao et al., 2021) in R (version 4.2) to perform quality control and quantitative analyses. Cells were filtered out when the number of unique features (genes) were <200 or >5000. Additionally, cells were excluded if >5% of their identified genes were mitochondrial. UMAPs for each scRNA-seq dataset were generated in Seurat iteratively to ascertain the optimal number of principal components and clustering resolution. Cellular identification was performed using the SingleR version 1.10.0 (Aran et al., 2019) in R, with PBMC datasets derived from Celldex version 1.6.0 (Aran et al., 2019) as references. Differential expression and sub-clustering analyses were also performed using Seurat. The final data were visualized and interpreted using Cellxgene VIP and managed internally on a CellDepot repository software (Lin et al., 2022).

Murine Lupus Studies

All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals and all local and national laws and regulations regarding animal care, using protocols approved by the local Institutional Animal Care and Use Committee.

Female MRL/lpr mice or MRL control mice were purchased from Jackson Laboratories (Maine, USA) and treatment was initiated at 8 weeks of age. Mice were dosed once per day via oral gavage with 0.25 mg/kg Dex and 0.5 mg/kg of CMPD2 formulated in 0.1 M sodium citrate buffer (pH 3.0) as the vehicle. After 12 weeks of treatment, mice were euthanized via CO₂ asphyxiation and blood was collected from the vena cava into EDTA-treated tubes.

For monitoring of proteinuria, urine was collected in the afternoon on three-consecutive days every-other week by bladder massage and the samples were pooled. Urine levels of albumin and creatinine were determined using the ADVIA 1800 Clinical Chemistry Analyzer (Siemens,
Berlin, Germany). The urinary albumin-to-creatinine ratio was calculated as the ratio of milligrams of albumin per gram of creatinine per deciliter of urine.

For detection of autoantibody levels, plasma was collected by centrifugation at 10,000 g for 10 minutes. Autoantibodies were measured in plasma samples using a custom-manufactured 5-plex Meso Scale Discovery plate (New Jersey, USA) that assessed antibodies against double-stranded DNA (anti-dsDNA), histone (anti-histone), Sjögren’s-syndrome-related antigen A (anti-Ro/SSA), Smith ribonucleoprotein (anti-SmRNP) and ribosomal P (anti-RiboP), as previously described (Vlach et al., 2021). Plasma samples were diluted 1:200 or 1:1000 with PBS containing 0.5% bovine serum albumin. Plasma samples from MRL/lpr mice with high autoantibody titers were pooled to create a standard curve that was used for quantitative comparison of the experimental samples and values were expressed as autoantibody units per milliliter of plasma (units/mL).

**Data Analysis Software**

Data were analyzed using GraphPad Prism (Dotmatics, Massachusetts, USA). Group medians were determined and statistical significance was tested using a Kruskal-Wallis test, ANOVA or T-test (as described in the figure legends). Synergistic effects were evaluated on Loewe matrix plots using Combenefit (SourceForge, California, USA), as described by (Di Veroli et al., 2016), and synergy scores were calculated from the area under the curve. Flow cytometry data were analyzed using FlowJo v10.8 (BD Biosciences, Oregon, USA) and the signaling model was created using BioRender (Ontario, USA).
Results

Synergistic Reduction of Cytokines in TLR7/8-Activated Human PBMCs by GC and TLR7/8i

It was first investigated whether GCs could reduce pro-inflammatory cytokine secretion from PBMCs activated by TLR7/8 agonists. Responsiveness to TLR7/8 activation was determined by measuring IL-6 and TNF-α secretion after overnight stimulation with the dual TLR7/8 agonist R848, the TLR7-selective agonist CL-087 or the TLR8-selective agonist motolimod.

A dose titration of Dex was performed, and at the highest concentration tested (10 µM), Dex reduced R848-mediated IL-6 and TNF-α secretion by ~40–50% (Fig. 1, A and B). However, when low doses of the TLR7/8i CMPD2 (3.9–7.8 nM) were combined with Dex, increased inhibition of both IL-6 and TNF-α was observed. Loewe matrix plots were generated to determine if this was a synergistic or combinatorial effect. The Loewe matrices for IL-6 (Fig. 1C) and TNF-α (Fig. 1D) clearly showed that low doses of the two agents synergized to reduce cytokine production in response to dual TLR7/8 activation. We also observed that the 50% inhibitory concentration (IC₅₀) of CMPD2 for blocking R848-induced IL-6 or TNF-α was reduced by Dex at low doses, suggesting truly synergistic interactions between Dex and TLR7/8i (data not shown).

