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Lipopolysaccharide and interferon-γ team up to activate HIF-1α via STAT1 in normoxia and exhibit sex differences in human aortic valve interstitial cells

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ABSTRACT

In early stages of calcific aortic valve disease (CAVD), immune cells infiltrate into the valve leaflets and release cytokines such as interferon (IFN)-γ. IFN-γ has context-dependent direct effects, and also regulates other immune pathways. The purpose of this study was addressing the effects of IFN-γ on human aortic valve interstitial cells (AVICs), focusing on the pathogenic processes underlying CAVD. Strikingly, under normoxic conditions, IFN-γ induced hypoxia inducible factor (HIF)-1α expression, an effect strongly potentiated by the Toll-like receptor (TLR)-4 ligand lipopolysaccharide (LPS). Immunodetection studies confirmed the nuclear translocation of HIF-1α. Gene silencing showed that HIF-1α expression is dependent on signal transducer and activator of transcription (STAT)-1 expression. Consistent with HIF-1α induction, the secretion of the endothelial growth factor was detected by ELISA, and downregulation of the antiangiogenic factor chondromodulin-1 gene was observed by qPCR. Results also disclosed IFN-γ as a proinflammatory cytokine that cooperates with LPS to induce the expression of adhesion molecules, prostaglandin E_2 and interleukins. Moreover, IFN-γ induced an osteogenic phenotype and promoted in vitro calcification that were markedly potentiated by LPS. Pharmacological experiments disclosed the involvement of Janus Kinases (JAK)/STATs as well as ERK/HIF-1α routes on the induction of calcification. Notably, *IFN-γ receptor 1* expression, as well as ERK/HIF-1α activation, and the subsequent responses were more robust in male AVICs. This is the first report uncovering an immune and non-hypoxic activation of HIF-1α via STAT1 in AVIC. The aforementioned results and the sex-differential responses may be potentially relevant to better understand CAVD pathogenesis.

1. Introduction

Calcific aortic valve disease (CAVD) is as an important health and economic burden due to its increasing prevalence and the lack of available pharmacological approaches [1]. CAVD is an active process in which ectopic calcification takes place by dystrophic calcification associated with cell damage and/or mature lamellar bone formation [2,3].

Inflammation is a recognized driver of calcification in CAVD and plays a pivotal role in the initial phases of the disease [4–7]. In this respect, Toll-like receptors (TLRs) are important players given their function as sensors of endogenous and exogenous molecules that trigger inflammatory responses in the valve [8]. Similar to atherosclerosis, innate immunity activation in valve tissue results in the infiltration of immune cells that amplify and perpetuate the inflammatory scenario [4,9,10]. Among the cytokines released in this setting is interferon (IFN)-γ, a pleiotropic cytokine known to be an activator of the various cell types involved in atherogenesis [11]. IFN-γ, the only member of the type II IFN family, signals through the heterodimeric receptor IFNGR, which activates the Janus kinases and signal transducer and activator of transcription (JAK/STAT) pathways that subsequently promote a wide range of effects [12]. Among these effects, IFN-γ is a well-known modulator of other immune pathways, i.e. TLR signaling [12].

Mature healthy valves are avascular structures, however the master regulator of angiogenesis, hypoxia inducible factor (HIF)-1α [13], has been recently reported to co-localize with angiogenesis and

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calcification nodules in stenotic valves [14]. Neoangiogenesis adjacent to cell-rich and calcific areas has been detected in stenotic valves where pro-angiogenic factors are upregulated while anti-angiogenic factors are downregulated [13–18]. To date, scarce molecular association between HIF-1α and valve calcification has been reported, but it is known that in an immune context, similar to hypoxia, IFN-γ and infectious agents have been shown to influence HIF-1α expression [19]. Prompted by these evidences and the reported sex differences in valve calcification [20–24], we explored the effects of IFN-γ in CAVD using human AVICs. Overall, we identified a novel immune non-hypoxic mechanism of activation of HIF-1α involving the cooperation of IFN-γ–LPS that exhibits robust sex-specific differences.

2. Material and methods

A full description of the methods is available in the Supplementary material online.

2.1. Human samples

Human tissues included 24 tricuspid aortic valves obtained from heart transplant recipients with no valve disease (control non-calcified valves, 21 males/9 females). Stenotic aortic valve leaflets were obtained from 22 patients with CAVD (nonrheumatic stenotic valves, 12 males/12 females). Diagnosis and indications for heart transplantation and valve replacement conformed to European guidelines. The study was conducted in accordance with the Helsinki Declaration and approved by the local ethical committee (protocol no. PI15-263). Informed consent was obtained from all patients. Clinical features of patients are shown in Supplementary Table S1.

2.2. Valve interstitial and endothelial cell isolation and culture

AVICs were isolated using collagenase digestion and cultured in M199 medium supplemented with antibiotic-antimycotic agents and 10% fetal bovine serum (FBS), as described [25,26]. The study included interstitial cells explanted from control aortic valves (male and female AVICs). Most cells in culture exhibited a myofibroblast phenotype with positive staining for α-smooth muscle actin. No evident morphological differences between sexes were observed.

Endothelial valve cells (VECs) for conditioned experiments were isolated with 2.5 mg/mL collagenase and cultured on gelatin-coated plates in Endothelial Growth Medium-2. Positive cells for the endothelial cell marker CD31 selected by cell sorting were used for the experiments.

2.3. RT-qPCR analysis

Total RNA was extracted from human valve tissue and AVICs. First-strand cDNA was synthesized by the reverse transcription reaction, and later amplified by real-time PCR. Technical replicates were performed. Basal transcript levels were expressed as relative to the housekeeping gene *GAPDH* value ($2^{-\Delta Ct}$, Ct = cycle threshold value). Relative transcript levels were expressed as value relative to *GAPDH* and to resting conditions data ($2^{-\Delta\Delta Ct}$).

