High-dose methylprednisolone mediates YAP/TAZ-TEAD in vocal fold fibroblasts with macrophages

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Article

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Abstract

The pro-fibrotic effects of glucocorticoids may lead to a suboptimal therapeutic response for vocal fold (VF) pathology. Targeting macrophage-fibroblast interactions is an interesting therapeutic strategy; macrophages alter their phenotype to mediate both inflammation and fibrosis. In the current study, we investigated concentration-dependent effects of methylprednisolone on the fibrotic response, with an emphasis on YAP/TAZ-TEAD signaling, and inflammatory gene expression in VF fibroblasts in physical contact with macrophages. We sought to provide foundational data to optimize therapeutic strategies for millions of patients with voice/laryngeal disease-related disability. Following induction of inflammatory (M(IFN/LPS)) and fibrotic (M(TGF)) phenotypes, THP-1-derived macrophages were seeded onto HVOX vocal fold fibroblasts. Cells were co-cultured +/-0.3-3000nM methylprednisolone +/- 3μM verteporfin, a YAP/TAZ inhibitor. Inflammatory (*CXCL10*, *TNF*, *PTGS2*) and fibrotic genes (*ACTA2*, *CCN2*, *COL1A1*) in fibroblasts were analyzed by real-time polymerase chain reaction after cell sorting. Ser211-phosphorylated glucocorticoid receptor (S211-pGR) was assessed by Western blotting. Nuclear localization of S211-pGR and YAP/TAZ was analyzed by immunocytochemistry. Methylprednisolone decreased *TNF* and *PTGS2* in fibroblasts co-cultured with M(IFN/LPS) macrophages and increased *ACTA2* and *CCN2* in fibroblasts co-cultured with M(IFN/LPS) and M(TGF). Lower concentrations were required to decrease *TNF* and *PTGS2* expression and to increase S211-pGR than to increase *ACTA2* and *CCN2* expression and nuclear localization of S211-pGR. Methylprednisolone also increased YAP/TAZ nuclear localization. Verteporfin attenuated upregulation of *CCN2*, but not *PTGS2* downregulation. High concentration methylprednisolone induced nuclear localization of S211-pGR and upregulated fibrotic genes mediated by YAP/TAZ activation.

INTRODUCTION

The vocal fold (VF), an essential apparatus for phonation, vibrates hundreds of times a second.[1] Due to its role and anatomical location, the VF is inherently exposed to mechanical and environmental stress. As many as 20 million people report voice disorders annually in the US.[2] Inflammation is broadly associated with dysphonia and is likely etiologic for benign vocal fold lesion development. Glucocorticoids (GCs) are frequently administered to manage voice disorders because of their anti-inflammatory functions and affordability.[3–6] However, recent reports suggest clinical outcomes of GC therapy to be variable.[7–9] Diverse functions of GCs likely underlie the disparate outcomes.[10–12] Of note, fibrotic effects of GC signaling have been reported in several organs and VF fibroblasts.[13–15] Optimizing GC therapy to minimize fibrosis while limiting inflammation has the potential to benefit millions of patients.

In the inflammatory milieu, dysfunctional cooperation between tissue-resident and infiltrated hematopoietic cells can drive pathological tissue responses, such as chronic inflammation and fibrosis.[16] Previous studies revealed the significance of intercellular communication between fibroblasts and macrophages in pathologies across multiple organs, including the VF.[17–20] Differential macrophage

phenotypes are induced via exposure to stimuli through the shift from inflammatory to fibrotic environments.[17,21,22] Inflammatory stimuli, such as interferon-gamma (IFN- γ) and lipopolysaccharide (LPS), induce the inflammatory M1 phenotype.[17,23] Interleukin (IL)-4, IL10, and transforming growth factor-β (TGF-β) drive the anti-inflammatory/fibrotic M2 phenotype.[23–25] However, various subtypes beyond the dualistic classification to the M1 and M2 are induced by individual stimuli,[17] likely related to organ-specific responses to macrophages.[26,27] For example, in VF fibroblasts, fibrotic genes were not activated by paracrine signaling from IL4-stimulated typical M2 macrophages,[27] which elicited a fibrotic response in non-VF fibroblasts.[28,29] Conversely, TGF-β-stimulated macrophages induced a fibrotic response in VF fibroblasts. Independently, physical contact and paracrine signals from macrophages differentially activated VF fibroblasts.[27] To that end, understanding interactions between VF fibroblasts and macrophages is foundational to optimally treat VF disease.

Recently, refinement of GC dose has emerged as a possible strategy to improve GC therapy.[15,30] Our previous work with indirect co-culture models found fibrotic and inflammatory responses of VF fibroblasts triggered by macrophage-derived paracrine signals were promoted and inhibited by 'high' and 'low' concentrations of methylprednisolone, respectively.[31] Based on this finding, we hypothesized minimizing GC concentrations to sufficiently inhibit inflammation improves efficacy of GC therapy. However, previous co-culture studies employed a cell culture insert to allow only paracrine signaling. Considering the *in vivo* environment in which macrophages directly engage fibroblasts,[32] co-culture models with direct intercellular communication further support the translation of *in vitro* findings to support *in vivo* investigation. In addition, mechanisms underlying concentration-dependent negative and positive gene regulation remain unknown.