We next tested for synergy between Dex and TLR7/8i in PBMCs stimulated with CL-087 (TLR7 agonist) or motolimod (TLR8 agonist). Similar to the effects observed with R848 (IL-6 p = 0.0098 and TNF-α p = 0.0355), following TLR8 activation the IC₅₀ of Dex on TNF-α was reduced with CMPD2 (p = 0.0035), with a similar trend for IL-6, but this was not observed following TLR7 activation (Fig. 1E). Evaluation of Combenefit scores (synergy metric) showed that the Dex and TLR7/8i co-treatment had a more robust effect on both IL-6 and TNF-α under
R848 (TLR7/8) stimulation compared with CL-087 (TLR7) stimulation (Fig. 1F; IL-6 p = 0.0004; TNF-α p = 0.0003). No meaningful reduction in cell viability was observed with the co-treatment (data not shown). These results suggest that TLR7/8 inhibition can potentiate the anti-inflammatory impact of GCs in human PBMCs, but there are different effects on the TLR7- and TLR8-mediated pathways.

**GC and TLR7/8i Synergy is Cell-Type Dependent**

We hypothesized that the observed differences in Dex and TLR7/8i synergy following TLR7- or TLR8-specific stimulation could be partly attributable to cell type-specific responses to TLR7 and TLR8 activation. To test this hypothesis, we performed flow cytometry analysis of PBMCs stimulated with R848 and analyzed immune cell activation and cytokine production by different cell types (gating scheme shown in Supplemental Fig. 1). We found that IL-6 and IL-1β were strongly induced by R848 in classical monocytes (CD14+CD16−) and mDCs (Fig. 2; Supplemental Fig. 2, A–C). TNF-α was also highly induced in these cell types as well as pDCs (Fig. 2; Supplemental Fig. 2, A–C) and IFN-α was modestly yet exclusively expressed in pDCs (Supplemental Fig. 2D and E). IL-6, TNF-α and IL-1β were greatly reduced in classical monocytes and mDCs by co-treatment with Dex and TLR7/8i compared to single treatments (Fig. 2, A–C; Supplemental Fig. 2, A–C). However, IFN-α, TNF-α and IL-1β were not reduced significantly in pDCs by the co-treatment as compared to single treatments (Fig. 2A and D; Supplemental Fig. 2C–E).

As TNF-α was the only cytokine induced by R848 in all three cell types, we compared the frequency of TNF-α-positive cells and found that the co-treatment benefit was seen in monocytes and mDCs but not pDCs (Fig. 2, B–D). As pDCs only express TLR7, the lack of synergy in pDCs may be explained by differential expression of TLR7 and TLR8 (Bender et al., 2020). We,
therefore, evaluated Dex and TLR7/8i synergy in B cells that also only express TLR7. B cell activation, as measured by CD69 expression, was not reduced significantly by low dose co-treatment compared to the single treatments (Supplemental Fig. 3A and B).

To exclude the effect of cell-cell interactions in the response to Dex and CMPD2, we next studied purified populations of pDCs and B cells. In purified pDCs, co-treatment with Dex and CMPD2 did not synergize to block R848-mediated IL-6 secretion (Supplemental Fig. 4A and B). In addition, in purified B cells, TLR7 agonist-mediated IL-6 secretion was not synergistically blocked by Dex and CMPD2 (Supplemental Fig. 4C and D). Taken together, these results show that Dex and CMPD2 synergize to reduce cytokine production in a cell-type specific manner and that the TLR7 pathway may be less sensitive than the TLR8 pathway to the co-treatment.

**Synergistic Reduction of Monocyte Activation by GC and TLR7/8i**

Since a strong synergistic effect was observed in monocytes in our PBMC flow cytometry experiments, we proceeded to study the effect in more detail using monocytes purified from PBMCs. Similar to the effect seen in PBMCs, R848-induced secretion of IL-6 and TNF-α was synergistically blocked by co-treatment with Dex and CMPD2 in purified monocytes (Supplemental Fig. 5). When stimulated with TLR7- or TLR8-specific agonists, Dex and CMPD2 were not found to synergize in TLR7 agonized cells, but a synergistic reduction of IL-6 was observed in TLR8 agonized cells (Supplemental Fig. 6). The differential effect of the dual TLR7/8 and specific TLR7 and TLR8 agonists on IL-6 and TNF-α secretion in monocytes was confirmed by analysis of Dex IC50 (Fig. 3A) and synergy scores (Fig. 3B). Higher doses of CMPD2 (62.5–125 nM) were required to obtain maximal synergy with low dose Dex in monocytes compared to PBMCs, which may be due to lower potency of CMPD2 against TLR8 compared to TLR7 (Vlach et al., 2021). Additionally, we observed that the reverse was true; low
doses of Dex increased the potency of CMPD2 against R848 agonism and the IC$_{50}$ of CMPD2 against TNF-α was reduced two-to-three fold by Dex (data not shown).