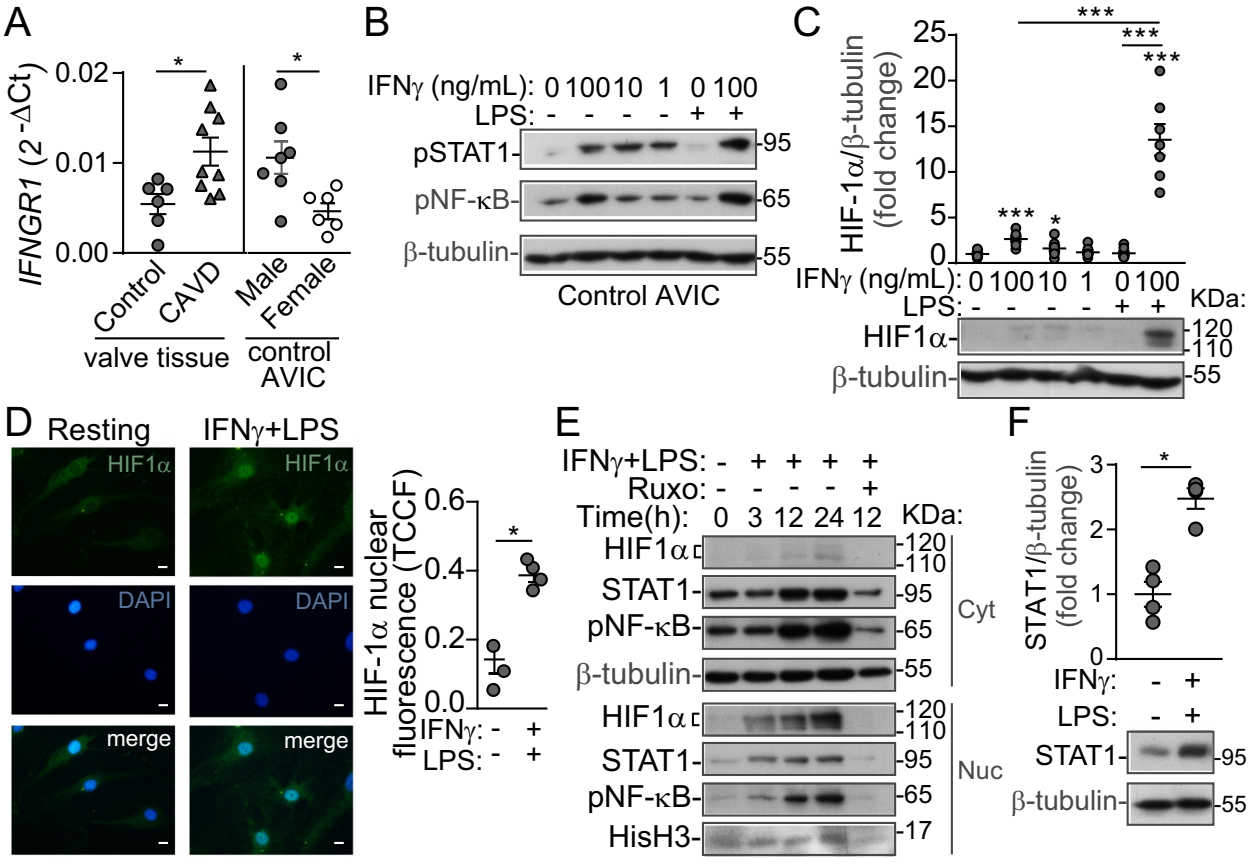

Fig. 1. Combination of IFN-γ and LPS induces HIF-1α expression and nuclear translocation. (A) Basal *IFNGR1* transcripts in control (circle) and CAVD tissue (triangle); n = 6 and 9, respectively, and in control AVIC, n = 8 in each group. (B–C) Male control AVICs were activated for 24 h and lysates were assayed by Western blot, n = 7. (D) HIF-1α immunostaining of cells activated for 24 h. Green, FITC; blue, DNA staining; white line, 50 μm. (E) Cytoplasmic and nuclear extracts from control AVICs pretreated with ruxolitinib and activated were analyzed by Western blot; n = 3. (F) Lysates from AVICs activated for 24 h were assayed for STAT1 protein expression; n = 4. Gray dots, control male AVIC; white dots, female AVIC. In the study, n designates the number of cell isolates from different donor valves used for the experiment. Concentrations: IFN-γ, when not indicated, 100 ng/mL; LPS, 1 μg/mL. * refers to statistical significance as compared to resting conditions or to the indicated by the line underneath *p < 0.05, **p < 0.01, ***p < 0.001.

2.4. Protocol for cell activation

AVICs were activated in M199 medium-2% FBS. Stimuli included recombinant human IFN-γ (specific activity ≥ 2×10^7 IU/mg), human IFN-α (specific activity ≥ 1.8×10^8 IU/mg) and LPS from *Escherichia coli* O111B4. In pharmacological experiments, cells were pre-incubated for at least 30 min with the indicated inhibitors.

2.5. Immunodetection of HIF-1α, proinflammatory molecules, and signaling routes

Whole cell lysates and cytoplasmic and nuclear extracts were analyzed by Western blot as described [24,26,27]. Leaflet tissue homogenization was carried out using a mortar and pestle in liquid nitrogen, and resuspended in RIPA buffer with protease inhibitors. Densitometry data were presented as arbitrary units normalized to β-tubulin and to resting conditions. Immunocytochemistry detection of HIF-1α was performed in cells activated for 24 h by using anti-human HIF-1α and Alexa Fluor® 488-conjugated antibodies (Supplementary Table S2).

2.6. Secretion of pro-angiogenic, proinflammatory, and matrix remodeling molecules by ELISA

Supernatants of cells activated for 24 or 48 h were assayed by ELISA. The absorbance at 450 nm was measured using a microplate reader. Data were normalized to total protein content.

2.7. In vitro calcification assays

Experiments were performed using a high phosphate concentration, as described [24]. Briefly, cells were incubated in calcification medium (M199, antibiotic-antimycotic, 3 mM inorganic phosphate, and 1% FBS) for 15 days. Mineralization was assessed by two independent methods using technical replicates. Calcification nodule formation was evaluated by Alizarin red staining (ARS) [24] and calcium deposition by the Quantichrom™ Calcium assay kit. Data were normalized to total protein content.

2.8. Proliferation assay

Cells activated in phenol-red free medium for 12 days were analyzed by MTT assay as described [24]. Briefly, MTT reagent was added to a final concentration of 1 mg/mL and incubated at 37 °C for 4 h. Purple formazan crystals were photometrically quantified. Data were expressed as the net $Absorbance_{570}$-$Absorbance_{630}$.

2.9. Flow cytometry-based apoptosis detection

Apoptosis was analyzed in cells activated in calcification medium for 7 days as described [24]. Briefly, cells stained with FITC-ApoScreen® annexin V and ApoScreen® propidium iodide were analyzed by flow cytometry. Data were expressed as % of annexin V-positive cells (quadrants B1 + B2).

2.10. Gene silencing by siRNA interference

AVIC were transfected with Dharmafect as in previous studies [27]. Ambion® Silencer® select pre-designed and validated siRNAs duplexes for *STAT1*, *STAT3*, and *HIF1A* genes, and siRNA negative control were used at 10 nM. Gene knockdown was confirmed by Western blot. 72 h post transfection, cells were used for the corresponding experiments.

2.11. AVIC-conditioned medium (CM) assays

AVICs were stimulated or not in M199-2% FBSi for 3 h. After the removal of stimuli, cells were incubated for 24 h. AVIC-CM (untreated or LPS + IFN-γ) were added to VEC monolayers and incubated for 24 h. Then, VECs were harvested for analysis. Non-conditioned medium was used as a control.

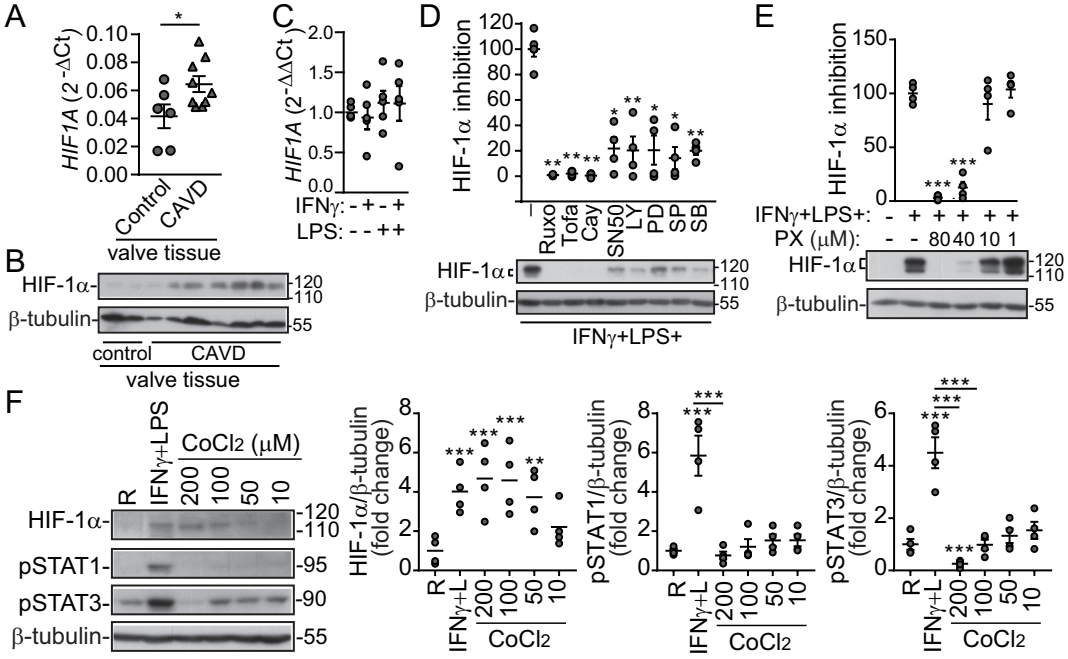

Fig. 2. HIF-1α is present in calcified valves and is inhibited by PX478 in activated AVICs. (A–B) Valve tissue from control/stenotic valves was analyzed for *HIF1A* mRNA and protein expression. (C) Relative *HIF1A* gene expression at 24 h, n = 5. (D–E) Male AVICs pretreated with the indicated inhibitors before activation for 24 h were assayed for protein expression. Data were normalized to IFN-γ + LPS value (100%), n = 4. (F) Cells treated with either immune or hypoxic stimulus for 24 h were analyzed by Western blot, n = 4. Cay indicates 5 μM CAY10614; LY, 50 μM LY294002; PD, 50 μM PD98059; PX, the HIF-1α inhibitor PX478; R, resting conditions; Ruxo, 6 μM ruxolitinib; SB, 10 μM SB203580; SN50, 50 μg/mL NF-kB SN50; SP, 10 μM SP600125; Tofa, 6 μM tofacitinib. Concentrations and symbols as in Fig. 1.

2.12. Statistical analysis

Values are presented as mean ± SEM. P denotes the p adjusted value, and significance was accepted for $p < 0.05$. Repeated measures ANOVA, one-way and two-way, analyses were performed. In case of data with non-normal distribution or unequal variances, log-transformation was applied previous to ANOVA analysis. If $p < 0.05$ for interaction between factors (sex/treatment), a *post hoc* analysis was performed by using the Benjamini–Hochberg procedure that applies corrections for multiple comparisons by controlling the false discovery rate (FDR). Patient features were analyzed as indicated in Supplementary Table SI. Statistical analysis was performed using GraphPad Prism 7 software.

