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Research Article

Novel role of Hippo effector YAP1 as a rheostat controlling inflammation in dendritic cells

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Abstract

Dendritic cells (DCs) function within tissues that experience wide fluctuations in oxygen availability, and these hypoxic environments exert a strong influence on innate immune activity. In this context, we identify the Hippo pathway effector YAP1 (Yes-associated protein) as a central regulator of human monocyte–derived DC responses under low-oxygen conditions. YAP1 is expressed in DCs and undergoes dynamic modulation following lipopolysaccharide stimulation, affecting signaling processes in both the cytoplasm and nucleus. Loss of YAP1 function—through pharmacological inhibition or gene knockdown—amplifies reactive oxygen species (ROS) accumulation during hypoxia and drives robust activation of the p38 mitogen-activated protein kinases (MAPK) pathway, resulting in increased interleukin (IL)-1β expression. In contrast, stabilization of YAP1 using a phosphorylation-resistant S366A mutant enhances transcription of IL-6 and tumor necrosis factor alpha, maintains activation of p38, nuclear factor-κB, and ERK pathways, and augments IL-1β release through a cytokine-dependent autocrine loop. Together, these findings position YAP1 as a homeostatic rheostat that balances DC inflammatory responses in hypoxic environments. By limiting oxidative-stress-induced inflammation while simultaneously supporting cytokine-driven amplification of immune signaling, YAP1 provides a mechanism through which DCs integrate environmental and inflammatory cues. This dual regulatory role highlights YAP1 as an important mediator of innate immune adaptation to tissue hypoxia. Moreover, understanding how YAP1 shapes DC function offers new insight into the mechanisms underlying inflammatory and autoimmune disease pathogenesis, where dysregulated oxygen sensing and cytokine production contribute to chronic immune activation.

Keywords: DCs, hypoxia, IL-1β, MAPK, YAP1

1. Introduction

Dendritic cells (DCs) are the most powerful antigen-presenting cells, capable of inducing primary immune responses, thus enabling the development of immunological memory. By sensing pathogens, cytokines, and environmental stress signals, DCs orchestrate both innate and adaptive immunity, bridging the recognition of danger signals with the activation of T cells.[1,2] Although DCs constitute a heterogeneous group of cells, they all share the ability to modulate innate responses via cytokine production and release and to initiate antigen-specific responses.[3] Following full maturation due to the activation of specific receptors involved in antigen recognition (such as Toll-like receptor 4) DCs migrate to secondary lymphoid organs (eg lymph nodes) that are characterized by a lower pO_2.[4,5] Stabilization of hypoxia-inducible factors (HIFs) under low-oxygen conditions modulates gene expression programs that govern glycolysis, survival, and cytokine production, highlighting the sensitivity of DCs to oxygen availability.[6] Inflammatory stimuli activate pattern recognition receptors, including Toll-like receptor 4 (TLR-4), which leads to downstream signaling through the mitogen-activated protein kinases (MAPK) and nuclear factor-κB (NF-κB) pathways.[7] Recently, the Hippo-YAP1 pathway has emerged as a crucial regulator of innate immunity, as well as the regulation of bone marrow stromal cells.[8–10] The Hippo signaling pathway is a conserved regulator of tissue growth, cell proliferation, and stress responses,[11] with its downstream effector YAP1 (Yes-associated protein) acting as a transcriptional co-activator controlling gene expression programs essential for cell survival and homeostasis.[12] While YAP1 has been extensively studied in the context of organ development, cancer, and tissue regeneration,[13,14] its expression and functional role in immune cells, particularly DCs, remain largely unexplored. Emerging evidence suggests that YAP1 can intersect with inflammatory signaling pathways and regulate cellular responses to oxidative stress, implying a potential role as a modulator of immune function.[15,16] At its core, the Hippo involves a kinase cascade wherein the upstream kinases MST1/2 (mammalian Ste20-like kinases) phosphorylate and activate the downstream kinases LATS1/2.[17] Activated LATS1/2, in turn, phosphorylates the transcriptional coactivators YAP1 and TAZ (transcriptional coactivator with PDZ-binding motif), marking them for cytoplasmic retention or proteasomal degradation.[18] When the pathway is inactive, YAP1/TAZ translocate into the nucleus, where they interact with transcriptional enhanced associate domain (TEAD) transcription factors to drive the expression of genes involved in cell growth, survival, and migration.[19] Mechanistically, the pathway integrates diverse extracellular signals, including mechanical cues, cell polarity, and metabolic status, to modulate tissue homeostasis and regeneration.[12,20,21] Dysregulation of the Hippo-YAP1 pathway has been implicated in cancer progression, fibrosis, and other pathological conditions, highlighting its therapeutic potential as a target in

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disease intervention.[22] Given the centrality of MAPK and NF-κB pathways in DC activation and the sensitivity of these cells to hypoxic stress,[23] we hypothesized that YAP1 might serve as a key regulatory node linking the Hippo pathway with DC inflammatory responses. To the best of our knowledge, YAP1 expression in human DCs has not been reported, and the mechanisms by which it could integrate hypoxic and inflammatory signals remain unknown. In this study, we examined the expression and regulation of YAP1 in human monocyte–derived DCs under normoxic and hypoxic conditions, with or without lipopolysaccharide (LPS)-induced maturation. We investigated how YAP1 modulates MAPK signaling, cytokine production, and oxidative stress, aiming to uncover its role as a fine-tuner of DC inflammatory responses. Our findings reveal that YAP1 operates through distinct, context-dependent mechanisms to regulate p38 phosphorylation and interleukin (IL)-1β production, highlighting a dual role in controlling both stress kinase activity and cytokine-driven amplification of inflammation. This work establishes YAP1 as a previously unrecognized regulator of DC function, providing new insights into the integration of hypoxic and inflammatory cues in innate immunity and suggesting potential implications for the modulation of immune responses in pathological conditions such as chronic inflammation and infection.

2. Materials and methods

2.1 Reagents

RPMI 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin, and L-glutamine were obtained from Euroclone (Devon, United Kingdom). Fycoll was sourced from Cederlane Laboratories, and Percoll from Amersham Biosciences (Pittsburgh, PA, United States). Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 were provided by ProSpec TechnoGene (East Brunswick, NJ, United States). All reagents were confirmed to contain endotoxin levels below 0.125 EU/mL, as determined by the Limulus amebocyte lysate (LAL) assay (Cambrex, East Rutherford, NJ, United States). LPS from *Escherichia coli* strain 055:B5 was purchased from Sigma-Aldrich (Milan, Italy) and used at a final concentration of 0.1 μg/mL for 24 h. Peptide 17 was purchased from Selleckchem (United States) and used at a final concentration of 100 nM and incubated for 30 min before adding LPS as previously described.[24] Briefly, Peptide 17 is a YAP1-TEAD protein–protein interaction inhibitor that prevents YAP1 target gene expression.

2.2 Cell cultures

DC2.4 cell line was purchased from Merck (Whitehouse Station, NJ, United States) and cultured according to the manufacturer's instructions. THP-1 cell line was kindly donated by Francesca Chiarini from the CNR Institute of Molecular Genetics "Luigi Luca Cavalli-Sforza" Unit of Bologna, 40136 Bologna, Italy, and cultured according to the ATCC guidelines.