To further characterize the impact of GC on monocytes under TLR7/8 activation, we investigated the expression of GC-induced proteins CD163 and VSIG4. R848 stimulation reduced the Dex-induced expression of CD163 and VSIG4 (Fig. 3C and D). Co-treatment with low dose CMPD2 and Dex did not significantly increase CD163/VSIG4 levels compared to single treatments; however, there was a clear trend in the accumulation of double CD163 and VSIG4 positive monocytes with the co-treatment in the absence or presence of R848 (Fig 3C and D). CD163 and VSIG4 are both known to dampen inflammation so their increased levels with Dex and CMPD2 co-treatment, although not statistically significant, may still have meaningful anti-inflammatory benefits (Li et al., 2017; Svendsen et al., 2020).

Overall, these results suggest that higher doses of CMPD2 may be required to completely restore Dex-induced signature genes in the context of strong TLR7/8 activation. Further, the synergy between Dex and CMPD2 is strong in monocytes, and more so for TLR8 than for TLR7 stimulation.

**Gene Expression Analysis of R848-Stimulated PBMCs in the Presence of GC and TLR7/8i**

To gain further mechanistic insight into how GCs and TLR7/8i may work synergistically, we performed gene expression analysis on R848-stimulated PBMCs in the presence of Dex alone at a concentration previously demonstrated to be suboptimal (200 nM) or in combination with CMPD2 (5–15 nM). With R848 stimulation, there was a significant induction of multiple inflammatory genes (Fig. 4; Supplemental Fig. 7). Notably, there was an increase in IFNs, with *IFNB1* and *IFNA2* being the most highly induced. There was also induction of NF-κB-regulated
cytokines such as \textit{IL6}, \textit{TNF} and \textit{IL1B}. As the analysis was performed only 1.5 hours after R848 stimulation, there was a modest increase in IFN-response genes.

With sub-optimal Dex treatment only, the induction of GC-response genes \textit{CD163}, \textit{DUSP1}, \textit{IRAK3} and \textit{TSC22D3} (also known as glucocorticoid-induced leucine zipper, GILZ) was observed (Fig.4; Supplemental Fig. 7). Induction of these genes was negatively regulated by R848. R848-stimulated cells treated with CMPD2 or Dex alone showed some inhibition of NF-\kappa B-regulated cytokines compared to no treatment, but co-treatment with CMPD2 and Dex further decreased NF-\kappa B cytokines. Co-treatment with Dex and CMPD2 showed little synergy for IFNs or the downstream IFN response gene signature. In contrast, the co-treatment reversed the suppression of GC-response genes induced by R848 (e.g., \textit{CD163} and \textit{TSC22D3}).

ScRNA-seq was performed to gain deeper insight into the cell-type specific effects of R848 stimulation for 1.5 hours following pre-treatment with GC and/or TLR7/8i for 15 minutes. Clustering analysis generated 19 different cell type clusters (Fig. 5A). The effects of R848 and Dex in the presence of CMPD2 was assessed for each cell type using genes representative of different pathways or processes: Type-I IFNs (\textit{IFNA2}, \textit{IFNA14}, \textit{IFNB1}), IFN-response genes (\textit{IFIT1}, \textit{MX1}), NF-\kappa B-regulated cytokines (\textit{IL1B}, \textit{IL6}, \textit{TNF}), GC-response genes (\textit{DUSP1}, \textit{TSC22D3}), and the NF-\kappa B negative feedback gene (\textit{TNFAIP3}, also known as \textit{A20}).

Both classical and non-classical monocytes and DCs showed significant induction of NF-\kappa B-regulated cytokines by R848 (Fig. 5B; Supplemental Fig. 8). In all cell types there was greater cytokine inhibition with CMPD2 alone compared with Dex alone, and gene expression was further decreased with the co-treatment. Although cytokine expression was much lower in B cells, a similar pattern of inhibition was observed.
DCs were the only cell type that showed expression of Type-I IFN genes (Fig. 5B; Supplemental Fig. 8). IFNA2, IFNA14 and IFNB1 expression was reduced by both Dex and CMPD2 alone and the co-treatment decreased IFNB1 expression further, although this was not statistically significant. Consistent with the minimal impact on IFN production, the expression of IFN-response genes was reduced minimally by CMPD2 and slightly increased by Dex, with no effect of the co-treatment observed.