3. Results

3.1. IFN-γ–LPS interplay promotes an immune and non-hypoxic induction of HIF-1α in control AVIC

We first examined IFN-γ receptor 1 (*IFNGR1*) gene, which was expressed in both valve tissue and interstitial cells and upregulated in calcified valves (Fig. 1A). The receptor is functional since IFN-γ activated several signaling routes in AVICs, i.e. the transcription factors STAT1/3 and NF-κB, as well as Akt and MAP kinases (Supplementary Fig. S1, Fig. 1B). Strikingly, even under normoxic conditions, IFN-γ upregulated HIF-1α protein in a dose-dependent manner, being the effects exacerbated by the TLR4 ligand LPS (Fig. 1C). Next, the functional relevance of non-hypoxic activation of HIF-1α was explored by analyzing its nuclear translocation. Immunostaining and Western blot detection of HIF-1α confirmed its nuclear localization upon combined IFN-γ/LPS treatment (Fig. 1D–E). Strikingly, upon activation, STAT1 protein was significantly upregulated and was present in the nuclei, being the effect abrogated by the JAK inhibitor ruxolitinib (Fig. 1E–F). In accordance with previous reports [14], HIF-1α transcripts and protein were detected and upregulated in CAVD tissue (Fig. 2A–B). Its transcript levels were not altered upon stimulation (Fig. 2C), arguing for a posttranscriptional regulation consistent with its stabilization by prolyl hydroxylase inhibition [13]. A pharmacological approach revealed that HIF-1α protein induction was receptor dependent, since was abrogated by IFNGR and TLR4 signaling inhibitors (Fig. 2D). In addition, PX-478 [28], a selective inhibitor of HIF-1α-dependent transcription and translation with fewer side effects than other agents,

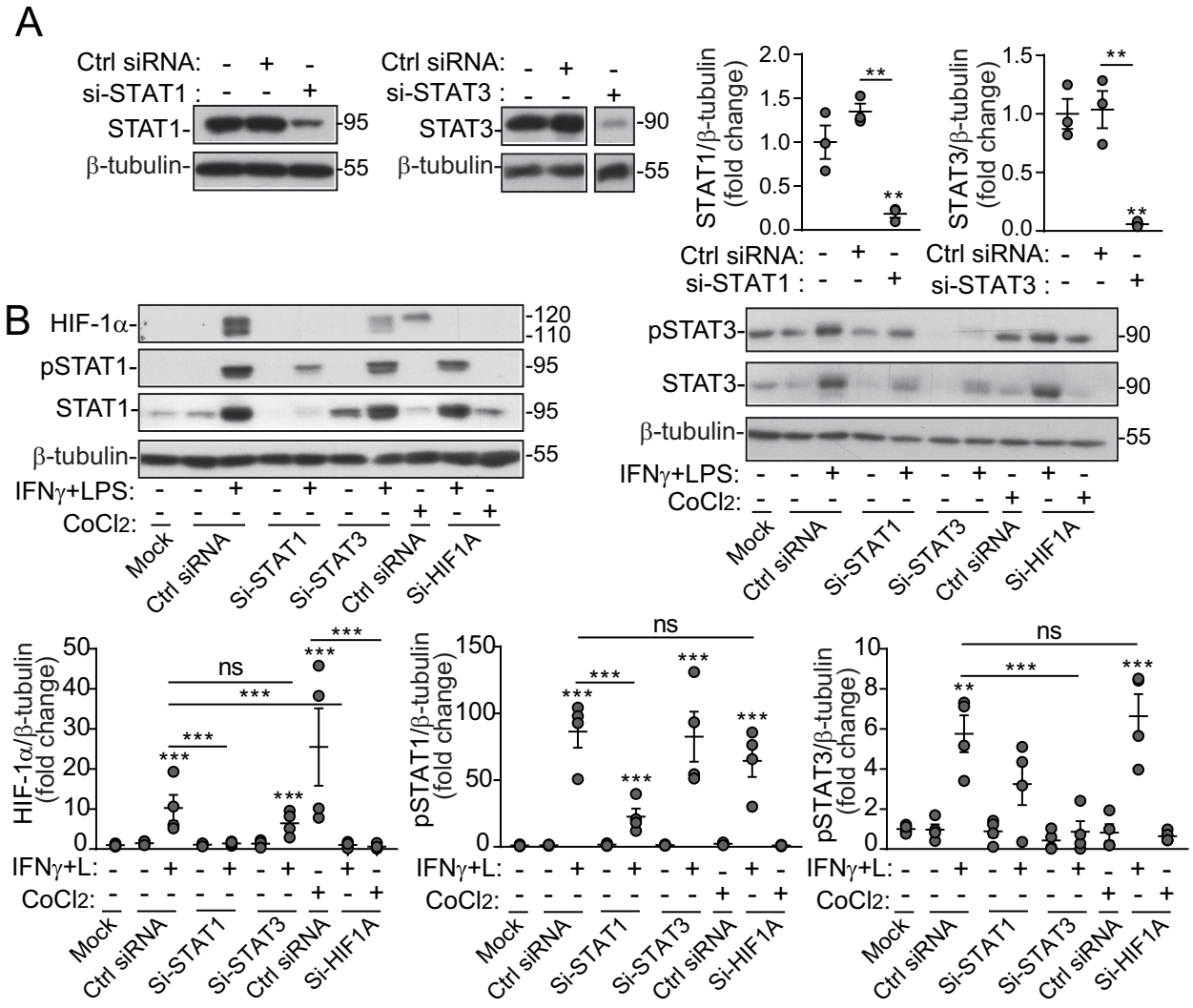

Fig. 3. STAT1, but not STAT3, is upstream of HIF-1α. Gene silencing experiments were performed as described in Methods. siRNA duplexes for *STAT1* (si-STAT1), *STAT3* (si-STAT3), and *HIF1A* (si-HIF1A) genes, siRNA negative control (Ctrl siRNA) and transfection reagent (mock) were used. (A) Western Blot analysis confirmed *STAT1/3* gene knockdown at 72 h; n = 3 independent cell isolates. (B) Cells transfected as indicated for 72 h were activated with IFNγ + LPS (IFNγ + L) or 100 μM of the HIF-1α inducer $CoCl_2$ for 24 h; n = 4 cells from different male donors. * refers to statistical significance as compared to mock values, or as indicated by the line below.

inhibited HIF-1α expression in a dose-dependent manner (Fig. 2E). Altogether, data point to a non-hypoxic and immune-mediated mechanism of HIF-1α activation.

3.2. IFN-γ in combination with LPS promotes HIF-1α via STAT1

To further elucidate the mechanism leading to HIF-1α induction, we performed gain and loss of function experiments (Figs. 2F, 3A–B, Supplementary Fig. S2). A hypoxic stimulus, $CoCl_2$ was used as a positive control for HIF-1α (Figs. 2F, 3B). Silencing of *STAT1*, but not of *STAT3*, gene inhibited HIF-1α protein induction upon activation (Fig. 3B, Supplementary Fig. S2). Conversely, *HIF1A* gene silencing did not affect phosphorylation of STAT1. Consistently, $CoCl_2$, a chemical inducer of HIF-1α expression, did not phosphorylate STAT1 (Fig. 2F). Together, data indicate that STAT1 signaling is upstream of HIF-1α.

3.3. IFN-γ induces pro-angiogenic molecules in AVICs via HIF-1α exhibiting sex differences

Next, prompted by our previous results on type I IFN in AVIC [24], potential sex specific effects in response to type II IFN were explored. Notably, HIF-1α protein induction was maintained and observed after 48 h stimulation to a major extent in male AVICs (Fig. 4A). Then, we investigated pro-angiogenic molecules downstream HIF-1α that are upregulated in CAVD tissue, i.e. vascular endothelial growth factor-A (*VEGFA*), and anti-angiogenic genes downregulated in CAVD tissue, i.e. *chondromodulin*-I (*CNMD*) (Fig. 4B). We found that IFN-γ promoted the secretion of VEGF-A in control AVICs, an effect potentiated by LPS (Fig. 4C–D). In keeping with JAK and HIF-1α mediated effects, VEGF-A secretion was blocked by pharmacological inhibition with ruxolitinib and PX-478 (Fig. 4D). Likewise, the secretion of MMP1, known to be associated with angiogenesis [29], was induced upon activation (Fig. 4E). Furthermore, *CNMD* gene, known to maintain valvular function by preventing angiogenesis [18], was downregulated upon IFN-γ activation (Fig. 4F), and is expressed in CAVD tissue (Fig. 5).