Based on the currently known biochemistry of GC signaling, unrelated to concentration-dependency, complex reactions of the GC receptor (GR) are thought to be a source of diverse GC functions.[10,33] GR interacts with numerous proteins. The GC/GR complex binds and inhibits other transcription factors in the cytoplasm. Alternatively, GR translocated to the nucleus binds to both negative and positive gene regulatory elements. Various post-translational modifications (phosphorylation, acetylation, SUMOylation) are involved in GR distribution and recruitment to gene regulatory elements. Additionally, accessibility to negative and positive gene regulatory elements is putatively altered by dimerization of GR concentrated in the nucleus.[10]

Despite diversity of GR interactions with other signaling pathways,[11] recent transcriptomic analysis on VF fibroblasts highlighted the impact of GR on the Hippos signaling pathway, which has a key role in fibrosis.[34–37] In this pathway, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) are the core.[38] Activated YAP/TAZ enters the nucleus and primarily serves as co-activators of TEA domain transcription factors (TEADs) to induce TEAD-dependent transcription. *CCN2*, a fibrotic gene induced by high-concentration GCs, is a target of YAP/TAZ-TEAD signaling;[39] this finding

underlies the hypothesis that YAP/TAZ-TEAD signaling is specifically activated by high-concentration GCs.

In the current study, a direct co-culture model was employed to further confirm concentration-dependent effects of methylprednisolone to alter fibrotic and inflammatory responses of human macrophages and VF fibroblasts. We additionally explored nuclear localization of GR and YAP/TAZ in this model to interrogate mechanisms underlying concentration-dependent effects of GCs. Ultimately, we seek to refine GC therapy corroborated by mechanistic insight, to benefit millions of patients with voice-related disability.

RESULTS

Methylprednisolone altered inflammatory genes in direct co-culture of human VF fibroblasts and macrophages

M(IFN/LPS) and M(TGF) stimulate inflammatory and fibrotic responses of VF fibroblasts.[27] Concentration-dependent effects of methylprednisolone on gene expression were assessed using direct co-culture models of human VF fibroblasts with GFP-expressing M(IFN/LPS) and M(TGF) macrophages (G-M(IFN/LPS) and G-M(TGF)). In human VF fibroblasts co-cultured with G-M(IFN/LPS) macrophages, three inflammatory genes (*TNF*, *PTGS2*, and *IL1B*) were downregulated by methylprednisolone in a concentration-dependent manner. However, *CXCL10*, another inflammatory gene, was unchanged (Fig. 1). In co-culture with G-M(TGF), methylprednisolone decreased *CXCL10* expression in VF fibroblasts and tended to inhibit expression of *TNF*, *PTGS2*, and *IL1B*. Methylprednisolone downregulated *TNF*, *PTGS2*, and *IL1B* in G-M(IFN/LPS) macrophages, and *CXCL10* in G-M(TGF) macrophages in a concentration-dependent manner. In addition, *TNF*, *PTGS2*, and *IL1B* tended to decrease in response to methylprednisolone in G-M(TGF) macrophages. Collectively, IC_{50} and IC_{90} to decrease inflammatory genes were 1.8–3.3 and 6.8-27nM in VF fibroblasts (**Table 1**) and 2.4–11.6 and 7.7-36.8nM in macrophages (**Table 2**).

Methylprednisolone altered fibrotic gene expression in direct co-culture of human VF fibroblasts and macrophages

Methylprednisolone increased *CCN2* and *ACTA2* expression in VF fibroblasts directly co-cultured with G-M(IFN/LPS) and G-M(TGF) macrophages in a concentration-dependent manner (Fig. 2). However, *COL1A1* expression was unchanged or decreased by methylprednisolone. *CCN2* in G-M(IFN/LPS) and G-M(TGF) macrophages was concentration-dependently upregulated by methylprednisolone in the direct co-culture model. *TGM2* and *FN1*, M2 markers associated with fibrosis, were also analyzed.[17,27,40] The effect on *FN1* expression was unclear. Weak tendencies of decreased and increased *TGM2* were observed in response to methylprednisolone in G-M(IFN/LPS) and G-M(TGF) macrophages, respectively. In fibroblasts, EC_{50} and EC_{90} of methylprednisolone to increase *CCN2* and *ACTA2* were 17–34 and 181-

249nM; IC_{50} and IC_{90} to decrease *COL1A1* were approximately 7 and 22nM (**Table 1**). In macrophages, EC_{50} and EC_{90} to increase *CCN2* were 21–28 and 151-316nM (**Table 2**).