The expression of GC-response genes and susceptibility to inhibition varied between cell types (Fig. 5B; Supplemental Fig. 8). Monocytes, DCs, natural killer cells and T cells showed induction of DUSP1 and TSC22D3 with Dex treatment, which was reduced by R848 and restored by co-treatment with CMPD2. The reduction of DUSP1 and TSC22D3 expression was greater with R848 in B cells and the addition of CMPD2 to Dex treatment effectively restored induction. Expression levels of the GC receptor NR3C1 were not found to explain the differences in cell-type sensitivity (Supplemental Fig. 9).

The response of the NF-κB negative regulator TNFAIP3 was complex. TNFAIP3 expression was induced by R848 in monocytes, DCs and B cells (Supplemental Fig. 8). This is likely to be a negative feedback mechanism and was further increased by Dex. CMPD2 reduced the potentiating effect of Dex in monocytes and B cells, but TNFAIP3 expression was further increased with the co-treatment in DCs, potentially due to complex regulation of R848 negative feedback mechanisms by CMPD2.

Overall, these results suggest synergy in cell types showing an NF-κB response, such as monocytes, and less of an effect on the IFNs and downstream pathways. Further, the gene expression results are generally consistent with the cytokine and flow cytometry results.
presented earlier that were conducted following prolonged R848 stimulation (Fig. 1; Fig. 2; Fig. 3C and D).

**GC and TLR7/8i Co-Treatment can Synergize Following IFN-α Pre-Treatment on PBMCs Stimulated with TLR7/8 Agonist**

Previous studies showed that IFN-α can reduce the potency of GCs (Guiducci et al., 2010; Northcott M, 2021). Thus, we sought to determine if pre-treatment with IFN-α impacts the response of PBMCs to TLR7/8 agonists and if potency of Dex is reduced further.

We found that IFN-α pre-treatment increased the responsiveness of PBMCs to R848, as measured by IL-6 secretion, perhaps due to increased expression of TLR7/8 (Fig. 6A and B; Supplemental Fig. 10A and C). At the highest Dex dose tested (10 μM), IL-6 was approximately 40% higher following R848 stimulation in IFN-α pre-treated cells compared with no IFN-α pre-treatment. Co-treatment with low doses of Dex and CMPD2, shown to be sufficient for synergy (Supplemental Fig. 10), blocked R848-induced IL-6 secretion in IFN-α pre-treated cells more than Dex or CMPD2 alone, although the magnitude of IL-6 induction by IFN-α in this system was modest (Fig. 6B). Synergy between Dex and CMPD2 was confirmed in the absence and presence of IFN-α pre-treatment by Combenefit analysis (Supplemental Fig. 10B and D), with similar synergy scores in R848-stimulated cells with and without IFN-α pre-treatment (Fig. 6C).

In summary, these results suggest that even in the context of a pre-existing pro-inflammatory environment, which can be envisioned in autoimmune diseases such as lupus, TLR7/8i is still able to potentiate the action of GCs to provide synergistic anti-inflammatory benefits.

**GC and TLR7/8i Combination Show in vivo Potency at Reducing Disease in the MRL/lpr Mouse Model of Lupus**
To determine if the *in vitro* results could be replicated *in vivo*, we tested the Dex and CMPD2 combination treatment in a mouse model of lupus. The MRL/lpr spontaneous model of lupus was chosen, where a mutation in the *FAS* gene leads to a lupus-like phenotype characterized by proteinuria and anti-dsDNA antibodies (Andrews et al., 1978). MRL/lpr mice were treated with low doses of Dex and CMPD2 alone (higher doses of both compounds as single agents were found to be efficacious previously) or in combination. While low doses of Dex and CMPD2 alone had a marginal impact on proteinuria, the combination treatment showed nearly complete suppression of proteinuria development (Fig. 7A and B). An improvement in survival was also observed (Fig. 7C). Titers of anti-dsDNA were strongly impacted by Dex alone, with only a marginal additional reduction in titers with the combination treatment (Fig. 7D). The synergistic effects observed on efficacy in a mouse model of lupus suggests a steroid-sparing effect that warrants further evaluation in patients.