2.3 Human monocyte–derived DC preparation and culture conditions

Human monocyte–derived DCs were generated from anonymous buffy coats provided by the South-East Tuscany Blood Establishment (AOUS, Siena, Italy), as previously described. This study was approved by the Ethical Committee of Azienda Ospedaliera Universitaria Senese and the University of Siena (protocol CAVSE 17022020), with written informed consent obtained from all participants. Monocytes were isolated using Ficoll–Percoll density gradient centrifugation from blood samples enriched in monocytes (>95% CD14$^+$). These monocytes were differentiated into immature DCs (>90% CD1a$^+$, <5% CD14$^+$) by culturing for 6 d in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine (Euroclone), 50 ng/mL GM-CSF, and 20 ng/mL IL-4 (PeproTech, Cranbury, NJ, United States) as previously reported.[25] Reagents were confirmed to have endotoxin levels <0.125 EU/mL via the LAL assay (Cambrex). DCs were incubated for 24 h at 37 °C under normoxic (21% O_2, 5% CO_2, and 74% N_2; pO_2 ~140 mmHg) or hypoxic (2% O_2, 5% CO_2, and 94% N_2; pO_2 ~14 mmHg) conditions using a Coy O_2-Controlled InVitro Glove-Box Hypoxia Chamber (Coy Laboratory Products, Grass Lake, MI, United States).[26] When indicated, DC terminal maturation was induced by treating cultures with 100 ng/mL LPS, as previously described.[24]

2.4 Immunoblotting and cytoplasmic/nuclear extraction

Cells were lysed in 40 μL of RIPA buffer (Cell Signaling Technologies, Danvers, MA, USA) containing a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Protein concentration was determined using the Micro BCA Protein Assay Kit (Rockford, USA). Cytoplasmic and nuclear fractions were prepared using the Abcam kit (ab113474) according to the manufacturer's instructions. Equal amounts of protein were loaded onto 4% to 20% Mini-Protean TGX gels (Bio-Rad, Hercules, CA, USA) for SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad). The membranes were incubated overnight at 4 °C with the appropriate primary antibodies: HIF-1α (BD Biosciences, San Jose, CA, 1:200, Cat. no. 610958), ph-NF-kB (Cell Signaling Technologies; 1:1,000, Cat. no. 3033), php38 MAP kinase (Thr180/tyr182; Cell Signaling Technologies; 1:1,000, Cat. no. 9211), php44/42 MAP kinase (Thr202/Tyr204; Cell Signaling Technologies; 1:1,000, Cat. no. 9101), YAP1 (Cell Signaling Technologies; 1:1,000, Cat. no. 14074), Histone H3 (Cell Signaling Technologies; 1:1,000, Cat. no. 5192), β-Tubulin (Cell Signaling Technologies; 1:1,000, Cat. no. 2146), and β-actin (Sigma-Aldrich, 1:50,000, Cat. no. A3854). Anti-mouse IgG-HRP (Cell Signaling Technologies; 1:2,000, Cat. no. 7076) and anti-rabbit IgG-HRP (Cell Signaling Technologies; 1:2,000, Cat. no. 7074) were used as secondary antibodies (Cell Signaling Technologies). The protein bands were analyzed using the ImageJ program. Protein levels were normalized against housekeeping proteins depending on the assay, and the treated samples were compared against nontreated controls, resulting in relative protein levels (fold change against control).

2.5 RNA extraction and reverse transcription quantitative polymerase chain reaction

Total RNA was extracted using the EuroGOLD Trifast reagent (Euroclone), and cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed using either SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) or iTaq SYBR Green Supermix (Bio-Rad Laboratories), depending on the experiment. mRNA expression levels were measured with the CFX Duet Real Time-PCR System (Bio-Rad) or the MiniOPTICON System (Bio-Rad), and data were analyzed using the iQ5 Optical System Software (Bio-Rad). The relative expression was quantified using the 2-ΔΔCT method, with β-actin as the housekeeping gene.[27] Primer validation was conducted, as previously described.[28]

2.6 Enzyme-linked immunosorbent assay

Supernatants collected from human monocyte–derived DCs or DC2.4 were evaluated for production of IL-1β by a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (Biotechne, United States). Briefly, standards and samples were properly diluted and incubated in 96-well plates for 2 h at 37 °C. Then, a biotinylated antibody specific to IL-1β was added for 2 h at 37 °C. After washing, avidin conjugated to horseradish peroxidase was added to each well for 30 min at 37 °C, and the development was performed using TMB substrate. The TMB reaction was stopped with a sulfuric acid solution, and the absorbance was measured at 450 nm using a MULTISKAN (Thermo Fisher Scientific, San Jose, CA, United States). To subtract high background signals, a reference measurement at 650 nm was performed.

2.7 ROS-ID hypoxia/oxidative stress detection kit

Reactive oxygen species (ROS) production in human monocyte–derived DCs or DC2.4 was measured using the ROS-ID Hypoxia/Oxidative stress detection kit (Enzo Life Sciences ENZ-51011) using only the ROS detection probe following the manufacturer's instructions. Briefly, cells were resuspended in PBS containing the oxidative stress (green) fluorescent probes and incubated at 95% humidity and 5% CO_2 for 30 min. Then, the cells were washed and resuspended in 100 μL of PBS. Five thousand events were acquired on a Guava Easy Cyte cytometer (Luminex Corp., Austin, TX, United States) and analyzed with FCS Express 6 software.

2.8 Confocal microscopy analysis

DC2.4 cells were seeded on an 8-well chamber slide and incubated at 37 °C and 5% CO_2 to allow cells to grow to confluence. LPS of 100 ng/mL was added where indicated, and cells were incubated for 24 h under normoxic or hypoxic conditions. The cells were then fixed with PBS-4% PFA methanol free (Thermo Fisher Scientific) for 10 min, permeabilized with PBS-0.25% Triton X-100 for 15 min, saturated with PBS-5% bovine serum albumin (BSA) for 60 min, and then incubated overnight at 4 °C with anti-ph-p38. Cells were finally incubated for 2 h at room temperature with secondary antibodies: goat anti-rabbit IgG DyLight 550 1:200 in PBS-1% BSA. Samples, mounted using Fluoroshield with DAPI (Sigma-Aldrich), were analyzed by confocal laser microscope (Leica TCS SP8) with 364 to 562 nm excitation and 458 to 576 nm emission filters for DAPI, DyLight 550, respectively. All images were processed using ImageJ software (NIH).

2.9 RNA interference

RNA interference was carried out by silencing YAP1 using specific YAP1-targeting shRNAs (NM_001130145.3) along with the MISSION shRNA Universal Negative Control #1 (SIC001), all obtained from Sigma-Aldrich, at a concentration of 46 nM. Transfection was performed using Lipofectamine RNAi MAX (Invitrogen, Paisley, United Kingdom), with shRNA-Lipofectamine complexes diluted in OPTI-MEM (1X) (Gibco, Thermo Fisher Scientific, Cleveland, OH, United States). The complexes were added to the cells, and DCs were incubated for 24 h under normoxic conditions. After transfection, DCs were exposed to either normoxic or hypoxic conditions for 24 h.