Ser211-phosphorylated GR was increased by low concentration methylprednisolone

Phosphorylation at Ser211 is thought to be most critical for ligand-induced activation of GR, as well as nuclear translocation.[41,42] We performed Western blotting to assess altered Ser211-phosphorylated GR (S211-pGR), as well as total and Ser203-phosphorylated GR (tGR and S203-pGR). Directly co-cultured fibroblasts and macrophages were collected together and protein levels were assessed as mixtures of co-cultured cells. S211-pGR levels were increased by methylprednisolone regardless of macrophage phenotype and peaked around 10nM methylprednisolone (Fig. 3). This concentration was closer to the concentration required for gene downregulation than upregulation. However, S203-pGR, an inhibito of nuclear GR localization,[42] and tGR were decreased by methylprednisolone. Similar results were observed in separately collected fibroblasts and macrophages from indirect co-culture (**Supplemental Figure S1**).

Nuclear localization of Ser211-phosphorylated GR was increased by high concentration methylprednisolone

We subsequently performed immunocytochemistry to assess nuclear localization of GR. Regardless of macrophage phenotype, tGR-positive staining in DAPI-positive nuclear regions was concentration-dependently increased by methylprednisolone in both fibroblasts and macrophages in co-culture. tGR staining in nuclear regions peaked at 3-30nM methylprednisolone and decreased at higher concentrations (Fig. 4). S211-pGR in nuclear regions was also increased by methylprednisolone and plateaued at 100-300nM methylprednisolone. These findings, collectively with qPCR data, suggested the concentration of methylprednisolone required to upregulate fibrotic genes was more related to S211-pGR nuclear localization than tGR.

Methylprednisolone induced Nuclear localization of YAP/TAZ in VF fibroblasts.

Distribution of YAP/TAZ was assessed by immunocytochemistry. Regardless of the phenotype of co-cultured macrophages, positive staining for YAP and TAZ increased in the nucleus of VF fibroblasts as methylprednisolone concentrations increased (Fig. 4). In contrast, methylprednisolone did not alter the distribution of YAP/TAZ in M(IFN/LPS) or M(TGF) macrophages.

Fibrotic gene expression induced by methylprednisolone was suppressed by inhibition of YAP/TAZ-TEAD signaling.

To assess involvement of YAP/TAZ in the negative and positive gene regulation of methylprednisolone, we pharmacologically inhibited YAP/TAZ-TEAD signaling in co-cultured fibroblasts in the presence of 30 or 1,000nM methylprednisolone. In VF fibroblasts co-cultured with M(IFN/LPS) macrophages, the decrease of *TNF* and *PTGS2* by 30nM methylprednisolone was not reversed by verteporfin (Fig. 5).

However, increased *CCN2*, a target gene of YAP/TAZ-TEAD signaling, induced by 1,000nM methylprednisolone was inhibited by verteporfin. Verteporfin also inhibited *ACTA2*. Similarly, *CXCL10* downregulation by 30nM methylprednisolone was not prevented by verteporfin in VF fibroblasts co-cultured with M(TGF) macrophages, whereas increased *CCN2* and *ACTA2* induced by 1,000nM methylprednisolone was ameliorated by verteporfin. These findings suggested *CCN2* upregulation mediated by high-concentration GCs was driven with support of YAP/TAZ-TEAD signaling, but GC-induced suppression of those inflammatory genes was independent of YAP/TAZ-TEAD signaling.

DISCUSSION

GC therapy persists as a reasonable therapeutic option due to potent anti-inflammatory effects as well as the affordability and safety data accumulated over decades of clinical use. However, GCs have diverse functions and unfavorable side effects may negatively affect clinical outcomes. Optimizing GC therapy by reducing unfavorable effects could benefit millions of patients, while developing a novel therapeutic would also advance clinical care. The current study provided incremental data regarding concentration-dependent negative and positive gene regulation by methylprednisolone, as well as insight regarding mechanism(s) underlying this effect.

The IC_{90} of methylprednisolone to reduce inflammatory gene transcription was lower than the EC_{90} to promote fibrotic transcription in the current direct co-culture model; these data are similar to mono-cultured and indirectly co-cultured macrophages and VF fibroblasts.[30,31] This finding further supports our hypothesis that reduced GC concentrations to a level sufficient to inhibit inflammatory response is preferable to minimize fibrotic side effects.[15] As noted in the previous indirect co-culture study,[31] the IC_{90} range of methylprednisolone in the co-culture model was comparable to peak plasma levels of free methylprednisolone (21.1nM) after oral administration,[43,44] but the EC_{90} values were lower than the concentration of methylprednisolone for injection (53-214mM).[45] *In vivo* investigation is warranted to optimize GC dosing for efficiently inhibiting inflammation without activating fibrotic response.