**Discussion**

Minimization of GC use is critical in the management of autoimmune and inflammatory diseases such as SLE, where serious complications (e.g., osteoporosis, cardiovascular disease, infections), organ damage and increased mortality are linked to GC exposure, the toxicity of which is dose related (Durcan et al., 2019; Apostolopoulos et al., 2020; Ruiz-Irastorza and Bertsias, 2020). Our results suggest a GC-sparing potential of TLR7/8i in the context of autoimmune diseases such as SLE. We demonstrated that TLR7/8 inhibition increases sensitivity to GCs, and in some cases the effect was found to be synergistic, not just combinatorial. Interestingly, this effect was found to be cell-type specific; there was a clear synergistic effect in monocytes, while a reduced effect was observed in DCs. Differences in the expression of the GC receptor could not account for the differences in GC sensitivity between the two cell types, suggesting that the cell-type specificity
observed is likely to be more complex.

One reason for the differential effects of the TLR7/8i and GC co-treatment could be the cell-type specific activity of GCs (Franco et al., 2019). GCs activate the GC receptor and mediate immune regulation through mechanisms that include transactivation and transrepression (Petta et al., 2016; Scheschowitsch et al., 2017; Escoter-Torres et al., 2019). It appears that both transactivation and transrepression may be enhanced in monocytes by the co-treatment since NF-κB cytokines are suppressed and R848-mediated inhibition of GC-response genes, such as TSC22D3, IRAK3 and CD163, is reversed. However, GC-response gene induction was reduced in pDCs and B cells, potentially because the epigenetic landscape does not allow induction of those specific genes. We also demonstrated that the IFN-regulatory factor (IRF) pathway and IFN induction were resistant to inhibition by the co-treatment in pDCs, suggesting that the IRFs, which promote IFN expression, may not have binding sites for the GC receptor. Further investigation of purified cell populations using chromatin immunoprecipitation sequencing or assay for transposase-accessible chromatin sequencing may allow us to determine whether genome-wide occupancy of the GC-GC receptor is substantially different in monocytes compared with DCs and B cells in the context of TLR7/8 inhibition.

Differential TLR7 and TLR8 expression (pDCs and B cells predominantly express TLR7, while monocytes and mDCs express both TLR7 and TLR8) and pathway regulation by GCs may also contribute to the cell-type specificity of the co-treatment. This is supported by the observed differences in the effects of GC and TLR7/8i treatment following stimulation with the TLR7 and TLR8 agonists. The signaling pathways downstream of TLR7 and TLR8 activate both overlapping and distinct gene expression programs (Bender et al., 2020) and there may be differences in the susceptibility of these pathways or genes to inhibition by GC. The fact that
both IL-6 and TNF-α were synergistically inhibited downstream of TLR8, but not TLR7, suggests that it is a pathway-specific difference and not a gene-specific difference in susceptibility.

Previous studies showed that GC therapy is less potent in patients with SLE who have a high Type-I IFN gene signature (Northcott M, 2021; Dankers et al., 2022) and in the context of TLR stimulation (Guiducci et al., 2010). We demonstrated increased potency of GC when combined with TLR7/8 inhibition in the context of IFN-α pre-treatment. This suggests that TLR7/8i treatment may have a GC-sparing effect in the clinical setting in patients with a high IFN gene signature. Although synergy for the inhibition of IFN production by pDCs was not observed, combination treatment with TLR7/8i and GC may still improve efficacy compared to GC therapy alone in patients with a high IFN gene signature due to greater reduction of the inflammatory processes that drive SLE development and progression.

The in vivo results showing the combined benefit of GC and TLR7/8 inhibition at sub-optimal doses in the MRL/lpr lupus mouse model suggest that combination treatment may be applicable to a range of autoimmune disorders. Disease development in the MRL/lpr model is driven by a mutation in the FAS gene, resulting in a loss of tolerance, primarily in T cells, and is not known to be driven by TLR activation. Our findings, as well as those from previous studies (Dudhgaonkar S, 2021), showing that a TLR7/8i is efficacious and has a combination effect with GCs in this model, suggests that TLR7/8 may contribute to autoimmune disease in ways not recognized previously. Similarly, efficacy for TLR7/8i was observed in the NZB/W F1 IFN-accelerated model (Vlach et al., 2021), which is also not known to be driven by TLR7/8. In both cases, there was some reduction in autoantibodies but a more marked reduction in proteinuria. It is possible that TLR7/8 inhibition is effective at reducing inflammation in the kidney by
impacting myeloid cell activation and that the combination treatment with GC further reduces activation of these GC-sensitive cells, leading to synergistic effects. A reasonable hypothesis that may be tested in future studies is that the GC and TLR7/8i combination treatment may reduce localized organ inflammation where there is infiltration of macrophages and monocytes that are activated by RNA-containing immune complexes or microRNA and extracellular vesicles released from dead or dying cells.