2.10 Generation of DC2.4 YAP1 S366A cell line

$YAP1^{S366A}$ DC2.4 cell line was generated via CRISPR-mediated homology-directed repair at the National Facility for Genome Engineering, Human Technopole (Milan, Italy). DC2.4 was maintained in DC2.4 medium, under standard culture conditions (37 °C, 5% CO_2). Briefly, 150 pmol of sgYAP1 were complexed with 122 pmol of Cas9 protein (Alt-R S.p. HiFi Cas9 Nuclease V3, IDT) and incubated at room temperature for 20 min to form RNPs. DC2.4 were detached, and 2×10^5 cells were nucleofected with RNP and 120 pmol of ssODN (IDT, Alt-R HDR Donor Oligo). The donor was designed to bear the desired modification (S366A) and 6 silent mutations to avoid re-cutting. Nucleofection was performed on a Lonza 4D Nucleofector using P3 Primary Cell Solution and the DN-107 program, supplemented with 4 μM Alt-R Cas9 Electroporation Enhancer (IDT). After nucleofection, cells were transferred to 12-well plates (Corning) containing prewarmed DC2.4 medium. Three days later, genomic DNA was extracted, and the targeted region was amplified and Sanger sequenced to evaluate the editing efficiency in the bulk population. Single-cell clones were subsequently isolated using limiting dilution. Sixty-one single-cell clones were picked and analyzed by Sanger sequencing to check for desired editing. Additionally, 1 clone that underwent the same genome editing procedures but did not show any modification in the YAP1 sequence was selected as a control clone to maintain the same passage number and culture conditions as the YAP1 S366A cell line. The sequences of ssODN, sgRNA, and primers for PCR and Sanger sequencing are listed in Table S1.

2.11 Statistical analysis

Data are shown as the mean ± SEM of at least 3 independent experiments. An unpaired 2-tailed Student's *t* test or 1-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons was performed with Graph-Pad Prism (San Diego, CA, United States). A 2-sided *P*-value of <0.05 was considered the threshold for statistical significance.

3. Results

3.1 The Hippo pathway is expressed in human monocyte–derived DC

First, we exposed human monocyte–derived DCs to a pO_2 of 140 mmHg/20% O_2 (normoxiaaerobic conditions) or 14 mmHg/2% O_2 (hypoxia, resembling the lymphoid or the inflammatory microenvironments), either in the presence or not of the MD-2/TLR-4 ligand LPS to induce final DC maturation. Figure 1A shows that hypoxia significantly enhanced the expression of the HIF-1α at the protein level, especially in the presence of LPS. This result established the hypoxic phenotype of DCs in our experimental conditions, as previously reported by our group.[29] This was accompanied by the significant increase of the MAPK ph-p38 and ph-p44/42 after LPS treatment in both normoxic and hypoxic conditions (Fig. 1B), suggesting that hypoxia and LPS can synergistically activate MAPKs, leading to enhanced inflammatory responses. Of interest, we examined whether YAP1 was expressed in DCs either in aerobic or hypoxic conditions. We should underline that, to our knowledge, YAP1 expression in DCs has not been reported yet in the literature. As shown in Fig. 1C, YAP1 is expressed in DCs, and the levels of YAP1 were modulated by LPS and hypoxia. Indeed, LPS significantly increases the expression of YAP1, indicating a correlation between YAP1 and its potential regulation of the inflammatory process. In addition, upon LPS