The GR response to different concentrations of methylprednisolone was complex. Our data suggest concentration-dependent differential effects on GR phosphorylation/nuclear localization contribute to the complex biochemistry of GC-GR signaling. Although our data are insufficient to detail molecular events associated with different concentrations of GCs, some similarities were observed between methylprednisolone concentrations required to alter GR status and gene expression. Ideally, these data may be foundational to unravel the complexity related to GC concentration. Concentrations required to fully increase nuclear localization of S211-pGR were 100-300nM, comparable to the EC_{90} for upregulation of fibrotic genes. In contrast, S211-pGR and nuclear localization of tGR reached a peak at 3-30nM methylprednisolone, close to the IC_{90} for gene downregulation. Therefore, it seems the nuclear level of GR without Ser211-phosphorylation and/or cytoplasmic S211-pGR is related to negative gene regulation via low GC concentration, and the nuclear localization of S211-pGR is related to positive gene regulation via high GC concentration. Nuclear localization of GR and its recruitment to positive gene

regulatory elements are restricted by various types of post-translational modifications. These phenomena may explain why increased S211-pGR by low concentration methylprednisolone did not lead to increased nuclear accumulation or *CCN2* upregulation. For example, phosphorylation at Ser203 and Ser226, and acetylation at Lys494 and Lys495 prevent nuclear localization and/or recruitment to positive gene regulatory elements.[10,42,46] Regarding Ser203 phosphorylation, the function of Ser211 phosphorylation to drive nuclear localization of GR is likely overcome by Ser203 phosphorylation to prevent nuclear localization of GR.[42] On the other hand, recruitment of GR to negative gene regulatory elements, presumably associated with low-concentration methylprednisolone, reportedly requires SUMOylation at Lys293 of GR.[47] In addition, concentration of ligand-activated GR in the nucleus is thought to be crucial for GR dimerization and GR-mediated transcription via positive gene regulatory elements.[10,48,49] In spite of our encouraging data, mechanisms underlying concentration-dependent negative and positive gene regulation are still unclear.

YAP/TAZ-TEAD signaling supports multiple fibrotic signaling pathways, such as SMAD, Wnt, and Rho.[50] Inhibition of YAP/TAZ-TEAD signaling, as well as neutralization of connective tissue growth factor (encoded by *CCN2*), reduced the fibrotic response in multiple animal models and VF fibroblasts.[35,36,51,52] In the current study, *CCN2* upregulation induced by high-concentration methylprednisolone was reversed by verteporfin, but downregulation of *CXCL10*, *TNF*, and *PTGS2* was not. This finding suggests the fibrotic response associated with YAP/TAZ-TEAD signaling is specifically induced by high-concentration GCs. Blocking YAP/TAZ in combination with GC therapy may be another possible strategy to reduce fibrotic response induced by GCs. However, with regard to inflammation, YAP/TAZ reportedly has positive and negative roles dependent on cell types and organs.[50,53] Notably, as shown in our immunocytochemistry results, YAP/TAZ and TEAD activities in hematopoietic cells are quite different from other cells.[50] The impact of YAP/TAZ and TEADs in inflammatory responses of VF-resident and hematopoietic cells requires investigation to potentially target YAP/TAZ for VF diseases.

In conclusion, concentration-dependent differential effects of methylprednisolone were broadly observed across *in vitro* co-culture models of human VF fibroblasts and macrophages. S211-pGR nuclear localization and the activation of YAP/TAZ-TEAD signaling were likely associated with fibrotic gene expression mediated by high-concentration methylprednisolone in VF fibroblasts.

MATERIALS AND METHODS

The Supporting Information provides more detailed methodological specifics.

Cells. HVOX human VF fibroblasts, created by our group,[54] and THP-1 human monocytic cells (ATCC, Manassas, VA) were expanded as described previously.[36] Green fluorescent protein (GFP)-expressing THP-1 cells were prepared by transfection of pAcGFP1-Actin (Takara Bio, Shiga, Japan). M(IFN/LPS) and M(TGF) macrophages were prepared by stimulating THP-1-derived macrophages with IFN-γ/LPS, and

TGF-β, and directly co-cultured with fibroblasts as described previously.[27] Cells were exposed to different concentrations of methylprednisolone and/or 3μM verteporfin for 24 hours.

Quantitative Real-Time Polymerase Chain Reaction (qPCR). HVOX fibroblasts and GFP-positive macrophages were separated by fluorescence-activated cell sorting (FACS). RNA extraction, reverse transcription, and real-time polymerase chain reaction were performed using commercially available kits. Expression levels relative to *GAPDH* were quantified by the ΔΔCt method.

Western blotting and Immunocytochemistry. Following co-culture of fibroblasts and GFP-negative macrophages, Western blotting and immunocytochemistry were performed as described previously.[27,37] Antibodies are shown in **Table S1.**

Data analysis. Data were collected from independently performed technical triplicate experiments, at least. The R *drc* package was employed on R studio to determine EC_{50}, IC_{50}, EC_{90}, and IC_{90}, and to fit data into sigmoid curves, when applicable.[55] Simple dot plots are presented for other data. Means of EC_x/IC_x, as well as standard deviations, were calculated from the EC_x/IC_x estimations of triplicate experiments.