Notably, sex differences in the response to a combination of IFN-γ/LPS were observed. Male cells exhibited higher induction of HIF-1α, as well as the ensuing secretion of VEGF-A and MMP1 (Fig. 4C, E), and *CNMD* gene downregulation (Fig. 4F). Then, we explored potential sex differences in valve tissue. Protein analysis of valve leaflets revealed higher STAT1 protein levels in valves from males, and a high variability in HIF-1α protein expression (Fig. 5A). Additionally, CAVD tissue from males exhibited higher expression of *IFNGR*1 and pro-angiogenic genes, and lower expression of anti-angiogenic genes than females (Fig. 5B–C).

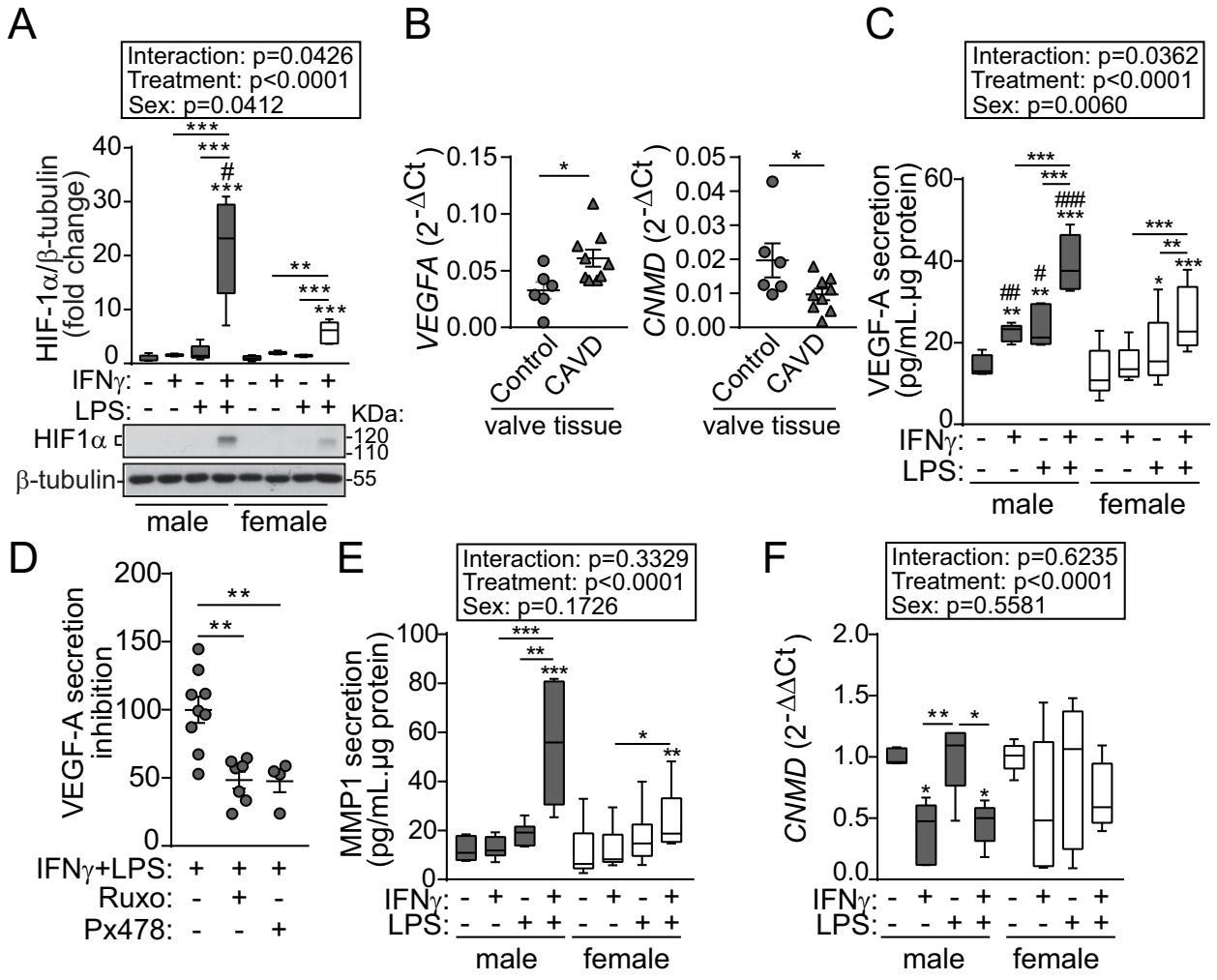

Fig. 4. IFN-γ in combination with LPS induces pro-angiogenic molecules via HIF-1α. (A) Activated male and female cells were assayed for HIF-1α expression upon 48 h activation; n = 5 in each group (B) Basal *VEGF* and *CNMD* transcript levels in control (circle) and CAVD tissue (triangle) (C–E) Supernatants from (A) were analyzed by ELISA. VEGF-A secretion, n = 5 in each group (C). Pretreatment with 6 μM ruxolitinib (Ruxo) and 40 μM PX-478 before male cell activation. Data were normalized to IFN-γ + LPS value (100%), n = 4 (D). MMP1 secretion at 24 h; n = 6 (E). (F) *CNMD* transcript levels upon 24 h stimulation; n = 5 in each group. Concentrations and color code as in Fig. 1. # indicates statistical significance between sexes for the corresponding treatment. $^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, $^{***,\#\#\#}p < 0.001$. 2-way ANOVA statistics for treatment/sex factors are shown above each panel in Figs. 4, 6–8.

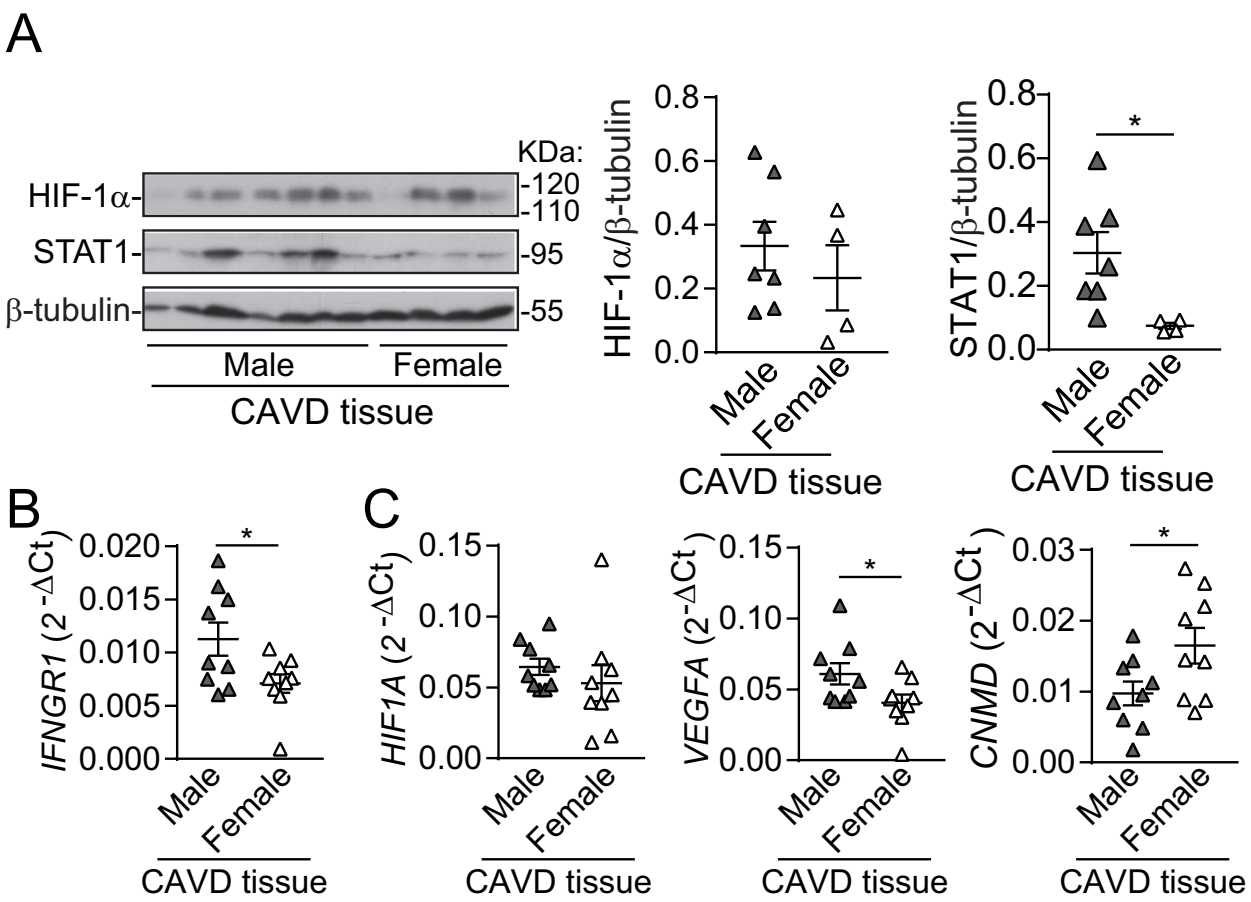

Fig. 5. Calcified valves from males express higher levels of STAT1 protein and *IFNGR1* and *VEGFA* transcripts than females. (A) Homogenized valve leaflets were analyzed by Western Blot for STAT1 and HIF-1α protein expression (normalized to β-tubulin levels); n = 7 valves from males and 4 from females. (B–C) Basal transcript levels of the indicated genes in CAVD tissue. n = 9 in each group. Color code, *, and symbols as in Fig. 1.