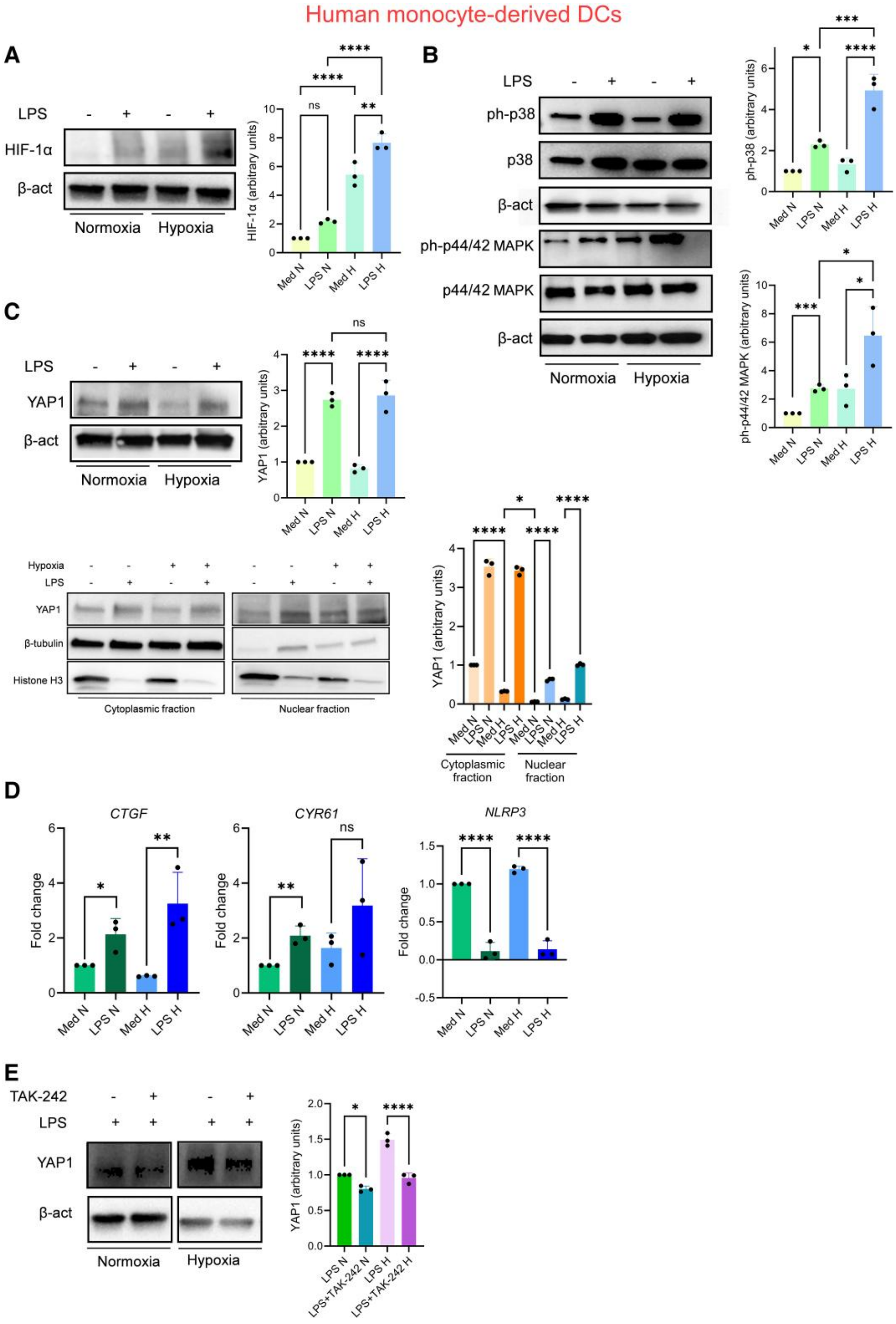

Fig. 1. YAP1 is expressed in human monocyte–derived DC. Protein expression levels of HIF-1α (A), ph-p38 and ph-p44/42 (B), YAP1 in the total lysate and in the cytoplasmic and nuclear fraction (C) after 24 h exposure of DCs to normoxia or hypoxia. (D) RT-qPCR analysis of Ctgf, Cyr61, and Nlrp3 after 24 h exposure of DCs to normoxia or hypoxia. (E) Protein expression levels of YAP1 after TAK-242 treatment. LPS was added where indicated for 24 h. β-Actin was used as a loading control, β-Tubulin and Histone H3 were used as loading controls for the cytoplasmic and nuclear fractions, respectively. β-Actin was used as a housekeeping gene for RT-qPCR analysis. Data represent SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$, $^{****}P < 0.0001$ using unpaired 2-tailed Student's t test or 1-way ANOVA followed by Tukey's test.

treatment, YAP1 levels were increased in both the cytoplasm and nuclear fractions. The expression and activation of the Hippo pathway in our inflammatory model were further confirmed by the significant increase of 2 canonical YAP1 target genes, such as *Ctgf* and *Cyr61*, together with the significant decrease of *Nlrp3* to counterbalance and limit the inflammatory response[30,31]

(Fig. 1D). To confirm that the modulation of YAP1 was mainly dependent on LPS stimulation and connected to an inflammatory microenvironment, we inhibited the LPS ligand TLR-4 with the specific inhibitor TAK-242 and tested the expression level of YAP1. TLR-4 inhibition decreases the expression of total YAP1 under normoxic and hypoxic conditions compared with LPS-stimulated cells, suggesting that the mechanism that regulates YAP1 stabilization after LPS stimulation is mediated by the TLR-4 receptor (Fig. 1E).

3.2 YAP1 regulates p38 phosphorylation and IL-1β production in human monocyte–derived DC

To elucidate the role of YAP1 in this context, we initially inhibited YAP1 using a commercially available inhibitor, Peptide 17, and then analyzed the expression of MAPK p38. Several lines of evidence implicate MAPK p38 in the pro-inflammatory response to bacterial agents and cytokines, as MAPK p38 and IL-1β expression are tightly related. Indeed, IL-1β is a key mediator in innate immune responses and inflammation. As shown in Fig. 2A, p-38 phosphorylation was decreased after LPS stimulation in the presence of peptide 17 in normoxic conditions, while it was increased in the hypoxic counterpart. This might suggest that YAP1-dependent regulation of p38 in an inflammatory context is restricted only to the hypoxic microenvironment. Of interest, YAP1 inhibition increased the expression of IL-1β in both normoxic and hypoxic conditions after LPS stimulation (Fig. 2B). To further confirm our data, we performed a transient knockdown of YAP1 and analyzed the expression of MAPK p38 and IL-1β. To this end a control nontargeting shRNA and 1 shRNA against YAP1 were used to test the efficacy in depleting YAP1. Figure 2C showed a significant reduction of ~50% in YAP1 protein expression level compared with the nontargeting shRNA and nontransfected cells (Med). No difference in YAP1 protein expression was observed between nontransfected cells (Med) and nontargeting shRNA (Scr). In line with the previous result obtained with peptide 17, Fig. 2D shows that p38 phosphorylation was increased in YAP1-knockdown cells only in LPS-stimulated cells under hypoxia, suggesting, once again, that YAP1-dependent regulation of p38 in an inflammatory context is primarily restricted to the hypoxic microenvironment. However, IL-1β gene expression and production were increased in YAP1-knockdown cells stimulated with LPS, in both normoxic and hypoxic conditions.

It has been reported that ROS plays a critical role in cytokine-induced p38 production.[32,33] More recently, it has been shown that YAP1 is a key regulator of oxidative stress response.[34] Figure 2F showed that ROS production was increased in shYAP1 DCs compared with Scr cells after LPS stimulation in both normoxic and hypoxic conditions. In addition, YAP1 knockdown was associated with a significant decrease in p44/42 MAPK and ph-NF-kB p65 phosphorylation in DCs stimulated with LPS in both normoxic and hypoxic conditions (Fig. 2E).

3.3 YAP1 is expressed in mouse DC (DC2.4)

To confirm our hypothesis on the involvement of YAP1 in the regulation of p38 phosphorylation and IL-1β production, we took advantage of an immortalized murine DC line (DC2.4)[35] to generate a stable cell line overexpressing YAP1 by mutating the serine at residue 366 into an alanine to prevent YAP1 phosphorylation (S366A) and therefore to increase its stabilization.[36] We first tested the expression of some parameters analyzed in human monocyte–derived DC, as shown in Figs. 1 and 2, using the DC2.4 cell line. This was done to assess the similarity between the 2 cell lines and to utilize the DC2.4 cell line for further experiments that are not feasible with primary cells due to the low transfection efficiency and the limited ex vivo viability of primary human DCs, together with the unfeasibility of establishing stable cell lines. DC2.4 cells show that hypoxia significantly enhanced the expression of HIF-α and YAP1 (Fig. 3A and B), especially in the presence of LPS, together with the significant increase in IL-1β mRNA expression after LPS challenge (Fig. 3C). Similar results were observed for p38 and p44-42 MAPK phosphorylation under normoxic and hypoxic conditions (Fig. 3D).

3.4 YAP1 stabilization increases the expression of ph-p38 and IL-1β production

We first tested the efficacy of YAP1 stabilization after S366A mutation, and as reported in Fig. 4A, western blot analysis of YAP1 showed that YAP1^{S366A} significantly increased in YAP1 protein level when compared with the Medium and the Crt cell lines. Of interest, YAP1^{S366A} significantly increased the expression of p38 phosphorylation after LPS stimulation in both normoxic and hypoxic microenvironments (Fig. 4B). These data were supported and confirmed by confocal microscopy analysis, which clearly showed a significant increase in p38 phosphorylation in the YAP1^{S366A} cell line compared with the Ctr, after LPS stimulation, both under normoxia and hypoxia (Fig. 4C). In addition, IL-1β gene expression and production were increased in YAP1^{S366A} compared with Ctr cells challenged with LPS, in both normoxic and hypoxic conditions (Fig. 4D). However, no significant changes were observed in terms of ROS production between YAP1^{S366A} and the Ctr after LPS stimulation, nor under normoxia, or hypoxia (Fig. 4E).