Declarations

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Contributions

RN, GJG, and RCB conceived the study. RN, MJG, and RB designed experiments. RN conducted most of the experiments and data analysis for figures 1-5 and tables 1 and 2. RB and GJG supported the experiments and data analysis for figures 1-5 and tables 1 and 2. RN wrote the manuscript. MJG and RCB supervised the study.

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Competing interests

The authors have no conflicts of interest or financial disclosures related to this work.

Data availability

All data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Tables

Table 1 and 2 are available in the Supplementary Files section.

Figures

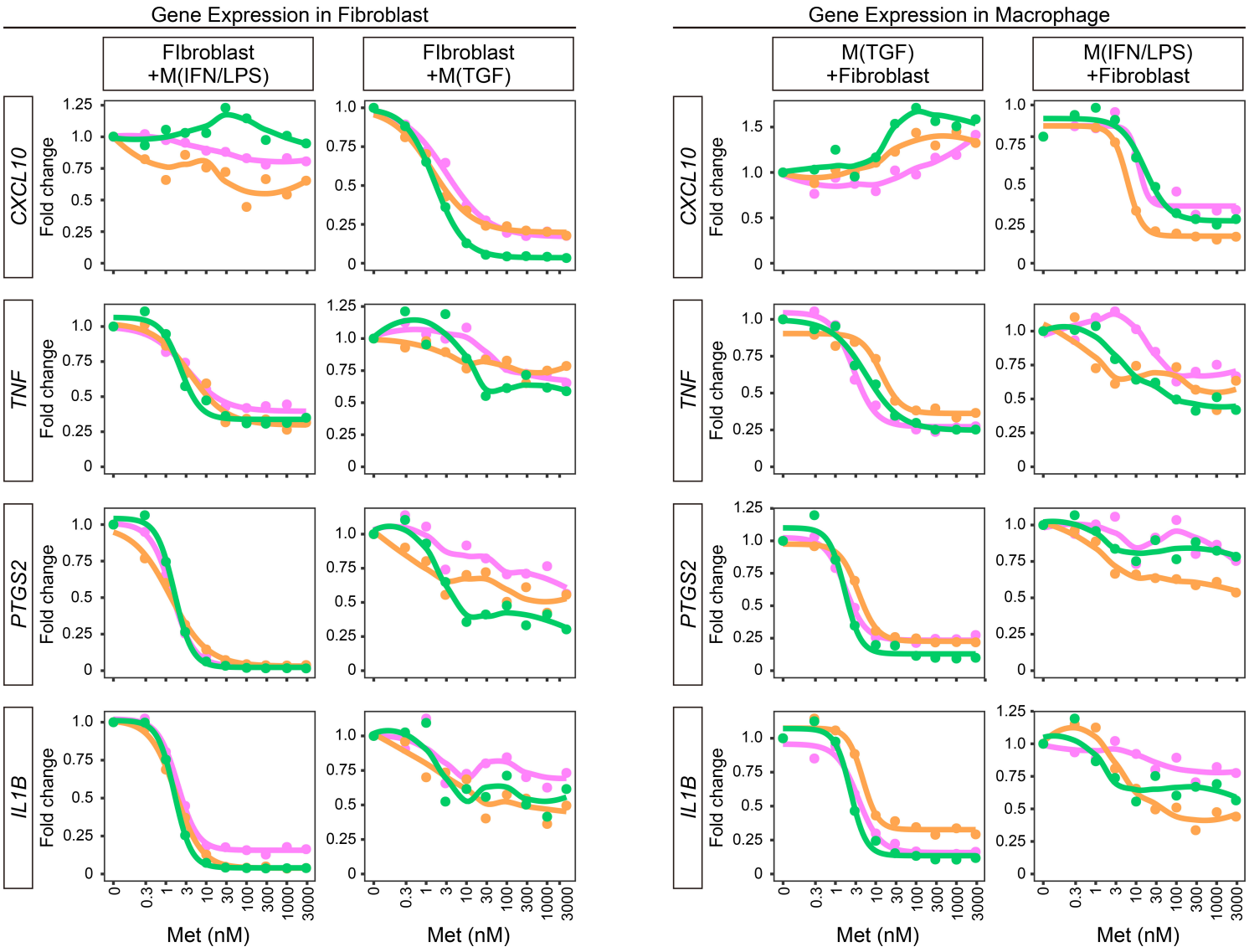

Figure 1

Inflammatory gene expression in fibroblast-macrophage co-culture. Human VF fibroblasts were directly co-cultured with G-M(IFN/LPS) or G-M(TGF) +/- 0.3-3,000nM methylprednisolone for 24 hours. Fibroblasts and macrophages were separated by FACS. Relative expression levels of *CXCL10*, *TNF*, *PTGS2*, and *IL1B* were determined by qPCR. Expression in cells unexposed to methylprednisolone was set to '1' and fold changes in expression levels were plotted. Data were obtained from independently performed technical replicates and are illustrated in different colors. Concentration-dependent curves are also presented for data applicable to both estimation of IC_{50}/EC_{50} and fitting to sigmoid curves in all triplicate experiments.