Taken together, our results suggest that differential activation of the TLR7 and TLR8 pathways in different cell types (depending on receptor expression) leads to cell-type specific GC resistance (see proposed model in Fig. 8). GCs tend to suppress NF-κB-response genes, but this is overridden in the presence of a Type-I IFN-induced response. Low doses of TLR7/8i may preferentially reduce TLR8-dependent NF-κB responses compared with TLR7-dependent IRF responses; therefore, TLR7/8i and GC combination treatment may synergize to overcome GC resistance in a cell-type specific manner (monocytes > mDCs > B cells and pDCs).

TLR7/8 inhibition is a promising strategy that may reduce innate and adaptive immune processes that contribute to and drive autoimmune diseases such as SLE. TLRs are primary defense molecules protecting against infection via pathogen recognition. Thus, TLR inhibition to reduce autoimmunity might come with a risk for increased infection, although there is redundancy in the immune system directed against infections. However, in a Phase 2 clinical trial of enpatoran in patients hospitalized with COVID-19 pneumonia (NCT04448756), no worsening of disease was observed with treatment, suggesting that in this context TLR7/8 inhibition did not impair viral defenses (McKinnon et al., 2022). Our preclinical evidence demonstrating synergy between TLR7/8i and GC suggests that TLR7/8i may have a further benefit by enabling GC dose reduction in situations where inflammation triggered by TLR7/8 activation reduces GC potency.
The safety, efficacy and potential GC-sparing effect of the TLR7/8i enpatoran is currently being evaluated in patients with SLE and/or cutaneous lupus erythematosus in the Phase II WILLOW trial (NCT05162586). Patients will undergo a mandatory GC tapering schedule to evaluate the effect of TLR7/8i on SLE disease control and clinically-meaningful GC reduction. Our preclinical results provide a hypothetical mechanism for a GC-sparing effect and will inform clinical pharmacodynamic/biomarker evaluation. The learnings from the preclinical studies and the ongoing clinical study will help develop future treatment paradigms for these two approaches and inform other rational combinational therapies for lupus and other autoimmune diseases, such as dermatomyositis and polymyositis, where TLR7/8 activation, Type-I IFN and GC biology intersect.
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Conflicts of Interest: A.D., A.P., N.G., E.T., A.T.B. and B.V are employees of EMD Serono, Billerica, MA, USA who funded the study and are developing a TLR7/8 inhibitor (enpatoran). M.P. and M.D.C. were employees of EMD Serono, Billerica, MA, USA when the study was conducted. E.F.M. has received grants/research support from AbbVie, Amgen, Biogen, AstraZeneca, Bristol Myers Squibb, Janssen, Eli Lilly, EMD Serono, Genentech, GSK, and Union Chimique Belge; and consultant fees from EMD Serono, AstraZeneca, Biogen, Bristol Myers Squibb, Eli Lilly, Genentech, GlaxoSmithKline, Janssen, Novartis, AbbVie, Galapagos and IGM.

Data Availability Statement: ScRNA-seq data have been uploaded to the GEO database (accession #GSE235927). Any requests for additional data by qualified scientific and medical researchers for legitimate research purposes will be subject to the healthcare business of Merck KGaA, Darmstadt, Germany’s (CrossRef Funder ID: 10.13039/100009945) Data Sharing Policy. All requests should be submitted in writing to the healthcare business of Merck KGaA, Darmstadt, Germany’s data sharing portal. When the healthcare business of Merck KGaA, Darmstadt, Germany (CrossRef Funder ID: 10.13039/100009945) has a co-research, co-development, or co-marketing or co-promotion agreement, or when the product has been out-licensed, the responsibility for disclosure might be dependent on the agreement between parties. Under these circumstances, the healthcare business of Merck KGaA, Darmstadt, Germany will endeavor to gain agreement to share data in response to requests.

Authorship Contributions

Participated in research design: Deshmukh, Bender, Vaidyanathan.

Conducted experiments: Deshmukh, Pereira, Tzvetkov, Przetak, Bender, Vaidyanathan.
Contributed new reagents or analytic tools: Not applicable

Performed data analysis: Deshmukh, Pereira, Geraci, Tzvetkov, Przetak, Catalina, Bender, Vaidyanathan.