3.5 YAP1 correlates with p38 phosphorylation and IL-6 and tumor necrosis factor alpha expression

Since we did not observe any difference in ROS production in YAP1^{S366A} cells compared with the Ctr cells, we investigated other possible pathways and mechanisms that could lead to an increase in p38 phosphorylation and IL-1β. Of note, several studies have demonstrated that p38 phosphorylation can be induced by an increase in IL-6 and tumor necrosis factor (TNF) production in LPS-stimulated cells in both normoxia and hypoxia. In our model, YAP1^{S366A} increased the expression of IL-6 and TNF mRNA levels (Fig. 5A). Mechanistically, p38 integrates these cues to potentiate inflammation by activating transcription factors (eg ATF2/AP-1, NF-κB crosstalk) that drive IL-6 and other target genes, creating an autocrine feed-forward loop that sustains p38 signaling.[37] Indeed, NF-κB activation was found to be dependent on p38 activity in different cell models, as NF-κB is an effector of p38. Figure 5B showed that NF-kB phosphorylation significantly increased in YAP1^{S366A} cells compared with the Ctr in LPS-stimulated cells in both normoxia and hypoxia. In line with previous studies,[38] YAP1 stabilization significantly increased p-44/42 phosphorylation compared with the Ctr in LPS-stimulated cells in both normoxia and hypoxia to maintain an inflammatory signal (Fig. 5C).

3.6 YAP1 functions as a fine-tuned regulator of DC inflammatory responses

Here, we report a schematic representation of the proposed mechanism of action of YAP1 in regulating p38 phosphorylation and IL-1β production in DC. Under physiological conditions, LPS increases and stabilizes YAP1, which activates YAP1 target genes in the nuclear fraction. YAP1 inhibition (left panel; eg Peptide 17 or shRNA) increases ROS accumulation, which drives p38

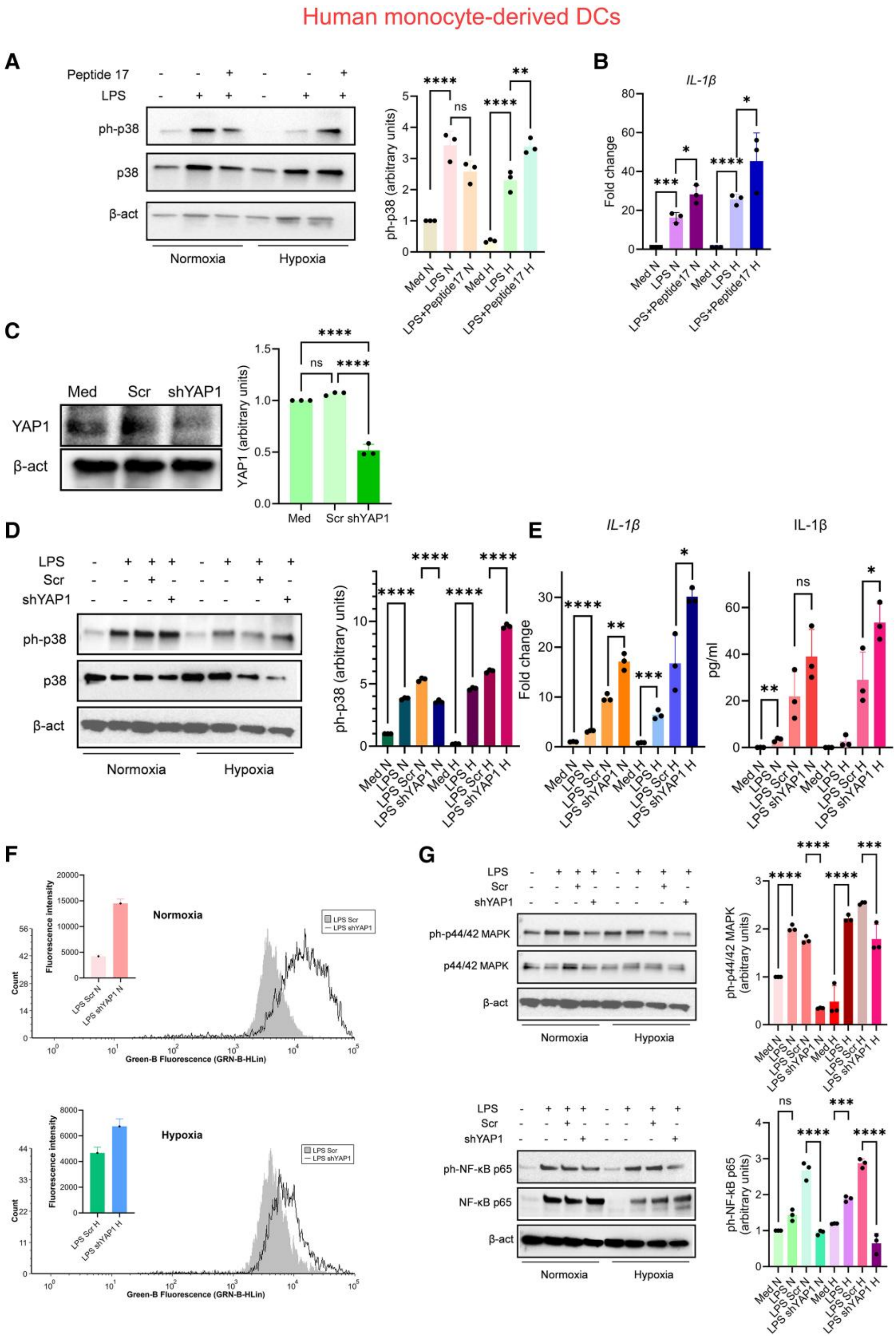

Fig. 2. YAP1 regulates p38 phosphorylation and IL-1β production in human monocyte–derived DC through ROS activation. (A) Protein expression levels of ph-p38 and (B) RT-qPCR analysis of IL-1β after Peptide 17 treatment and 24 h exposure of DCs to normoxia or hypoxia. Protein expression levels of YAP1 in DCs transfected with Ctr Silencer Select Negative Control shRNA, here referred as Scr. (Scramble), or shRNA YAP1 (C). (D) Protein expression levels of ph-p38, (E) RT-qPCR analysis of IL-1β, and IL-1β secretion levels as determined by ELISA test in DCs transfected with Scr or shRNA YAP1 and exposed to normoxia or hypoxia for 24 h. (F) ROS production by ROS-ID Hypoxia/Oxidative stress detection kit in DCs transfected with Scr or shRNA YAP1 and exposed to normoxia or hypoxia for 24 h. (G) Protein expression levels of ph-p44/42 and ph-NF-kB p65 in DCs transfected with Scr or shRNA YAP1 and exposed to normoxia or hypoxia for 24 h. LPS was added where indicated for 24 h. β-Actin was used as a loading control and as a housekeeping gene for RT-qPCR analysis. Data represent SEM ($n = 3$). *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.0001$ using unpaired 2-tailed Student's *t* test or 1-way ANOVA followed by Tukey's test.