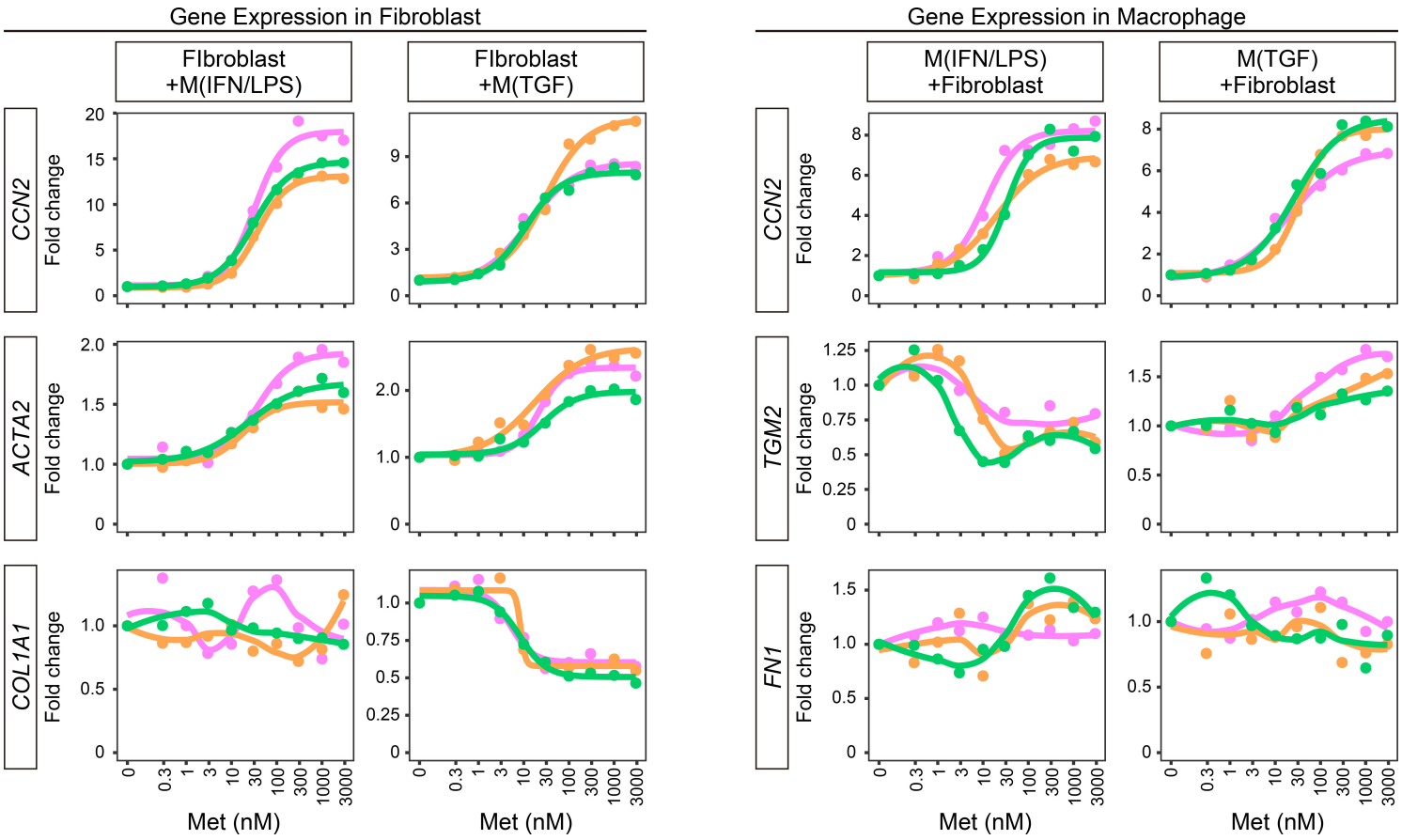

Figure 2

Fibrotic gene expression in fibroblast-macrophage co-culture. Human VF fibroblasts were directly co-cultured with G-M(IFN/LPS) or G-M(TGF) +/- 0.3-3,000nM methylprednisolone for 24 hours. Fibroblasts and macrophages were separated by FACS. Relative expression levels of *CCN2, ACTA2,* and *COL1A1* in fibroblasts, and *CCN2, TGM2,* and *FN1* in macrophages were determined by qPCR. Expression in cells unexposed to methylprednisolone was set to '1' and fold changes in expression levels were plotted. Data were obtained from independently performed technical replicates and are illustrated in different colors. Concentration-dependent curves are also presented for data applicable to both estimation of IC_{50}/EC_{50} and fitting to sigmoid curves in all triplicate experiments.