Together, data suggest IFNGR/STAT1-mediated sex mechanisms with potential relevance in CAVD.

3.4. IFN-γ is as a proinflammatory cytokine that cooperates with LPS in AVICs

Next, IFN-γ–mediated proinflammatory effects were investigated. IFN-γ triggered the induction of adhesion molecules VCAM-1 and ICAM-1 and its effects were enhanced by LPS (Fig. 6A). Likewise, crosstalk on the secretion of prostaglandin E_2, interleukin (IL)-6, and IL-8 was observed (Fig. 6B–C), whereas tumor necrosis factor-α secretion was undetectable (data not shown). Consistent with receptor-mediated effects, the crosstalk on inflammatory molecules was inhibited by blockade of JAK and TLR signaling (Supplementary Fig. S3).

3.5. IFN-γ promotes AVIC differentiation with sex differences

In line with its well-established role as a regulator of proliferation, IFN-γ reduced the proliferation rate of AVICs as shown in MTT assays (Supplementary Fig. S4A). Additionally, IFN-γ treatment for 12 days promoted morphological changes in AVICs (Supplementary Fig. S4B). Consistent with an effect on AVIC differentiation, data disclosed an IFN-γ–mediated osteoblastic differentiation phenotype characterized by upregulation of the pro-osteogenic genes bone sialo protein (*BSP2*) and bone morphogenetic protein (*BMP2*) (Supplementary Fig. S4C). Additionally, the mineralization inhibitor *MGP* gene was down-regulated upon IFN-γ activation (Supplementary Fig. S4D). Moreover, *BMP2* gene upregulation was abrogated by ruxolitinib (Supplementary Fig. S4E). Notably, the pro-osteogenic gene profile was sex-specific with higher upregulation of *BSP2* and *BMP2* genes in male than in female AVICs (Supplementary Fig. 4C).

3.6. IFN-γ acts as a procalcific factor via JAK and ERK/HIF-1α-dependent mechanisms exhibiting sex differences

The potential effect on calcification was explored under high phosphate conditions. AR staining and calcium deposition analysis revealed that IFN-γ induced calcific nodule formation and mineralization, with marked potentiation by LPS (Fig. 7A–B). In keeping with the involvement of IFNGR/JAK pathways in mineralization, IFN-γ–mediated calcification was abrogated with ruxolitinib (Fig. 7C–D). Notably, male cells were more prone to calcification (Fig. 7A). Next, we explored the pathways involved. $CoCl_2$, a chemical inducer of HIF-1α expression, but not of STAT1/3 activation (Fig. 2F), was able to promote AVIC calcification (Fig. 8A). Pharmacological inhibition with PX-478 further confirmed the partial contribution of HIF-1α to IFN-γ + LPS-mediated calcification (Fig. 8C).

Exploring other pathways, we observed that ERK activation was induced upon IFN-γ + LPS activation (Fig. 8B), and its blockade inhibited mineralization (Fig. 8C). As for the sex-specific mechanism, both ERK activation (Fig. 8B) and HIF-1α induction (Fig. 4A) were significantly higher in male as compared to female AVICs. Calcification assays with osteogenic differentiation medium also confirmed IFN-γ-mediated cell differentiation and the subsequent calcification (Supplementary Fig. S5). Overall, IFN-γ in combination with LPS promoted AVIC calcification in a higher extent in male cells via JAK/ERK/HIF-1α pathways.

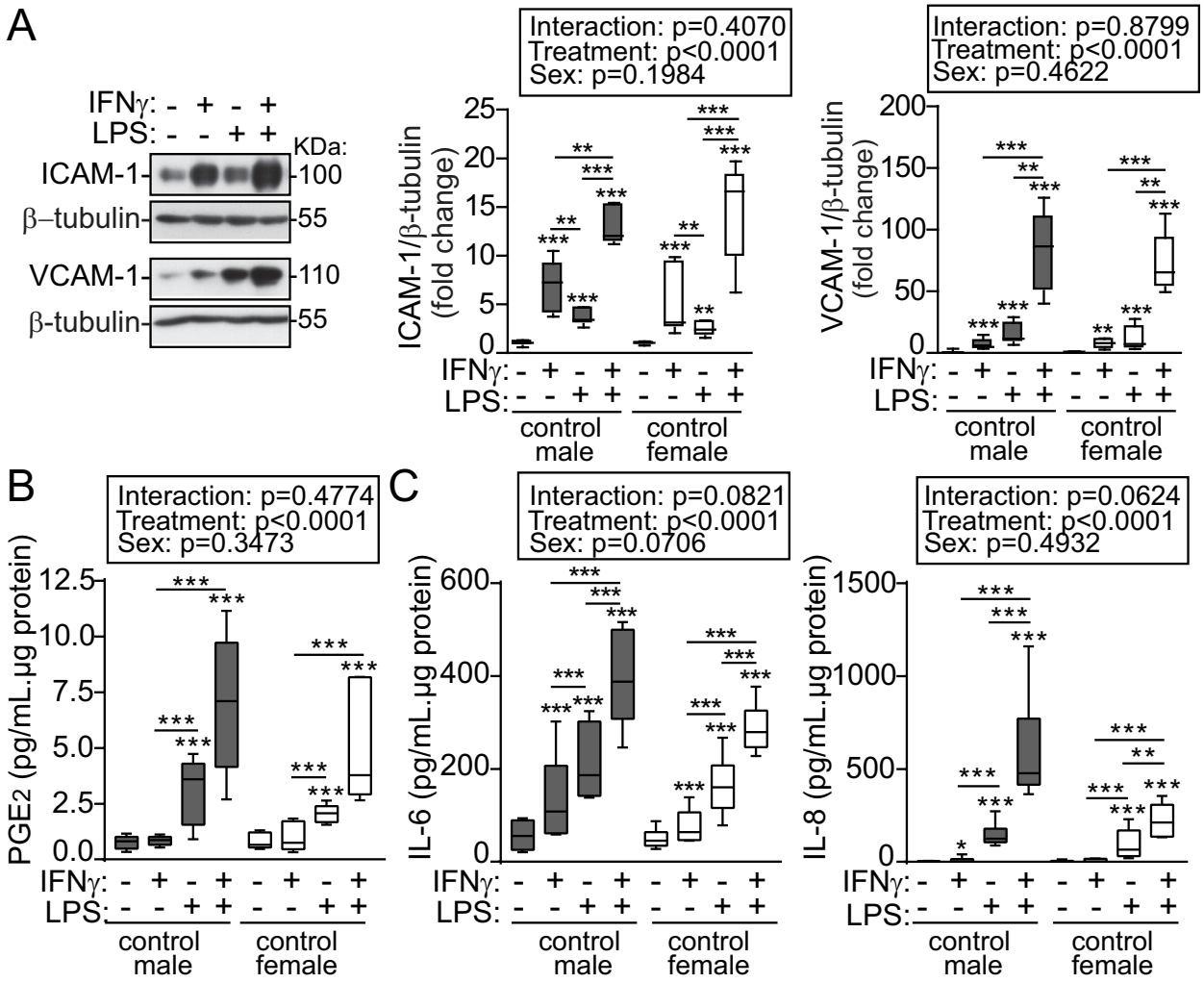

Fig. 6. IFN-γ induces proinflammatory molecules and further cooperates with LPS. (A-C) AVICs from males and females were activated. Cell lysates were assayed for ICAM-1 and VCAM-1 proteins; n = 5 in each group (A). Secretion of prostaglandin E_2 (PGE_2), IL-6, and IL-8 was analyzed by ELISA, n = 6 in each group (B–C). Concentrations, color code, and $*^{\#}$ as in Figs. 1, 4.