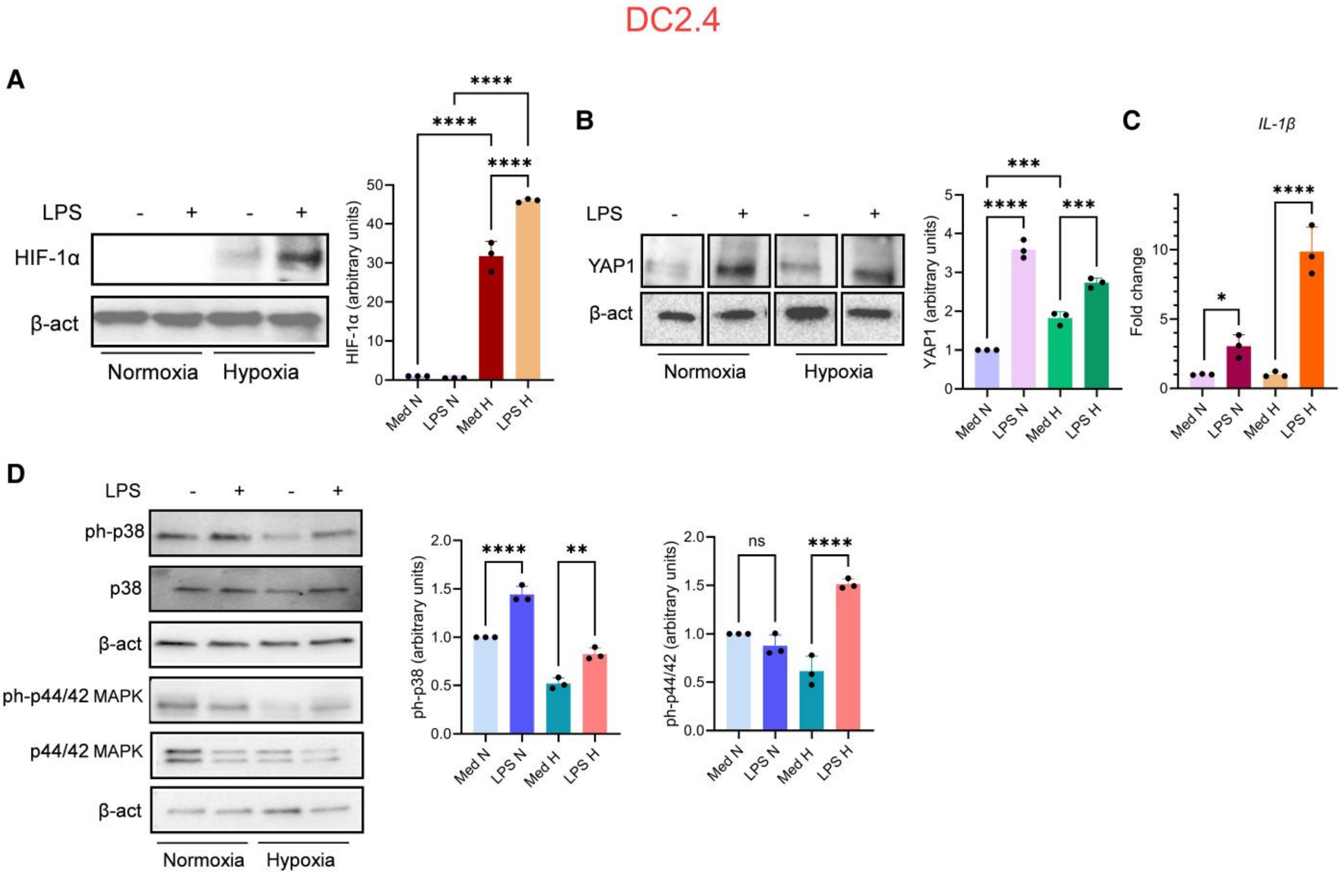

Fig. 3. YAP1 is expressed in the immortalized murine DC line (DC2.4). Protein expression levels of HIF-1α (A), YAP1, and RT-qPCR analysis of IL-1β (B), ph-p38, and ph-p44/42 (C) in DC2.4 after 24 to normoxia or hypoxia. LPS was added where indicated for 24 h. β-Actin was used as a loading control and as a housekeeping gene for RT-qPCR analysis. Data represent SEM ($n = 3$). $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$, $^{****}P < 0.0001$ using unpaired 2-tailed Student's *t* test or 1-way ANOVA followed by Tukey's test.

phosphorylation, particularly under hypoxia, resulting in elevated IL-1β expression ("ROS–p38 axis"). YAP1 stabilization (right panel; eg S366A mutation) enhances IL-6 and TNF production, which sustain p38 activation via autocrine/paracrine signaling and further amplifies NF-κB and ERK1/2 activity, leading to increased IL-1β release ("Cytokine feedback loop"). Both extremes of YAP1 activity converge on the same pro-inflammatory outcome, highlighting the necessity for finely tuned YAP1 regulation to maintain DC homeostasis (Fig. 6).

4. Discussion

This study provides novel insights into the role of YAP1 in DCs under varying oxygen tensions and its regulation by LPS-induced TLR-4 activation. Our findings highlight YAP1 as a key player in DC functionality, particularly in the context of hypoxia and inflammation, which are characteristic of lymphoid tissues and inflamed microenvironments. Hypoxia is a hallmark of lymphoid and inflamed tissues and has been reported to influence DC metabolism, maturation, and function.[39] As expected, hypoxic culture conditions induced HIF-1α stabilization and synergized with LPS to activate MAPK, consistent with previous studies linking hypoxia to heightened inflammatory responses.[40,41]

Within this context, we identified YAP1 expression in DCs and found that its levels were increased upon LPS stimulation in the whole cell lysate and both in the cytoplasm and nuclear fraction, suggesting its transcriptional activation. Importantly, YAP1 upregulation was associated with the induction of some of the canonical YAP1 target genes (eg *Ctgf* and *Cyr61*)[30] and the inflammasome component *Nlrp3*, indicating that Hippo pathway activation is integrated within the DC inflammatory program.[16] The observation that TLR-4 inhibition abrogated YAP1 induction under hypoxia supports the view that YAP1 stabilization is tightly coupled to inflammatory receptor signaling.

Functionally, our results uncover a dual role of YAP1 in regulating MAPK signaling and IL-1β production. Pharmacological inhibition and genetic silencing of YAP1 both resulted in enhanced p38 phosphorylation selectively under hypoxic conditions, while increasing IL-1β expression in both normoxic and hypoxic environments. These findings suggest that YAP1 normally restrains p38 activity in DCs exposed to hypoxia, thereby acting as a negative regulator of stress kinase signaling. At the same time, however, YAP1 deficiency consistently increased IL-1β production, implicating YAP1 as a fine-tuner of pro-inflammatory cytokine output. Given that ROS are known to activate p38,[42,43] our finding that YAP1 knockdown increased ROS production supports a model where YAP1 contributes to redox homeostasis in DCs. The increase in ROS is consistent with the observed decrease in p44/42 MAPK and phospho-NF-κB p65 in YAP1-knockdown cells (Fig. 2E), and supports a model in which YAP1 contributes to redox homeostasis, indirectly regulating MAPK signaling. However, ROS levels were unchanged in YAP1S366A cells (Fig. 4E), indicating that enhanced MAPK and cytokine responses in these cells occur via ROS-independent mechanisms. Specifically, YAP1