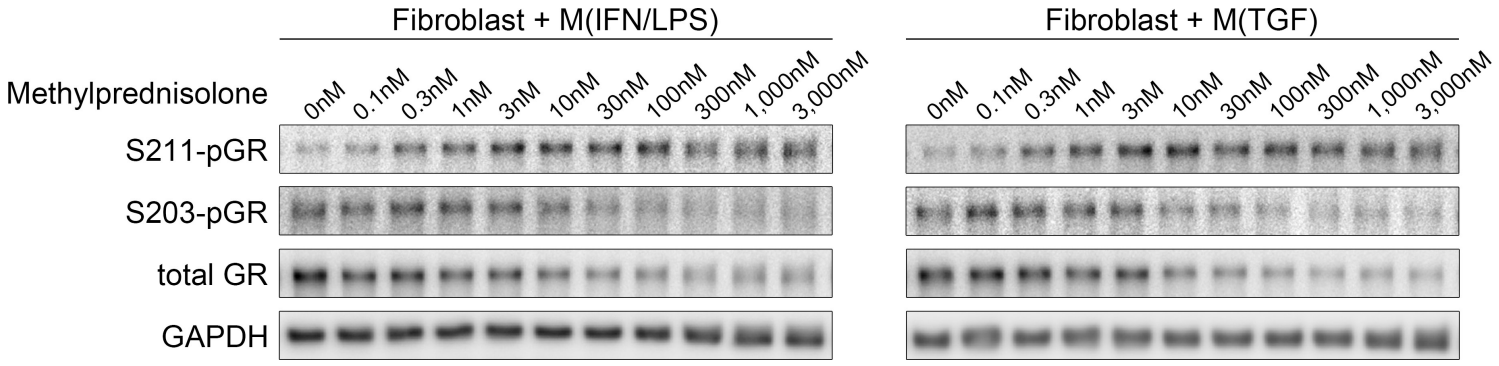

Figure 3

Western blots for S211-pGR, S203-pGR, and tGR. Human VF fibroblasts were directly co-cultured with M(IFN/LPS) or M(TGF) macrophages ± 0.1–3,000nM methylprednisolone for 24 hours. Proteins were extracted from the co-cultured cells without separating fibroblasts and macrophages.