Wrote or contributed to the writing of the manuscript: Deshmukh, Pereira, Geraci, Tzvetkov, Przetak, Catalina, Morand, Bender, Vaidyanathan.
References


Antagonist of Toll-like Receptors 7 and 8, in Healthy Volunteers. *Clin Pharmacol Drug Dev.*
Footnotes

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Figure Legends

Fig. 1. Dex and TLR7/8i synergistically reduce immune cell activation in PBMCs. (A–B) Representative dose response curve of Dex reducing IL-6 (A) and TNF-α (B) secretion either alone or with different doses of CMPD2 at 16 hours post-R848 stimulation. (C–D) Combenefit analysis showing Loewe matrix plots of Dex and CMPD2 interactions for IL-6 (C) and TNF-α (D) inhibition in R848-stimulated cells (data from multiple experiments pooled; total 17 donors for IL-6 and 12 donors for TNF-α). (E) IC$_{50}$ of Dex either alone or in the presence of CMPD2 (7.8 nM) for IL-6 and TNF-α in R848- and CL-087-stimulated cells, and for IL-6 (5 nM) and TNF-α (2.5 nM) in motolimod-stimulated cells. (F) Combenefit matrix score (area under curve) for IL-6 and TNF-α under R848- or CL-087-stimulation. Experiments were repeated at least three times with 2–4 donors. In (A) and (C), the data were normalized to % of the R848 stimulation control and 4 donors are pooled. Paired t-test: *p < 0.05, **p < 0.005, ***p < 0.0005.

Fig. 2. Cell-type specific effects of Dex and TLR7/8i at inhibiting cytokines in PBMCs. (A) Representative histogram showing mean fluorescence intensity for IL-6, TNF-α and IL-1β in classical monocytes, mDCs and pDCs stimulated with R848 (4–5 h) in the presence of 200 nM Dex and/or 5 nM CMPD2. Experiments were repeated three times with 2–4 donors. (B–D) Quantitation of flow cytometry dot plots from multiple experiments (n = 14 donors) showing TNF-α production in classical monocytes (B), mDCs (C) and pDCs (D) after stimulation with R848 for 4–5 hours (normalized % R848 stimulation control). Paired t-test: **p < 0.005, ***p < 0.0005.
**Fig. 3.** Dex and TLR7/8i work synergistically in monocytes. (A) IC$_{50}$ of Dex with/without 7.8 nM CMPD2 (or 62.5 nM for TLR8 agonist) for IL-6 and TNF-α in monocytes stimulated with R848, CL-087 or motolimod. NI indicates no inhibition; Dex IC$_{50}$ for IL-6 in R848- or motolimod-stimulated cells could not be calculated. For R848-stimulated cells, there was ~10% inhibition with the highest Dex concentration (10 µM) and ~65% inhibition with the Dex and CMPD2 co-treatment. For TLR8-stimulated cells, there was ~15% inhibition with 10 µM Dex and ~70% inhibition with Dex plus CMPD2. (B) Quantification of synergy scores from Combenefit Loewe matrix plots showing Dex and CMPD2 interactions for IL-6 and TNF-α inhibition in R848-, CL-087- and motolimod-stimulated monocytes (n = 4). (C) Representative flow cytometry dot plots of PBMCs gated for total CD14$^+$ monocytes showing expression of GC-induced signature markers CD163 and VSIG4 when unstimulated or stimulated with R848 in presence or absence of Dex and/or CMPD2. (D) Quantitation of frequency of CD163$^{hi}$ VSIG4$^+$ pan monocytes in PBMCs treated as described above. Data pooled from 12 donors and multiple independent experiments were performed. Paired t-test: *p < 0.05, **p < 0.005, ***p < 0.0005.

**Fig. 4.** NanoString analysis of R848-stimulated PBMCs treated with dexamethasone and/or CMPD2. PBMCs were pre-treated with CMPD2 (5 nM or 15 nM) or Dex (200 nM) for 15 minutes and were then stimulated with R848 for 1.5 hours. Cell lysates were prepared and analyzed by NanoString using a custom panel containing genes known to be regulated by TLR7/8 or GCs. The Log2 fold change was calculated versus untreated control cells. The heat map shows the results averaged for 2 donors. Notable gene expression changes are shown in boxes and labeled. The experiment was repeated twice.
**Fig. 5.** ScRNA-seq analysis of the cell types activated by R848 and the impact of Dex and TLR7/8i treatment. PBMCs were pre-treated with 200 nM Dex and/or 15 nM of CMPD2 for 15 minutes and then stimulated with 3 μM R848 for 1.5 hours. Cells were then collected and scRNA-seq was run. (A) The sequencing data was analyzed by Seurat and a UMAP projection was generated of the clustered cells. (B) Heat maps were constructed to show the expression signatures of different pathways for the cells in each cluster. The coloring indicates the mean expression level of all genes in each signature and is scaled per expression signature. Combined data from 3 separate experiments using cells from 6 donors are shown.