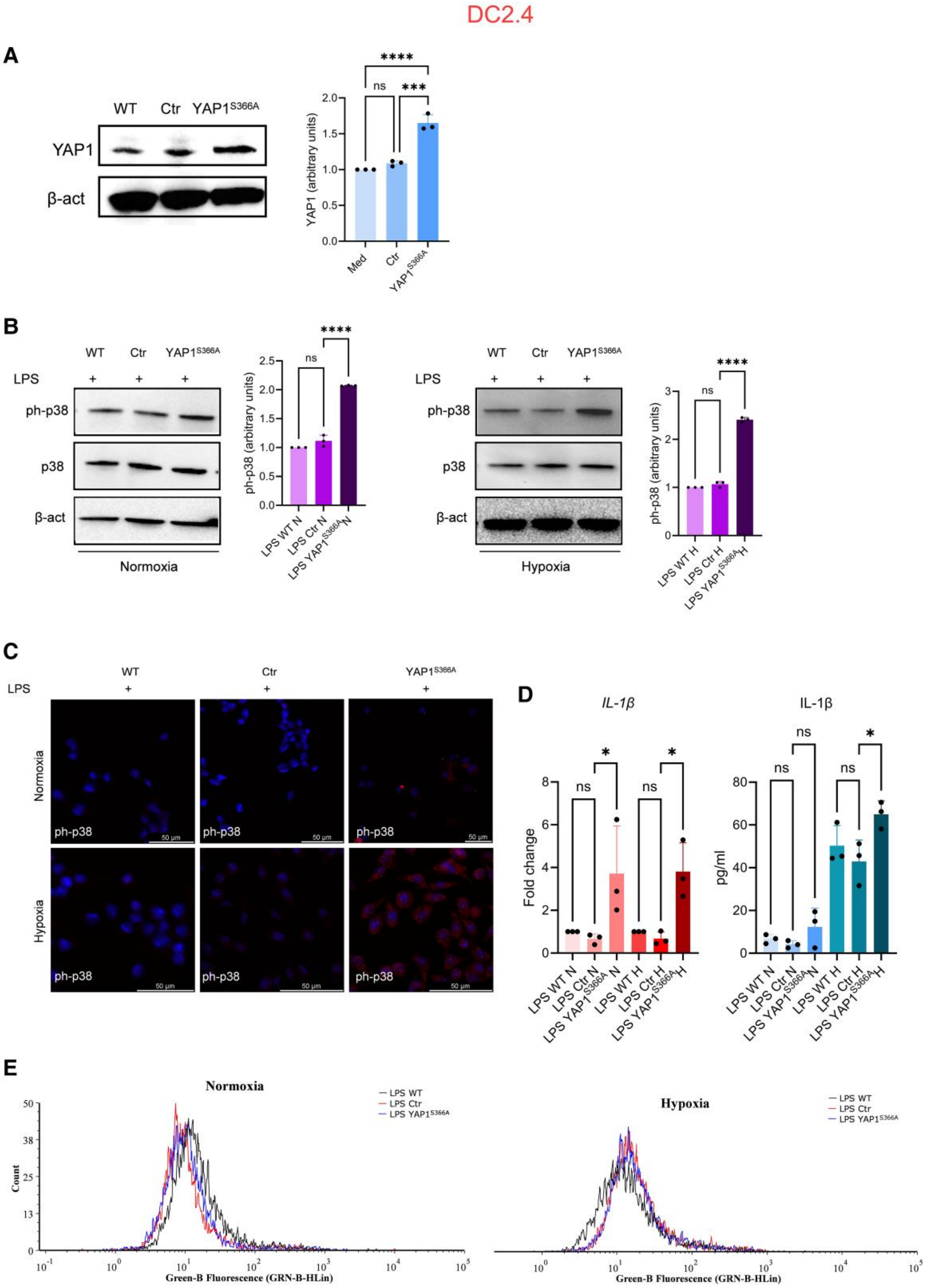

Fig. 4. YAP1 stabilization increases the expression of ph-p38 and IL-1β production (A) Protein expression levels of YAP1 in WT, Ctr, and YAP1^{S366A} DC2.4 cells. (B) Protein expression levels of ph-p38 in WT, Ctr and YAP1^{S366A} DC2.4 cells exposed for 24 h to normoxia or hypoxia. (C) Protein expression levels of ph-p38 in WT, Ctr, and YAP1^{S366A} DC2.4 cells exposed for 24 h to normoxia or hypoxia, as determined by confocal microscopy analysis (Scale bar: 50 μm). (D) RT-qPCR analysis of IL-1β and IL-1β secretion levels as determined by ELISA test in WT, Ctr, and YAP1^{S366A} DC2.4 cells exposed for 24 h to normoxia or hypoxia. (E) ROS production by ROS-ID Hypoxia/Oxidative stress detection kit in WT, Ctr, and YAP1^{S366A} DC2.4 cells exposed for 24 h to normoxia or hypoxia. LPS was added where indicated for 24 h. β-Actin was used as a loading control and as a housekeeping gene for RT-qPCR analysis. Data represents SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$, $^{****}P < 0.0001$ using unpaired 2-tailed Student's *t* test or 1-way ANOVA followed by Tukey's test.