3.7. IFN-γ promotes apoptosis in high phosphate conditions via JAK pathways exhibiting sex differences

Given that inflammatory stimuli promote AVIC apoptosis leading to dystrophic calcification, we next examined cell death. Annexin-V staining and flow cytometry analysis revealed that IFN-γ promoted AVIC apoptosis to a higher extent in male that was blocked by the JAK inhibitor ruxolitinib (Supplementary Fig. S6A–B).

3.8. Mechanistic differences between type I and type II IFN

Potential differences between IFNs were then explored given our recent data demonstrating type I IFN-induced calcification in AVICs [24]. Remarkably, immune activation of HIF-1α seems to be specific for IFN-γ (type II) (Fig. 9A). Notably, differences between IFNs were also observed in the pro-osteogenic gene differentiation profile, since *RUNX2* and the chondrogenic markers *SOX9* and *ACAN* were upregulated by IFN-α but not by IFN-γ (Fig. 9B). Moreover, the anti-apoptotic gene *BCL2* was downregulated by IFN-γ but not by IFN-α (Fig. 9C).

3.9. Human AVIC-conditioned medium induces inflammatory and angiogenic molecules in human VECs

The relevance of IFN-γ effects on the aortic valve was investigated in conditioned medium experiments. CM from IFN-γ + LPS-treated AVICs (Fig. 10A) promoted morphological and gene profile changes in VECs (Fig. 10B–D). These changes included (i) *eNOS* expression downregulation (Fig. 10C), known to be associated to endothelial dysfunction, (ii) the upregulation of genes associated to angiogenesis and inflammation, i.e. *VEGFA* and *IL6* (Fig. 10C), and induction of ICAM-1 and VCAM-1 proteins (Fig. 10D). Data are consistent with secreted factors by IFN-γ + LPS-stimulated AVICs with subsequent effects on VECs.

4. Discussion

Our data disclose a novel immune and non-hypoxic mechanism of HIF-1α activation involving interplay between IFN-γ and LPS that shows sex-differential responses in valve cells. The results also reveal the robust pro-angiogenic, proinflammatory, and procalcific effects of IFN-γ in AVICs.

The major sources of IFN-γ are natural killer and T lymphocytes activated upon viral infection. IFN-γ receptor is ubiquitously expressed [12], including aortic valve leaflets, as shown in the study. Exposure of AVICs to IFN-γ is a likely event under different conditions. First, exposure to IFN-γ released from infiltrating immune cells [9,10]. In fact, Nagy and colleagues recently reported that infiltrated $CD8^+$ T lymphocytes are activated in calcified human aortic valves and secrete

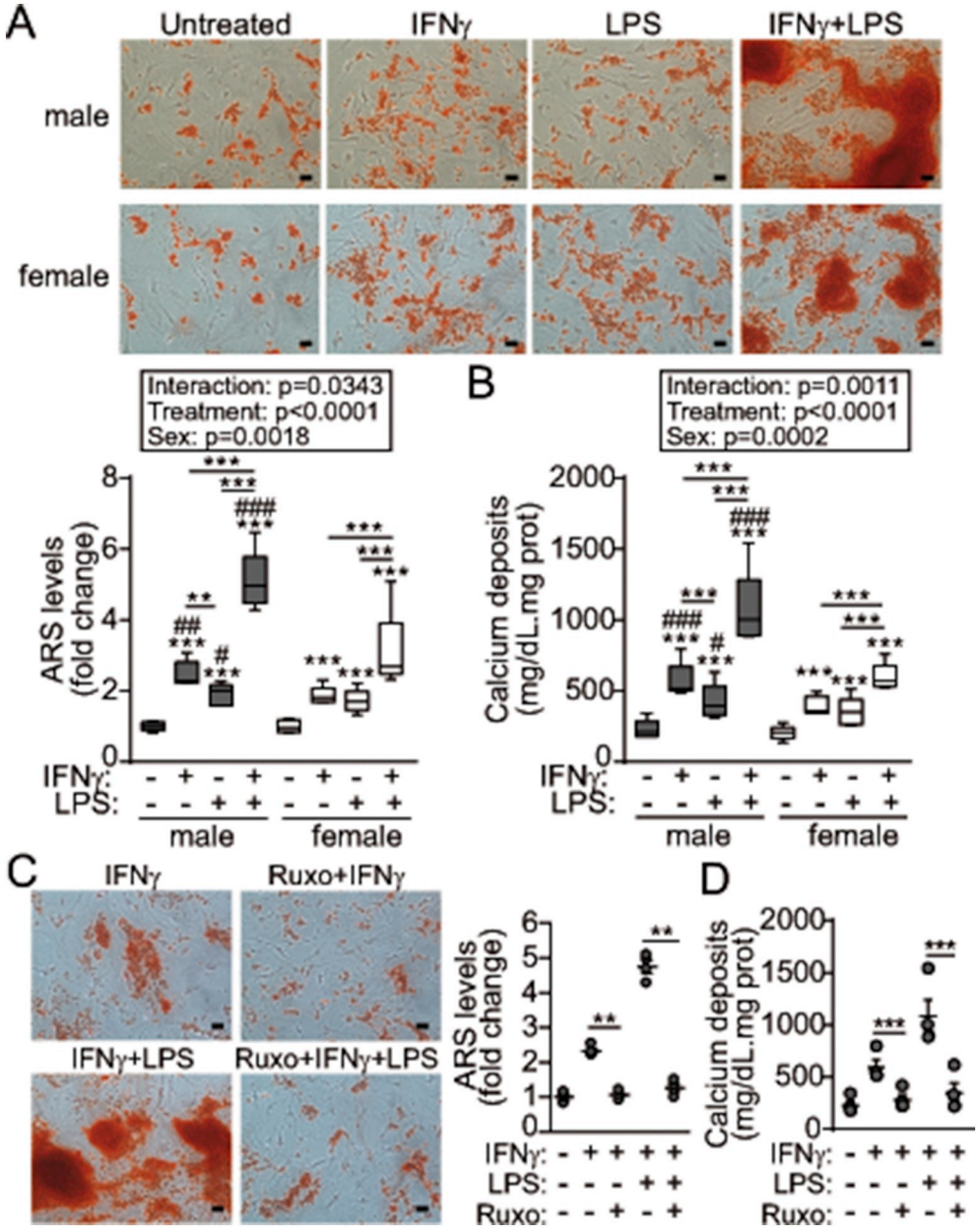

Fig. 7. IFN-γ induces AVIC calcification via JAK routes with sex-differential responses. (A–B) Male and female AVICs were stimulated in calcification medium for 14 days. Calcium nodules were detected by ARS and quantified (A) and calcium deposition was quantified (B); n = 5 in each group. (C–D) Cells were pretreated with 6 μM ruxolitinib (Ruxo). Images are representative of n = 4 male donors. Black line, 50 μm. Concentrations, color code, and $*^{\#}$ as in Figs. 1, 4.

active IFN-γ that subsequently impairs the calcium resorption potential of osteoclasts in calcified human aortic valves [30]. Second, activation of IFNGR and TLR4 by their cognate ligands conveyed by the blood stream may occur in the course of infections and/or after tissue damage.

4.1. IFN-γ in combination with LPS promotes HIF-1α- induction via STAT1

To our knowledge, no active mechanisms of HIF-1α expression have been reported in AVIC. The striking HIF-1α expression and neoangiogenesis found in stenotic valves may be thought at first glance as a consequence of valve thickening and the subsequent impairment of O_2 diffusion; however, additional mechanisms could account for these processes. Herein, we report a novel immune-driven and HIF-1α-dependent signaling pathway leading to a pro-angiogenic phenotype in human AVICs. This is reminiscent of the Warburg effect reported in macrophages during microbial infections, where NF-κB, by regulating HIF-1α activation, can increase glycolytic metabolism and the production of angiogenic factors, in addition to proinflammatory mediators [31].

A major finding of this study is that STAT1 is upstream of HIF-1α expression. This mechanism is supported by gene silencing experiments and JAK inhibition by ruxolitinib. Moreover, STAT1 is not only regulated by phosphorylation, but by increasing its protein expression levels upon immune stimulation. These data parallel a mechanism reported in human fibroblasts cell lines, where IFNs promoted upregulation of unphosphorylated STAT1 that prolonged the expression of IFN-induced immune regulatory genes [32].