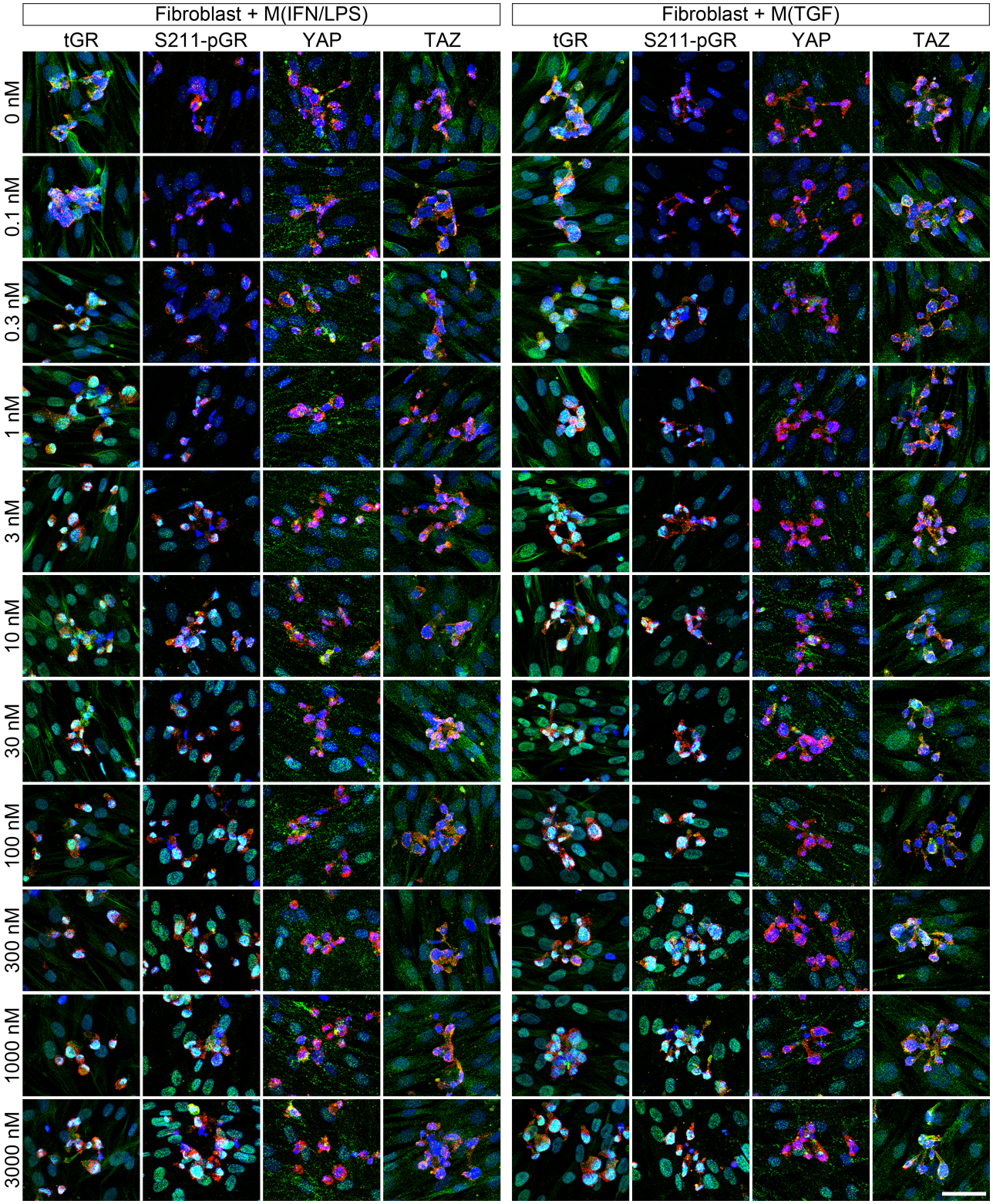

Figure 4

Immunocytochemistry of tGR, S211-pGR, YAP, and TAZ. Human VF fibroblasts were directly co-cultured with M(IFN/LPS) or M(TGF) macrophages ± 0.1–3,000nM methylprednisolone for 24 hours. Green: tGR,

S211-pGR, YAP, and TAZ. Red: Macrophages (CD11B and F4/80). Blue: Nuclei (DAPI). Bar: 50µm.

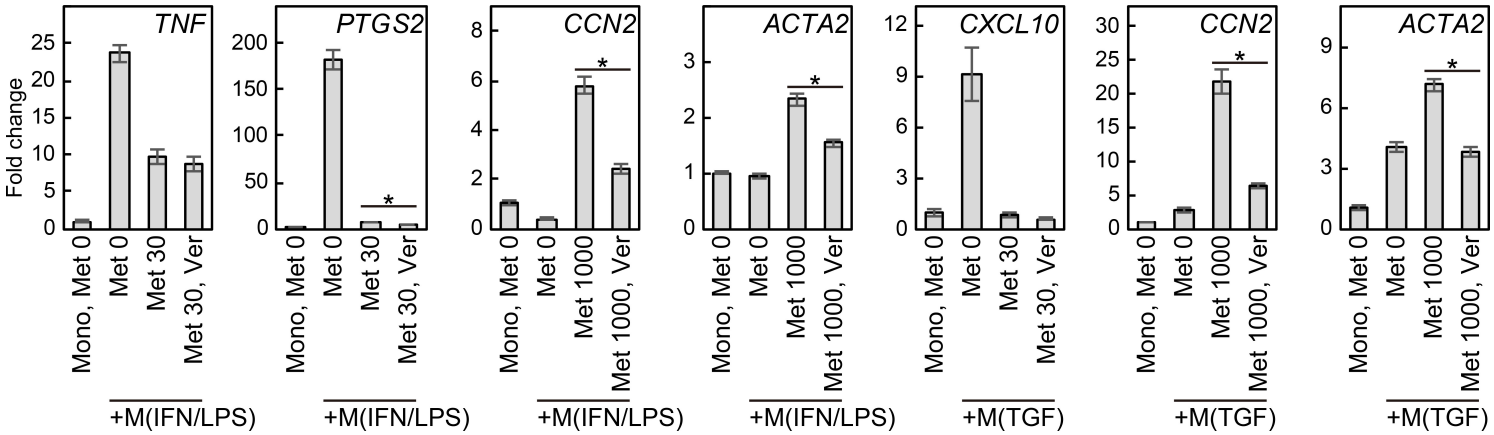

Figure 5

Effects of pharmacological YAP/TAZ-TEAD inhibition on methylprednisolone-induced gene regulation. Human VF fibroblasts were directly co-cultured with G-M(IFN/LPS) or G-M(TGF) macrophages +/- 30 or 1,000nM methylprednisolone and +/- 3µM verteporfin for 24 hours. Fibroblasts were isolated by FACS. Relative expression levels of *TNF*, *PTGS2*, *CCN2*, and *ACTA2* in fibroblasts co-cultured with G-M(IFN/LPS) macrophages, and *CXCL10*, *CCN2*, and *ACTA2* in fibroblasts co-cultured with G-M(TGF) macrophages were determined by qPCR. Expression in mono-cultured fibroblasts was set to '1.' Data are presented as mean ± SD. Co-cultured fibroblasts exposed to only methylprednisolone, and both methylprednisolone and verteporfin were subjected to statistical consideration with Student's *t*-test. Statistically significant differences are denoted as asterisks.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx
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- FigS1.docx
- SupplementalMaterialsandMethods.docx
- Table1.docx
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