**Fig. 6.** CMPD2 reverses the ability of IFN-α pre-treatment to reduce Dex potency. (A) Dose response curve of Dex impacting IL-6 in PBMCs with/without IFN-α pre-treatment at 16 hours post-R848 stimulation. (B) Representative bar graph of IL-6 secretion following treatment with 41 nM Dex and/or 7.8 nM CMPD2 in the presence or absence of IFN-α pre-treatment. (C) Quantification of synergy scores from Combenefit Loewe matrix plots (area under the curve) of Dex and CMPD2 interactions for IL-6 inhibition in R848-stimulated PBMCs either untreated or pre-treated with IFNα at 16 hours post-stimulation. Figures (A–C) show pooled data from 5 donors. In (A) and (B), the data are normalized to the R848 stimulation control. Paired t-test: **p < 0.005.

**Fig. 7.** Dex and TLR7/8 inhibition have synergistic effects in a mouse model of lupus. MRL/lpr mice were treated with low doses of Dex (0.25 mg/kg) and/or CMPD2 (0.5 mg/kg). (A)
Proteinuria tracked over time and (B) an area under the curve for the same. (C) Survival of mice over the disease course. (D) Titers of anti-dsDNA at BL (prior to treatment) and at the end of the study in different treatment groups, including HC MRL mice. Proteinuria and anti-dsDNA data are plotted as medians with 95% confidence intervals. N = 10 mice were used per treatment group. Data are representative of 2 independent experiments.

**Fig. 8.** Proposed model for synergistic effects of GC and TLR7/8i. TLR7/8 activation leads to stimulation of the NF-κB and IRF pathways, resulting in cytokine production and other inflammatory activities in several immune cell types. GC receptor activation effectively inhibits the NF-κB pathway but has less activity against IRF activation and IFN production. The cytokines most susceptible to GC inhibition are shown in red font. IFN downstream of TLR7 activation (and other pathways) can cause resistance to GCs. GC resistance may be overcome by the addition of a TLR7/8i. Activation of the IRF and NF-κB pathways by TLR7/8 ligands that are associated with autoimmune diseases leads to resistance against Dex-mediated anti-inflammatory effects that can be reversed by low dose TLR7/8i in a cell-type specific manner. Figure created using BioRender.com.
**Figure 1.**

(A) IL-6 (% control) in response to various concentrations of Dex.

(B) TNF-α (% control) in response to various concentrations of Dex.

(C) Combenefit score for IL-6 in response to TLR7/8 (R848) and TLR7 (CL-087) stimulation with different concentrations of CMPD2.

(D) Combenefit score for TNF-α in response to TLR8 (motolimod) stimulation with different concentrations of CMPD2.

(E) Comparative effectiveness of Dex at IC50 concentrations on IL-6 and TNF-α in response to TLR7/8 (R848), TLR7 (CL-087), and TLR8 (motolimod) stimulation.

(F) Comparison of Combenefit score for IL-6 and TNF-α in response to TLR7/8 (R848) and TLR7 (CL-087) stimulation.

**Notes:**
- The article has not been copyedited and formatted. The final version may differ from this version.
- The diagrams show the percentage control of IL-6 and TNF-α in response to various stimuli and concentrations of Dex.
- The Combenefit score is depicted for IL-6 and TNF-α in response to different TLR stimulations with varying concentrations of CMPD2.
- The effectiveness of Dex at IC50 concentrations is compared for IL-6 and TNF-α.
- The Combenefit score for IL-6 and TNF-α is compared for TLR7/8 (R848) and TLR7 (CL-087) stimulation.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
**Figure 6.**

(A) Graph showing IL-6 (% control) as a function of Dex (μM) for different treatments: Control, IFN-α, and Unstim.

(B) Bar graph comparing IL-6 (% control) for various treatments: No cytokine, IFN-α pre-Tx, R848, R848 + Dex, R848 + CMPD2, R848 + Dex + CMPD2.

(C) Bar graph showing Combenefit score for No pre-Tx and IFN-α pre-Tx conditions.
Figure 7.
Figure 8.