4.2. Immune activation under normoxic conditions induces pro-angiogenic molecules via HIF-1α- in AVICs

HIF-1α seems to be a key regulator of IFN-γ + LPS pro-angiogenic effects in AVICs. This transcription factor has been recently pointed out

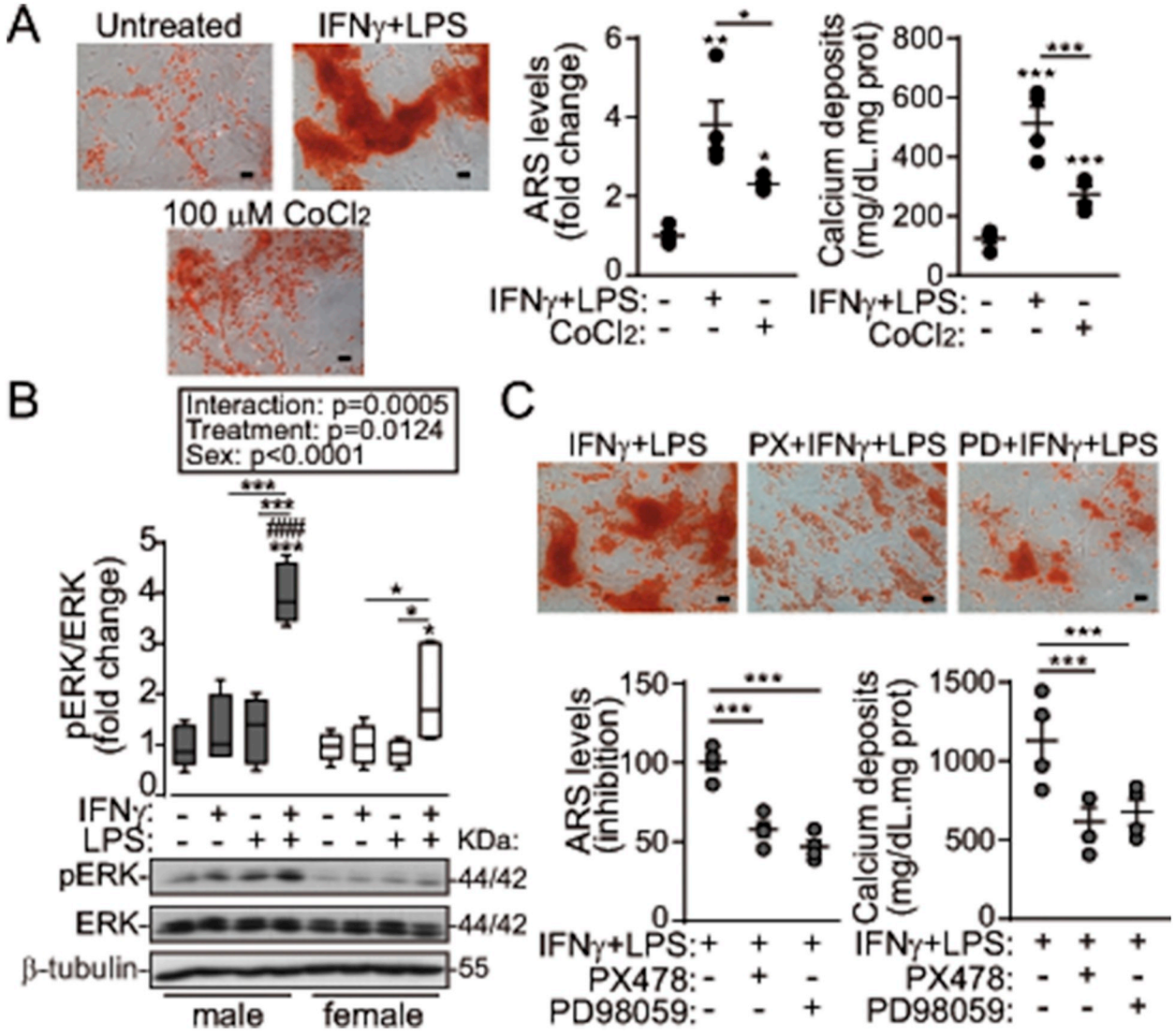

Fig. 8. IFN-γ induces AVIC calcification with the involvement of ERK/HIF-1α routes. (A) Cells were stimulated in calcification medium for 7 days. Representative ARS image and calcium quantification, n = 4 male donors. (B) Male/female cells were activated and ERK phosphorylation was analyzed. (C) Male AVICs pretreated with 33 μM PX478 or 50 μM PD98059 were stimulated in calcification medium for 10 days, n = 4. Concentrations, color code, and *# as in Figs. 1, 4.

as a mediator of hypoxia-induced secretion of matrix remodeling molecules in mitral valve interstitial cells [33], while our results demonstrate their secretion in the aortic valve cells following an immune and non-hypoxic activation. Data suggest an alternative mechanism in normoxia via activation of JAK/STAT1 and TLR pathways. In line with this, *M. tuberculosis* infection of IFN-γ-stimulated macrophages leads to a synergistic increase in HIF-1α protein levels that is functionally relevant [34]. Our findings also suggest that HIF-1α could also be relevant in the early stages of CAVD, when valve cells are not exposed to hypoxic conditions. In normoxia, prolyl-4-hydroxylases regulate HIF-1α expression, which is known to be translated continuously and subsequently degraded by ubiquitination [13]. Whether HIF-1α activation upon IFN-γ treatment is due to attenuation of hydroxylation is a likely hypothesis.

Even though IFN-γ is thought to be an anti-angiogenic agent by inhibiting endothelial cell growth and tube formation [12], some pro-angiogenic effects have also been found in human retinal and corneal cells [35]. These data are reminiscent of our previous study showing VEGF secretion upon crosstalk between LPS and the lipid mediator sphingosine-1-phosphate in human AVICs [27]. Remarkably, IFN-γ-mediated upregulation of pro-angiogenic molecules paralleled downregulation of the anti-angiogenic factor chondromodulin-1. This factor plays a protective role in valvular homeostasis, since its downregulation promotes neovascularization and valve calcification [18]. The pro-angiogenic phenotype in AVIC was abrogated by PX-478, currently in clinical trials for the treatment of solid tumors and lymphoma, due to its inhibitory effect of HIF-1α at different levels [28].

4.3. IFN-γ is a proinflammatory cytokine that potentiates LPS effects in AVICs

Consistent with its role in inflammation [12], IFN-γ exerted proinflammatory effects in AVICs. The present data also disclose a positive crosstalk between IFN-γ and LPS, thus supporting the notion that many functions of IFN-γ depend on the cross-regulation of cellular responses to other cytokines and inflammatory factors [36]. The present results are reminiscent of our recent report on the interplay between type I IFN and LPS [24]. However, the present study showed more prominent effects regarding type II IFN and TLR signaling, i.e. ERK activation, and proinflammatory molecule induction. Data are consistent with the reported role of IFN-γ as an amplifier of TLR signaling by promoting ligand-receptor interactions as well as downstream signaling routes and by inactivating TLR-induced feedback inhibitory loops [36,37]. Notably, in the cardiovascular system, IFN-γ and TLR4 ligands have been

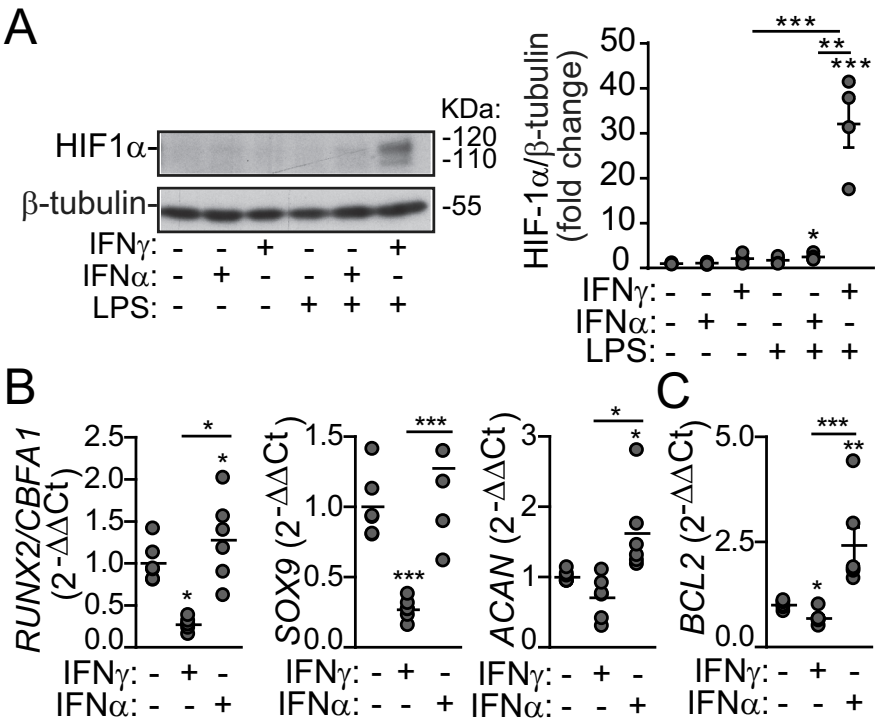

Fig. 9. Differences in the response to IFNs. Male AVICs were activated as indicated for 24 h. (A) Cell lysates were assayed for HIF-1α protein expression. (B–C) Total RNA was analyzed for the indicated genes, n = 4. Concentrations, * as in Fig. 1.

found synergizing to induce a pro-atherogenic state [38].

4.4. IFN-γ promotes AVIC calcification through JAK/ERK/HIF-1α pathways

IFN-γ is as a procalcific factor of human AVICs that accelerates osteogenesis, as evidenced by osteoblastic differentiation, calcific nodule formation and calcium deposition. This is reminiscent of the induction by IFN-γ of BMP2 expression in pancreatic cells [39] and of the necrotizing myopathy with dystrophic calcification observed in IFN-γ transgenic mice [40]. However, our results disclose novel effects of IFN-γ distinct from to the suppression of osteoclast function recently reported by IFN-γ [30].

HIF-1α is a regulator of IFN-γ + LPS procalcific phenotype in AVICs under hyperphosphatemic conditions. Its role on calcification is supported by hypoxic stimulus-induced mineralization and by pharmacological blockade significantly reducing AVIC mineralization. In line with this, HIF-1α has recently been reported to play a role in phosphate-induced vascular smooth muscle cell calcification [41]. Moreover, our data agree with a report showing that HIF-1α inhibitors prevent extraskeletal bone formation in several models of heterotopic ossification [42]. Furthermore, a role of HIF-1α on valve calcification is supported by a recent report in porcine and human valves demonstrating that disturbed flow increases ubiquitin E2 ligase C by regulating HIF-1α stabilization, thus leading to endothelial inflammation, endothelial-mesenchymal transition, and subsequent aortic valve calcification [43]. These data and our study support that HIF-1α induction via non-hypoxic mechanisms can mediate valve calcification.

ERK activation is involved in IFN-γ-mediated calcification in AVICs. This is consistent with previous reports pointing to a crucial role of ERK in AVIC osteogenesis [44]. In line with our data, these kinases have been found to play a role in the IFN-γ–mediated alterations in macrophage cholesterol homeostasis and pro-atherogenic gene expression [45]. The finding of HIF-1α regulated by ERK agrees with current notions on the role of this kinase as an enhancing factor of HIF-1α transcriptional activity [46].

The JAK1/2 inhibitor ruxolitinib, currently used for the treatment of primary myelofibrosis [47] and polycythemia vera [48], prevented IFN-γ-dependent inflammation, angiogenesis, and calcification. Moreover, ruxolitinib blocked HIF-1α and VEGF-A induction.

4.5. Differential responses to interferons

In a recent study we have shown type I IFN as inducers of inflammation and calcification [24]. The current study reveals striking differences between type I and II IFNs, including specific mechanisms on HIF-1α induction and distinct differentiation gene profile with consequences on the inflammatory and osteogenic responses. Our study discloses a specific induction of HIF-1α by IFN-γ that may be relevant to its higher potency on inflammation and mineralization. Additionally, a distinct set of differentiation markers is induced by IFNs, i.e. IFN-α, but not IFN-γ, upregulated the chondrogenic markers *SOX9* and *ACAN*, consistent with a potentially different differentiation fate. Whereas type I IFN is derived from many cell types, IFN-γ is produced by T lymphocytes and natural killer cells. In addition, type I and type II IFN bind to different receptors that activate different transcription factors, i.e. STAT1/2, type I IFN, and STAT1 dimers, type II IFN. These specificities help explain the distinct involvement of the different IFN type in inflammatory and osteogenic settings.

4.6. Sex differences on IFN-γ effects

Our data reveal sex differences in the extent of IFN-γ effects and agree with the sex differences reported in aortic valve calcification [20–23], as well as with recent data showing sex differential effects upon IFN-α activation of AVICs [24]. Sex groups were similar in terms of age, characteristics and comorbidities (Supplementary Table SI), and all specimens were processed using the same protocols. Basal gene and protein profile exhibited sex differences in valve tissue and derived cells. Higher expression of *IFNGR*, *VEGFA* and *CNMD* in valve tissue and derived AVICs from male than in female was detected. Also, STAT1 protein is more expressed in calcific valve tissue from males. Consistent

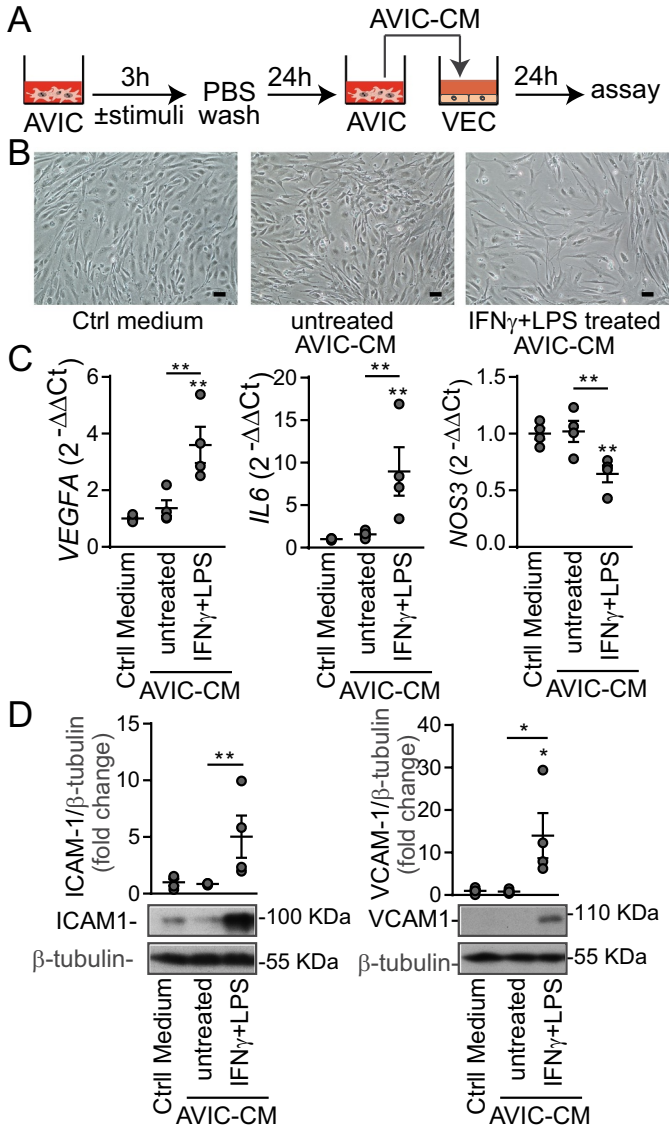

Fig. 10. Conditioned medium from IFN-γ + LPS-stimulated AVICs promote changes in VECs. (A) Protocol used for CM experiments in VECs. (B) Morphological changes in VECs induced by CM from stimulated AVICs, n = 4. (C–D) Gene and protein expression changes in VECs upon exposure to activated AVIC-CM, n = 4 independent valve donors. Black line, 50 μm. Concentrations, symbols as in Fig. 1. * refers to statistical significance as compared to non-conditioned (Ctrl) medium.

with this, male cells exhibited greater pro-angiogenic, procalcific and pro-apoptotic responses. As for the mechanism involved, sex-related dissimilarities on HIF-1α and ERK activation and chondromodulin-1 function might account for the lower responses observed in female AVICs.

4.7. Relevance of IFN-γ-mediated effects in the aortic valve context

Conditioned medium experiments revealed a role of IFN-γ-mediated effects on the aortic valve physiopathology. Factors secreted by activated AVICs promote both genotypic and phenotypic changes in VECs consistent with the induction of processes known to be relevant to CAVD pathogenesis, i.e. endothelial dysfunction, angiogenesis, differentiation and inflammation [1,49].

5. Conclusions

The study unravels a novel immune and non-hypoxic mechanism underlying HIF-1α activation via STAT1 in valve cells. Results also highlight the robust pro-angiogenic, proinflammatory, and pro-osteogenic effects of IFN-γ in the valve setting and disclosed sex differences and crosstalk with LPS. Further studies are needed to elucidate whether targeting JAK/HIF-1α pathways could be a potential treatment of CAVD at the early/middle stages.

Transparency document

The Transparency document associated this article can be found, in online version.

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Conflict of interest

None. The authors report no commercial or proprietary interest in any product or concept discussed in this article.

Declarations of interest

None

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dummy.2019.01.002.

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