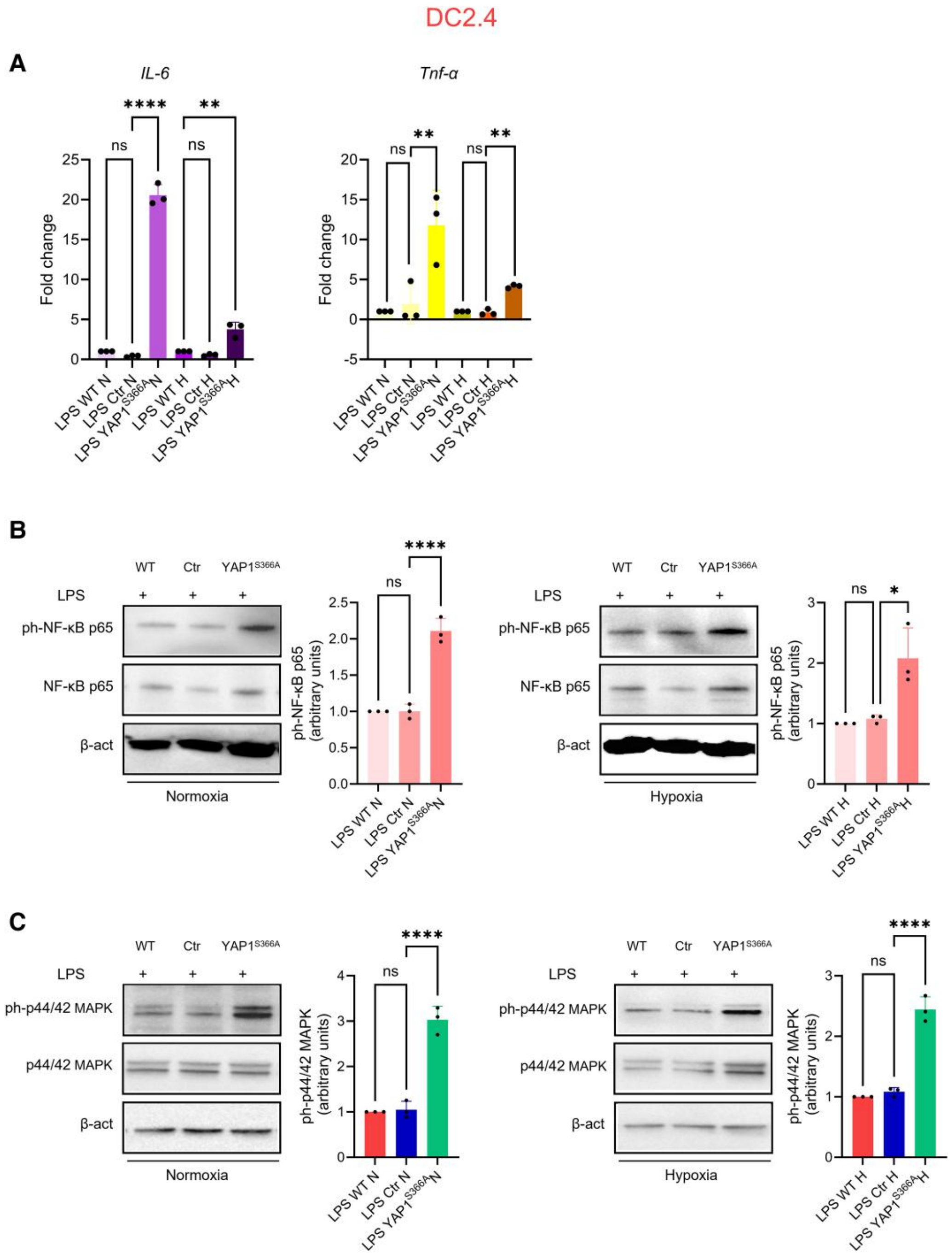

Fig. 5. YAP1 regulates p38 phosphorylation through IL-6 and TNF-α. (A) RT-qPCR analysis of IL-6 and TNF-α in WT, Ctr, and YAP1^{S366A} DC2.4 cells exposed for 24 h to normoxia or hypoxia. (B) Protein expression levels of ph-NF-κB in WT, Ctr and YAP1^{S366A} DC2.4 cells exposed for 24 h to normoxia or hypoxia. (C) Protein expression levels of ph-p44/42 in WT, Ctr, and YAP1^{S366A} DC2.4 cells exposed for 24 h to normoxia or hypoxia. LPS was added where indicated for 24 h. β-Actin was used as a loading control and as a housekeeping gene for RT-qPCR analysis. Data represent SEM ($n = 3$). *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.0001$ using unpaired 2-tailed Student's t test or 1-way ANOVA followed by Tukey's test.

stabilization increases IL-6 and TNF-α expression, cytokines known to sustain p38 activation through autocrine/paracrine signaling, suggesting a feed-forward loop that amplifies MAPK and NF-κB activity. Functionally, our results uncover a dual role of YAP1 in regulating DC responses: YAP1 deficiency promotes p38 activation via oxidative stress, whereas YAP1 stabilization enhances pro-inflammatory outputs through cytokine-driven feedback. Thus, YAP1 acts as a homeostatic regulator of MAPK signaling and IL-1β production, where both reduced and excessive YAP1 activity converge on enhanced p38 phosphorylation and cytokine production, although through mechanistically distinct pathways. Importantly, similar findings were obtained in THP-1 cells, a widely used model of human myeloid cells able to differentiate into DCs,[44,45] reinforcing the robustness and reproducibility of our results across experimental systems (Fig. S1).

This dual, context-dependent role of YAP1 suggests that its activity levels, rather than its presence, are a critical determinant of DC functions in diseases characterized by chronic inflammation (eg rheumatoid arthritis and inflammatory bowel disease). Consequently, therapeutic strategies aimed at regulating YAP1

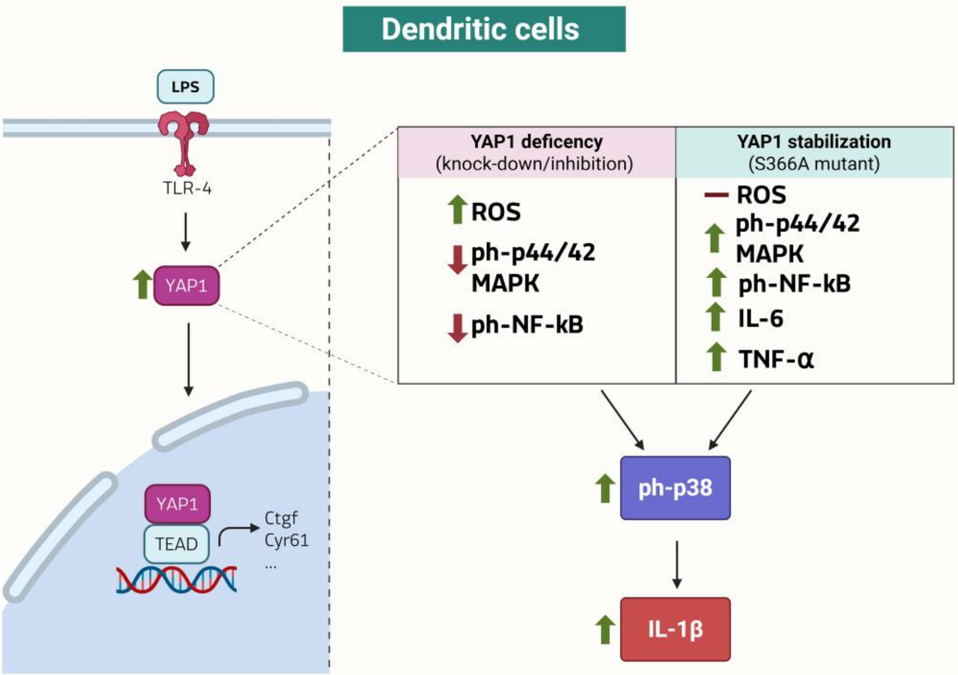

Fig. 6. Schematic representation of the proposed mechanism of action of YAP1 in regulating p38 phosphorylation and IL-1β production in DC. YAP1 modulates DC inflammation by controlling p38 phosphorylation and IL-1β production. LPS stabilizes YAP1 and activates target genes. YAP1 inhibition (eg Peptide 17 or shRNA) increases. ROS, driving p38 phosphorylation and IL-1β expression (ROS–p38 axis). YAP1 stabilization (eg S366A mutation) enhances IL-6 and TNF, sustaining p38 activation via autocrine/paracrine signaling and amplifying NF-κB/ERK1/2 activity, which further increases IL-1β (cytokine feedback loop).

activity in DC could represent a novel approach to treat complex inflammatory conditions. Indeed, quantitative differences in YAP1 expression or activity have been linked to distinct phenotypic outcomes in a range of biological contexts. For example, meta-analyses across multiple cancer types demonstrate that higher levels of YAP1 expression are associated with significantly worse overall and disease-free survival compared with lower expression groups, indicating that stratification by expression level can define “higher-risk” vs relatively benign states in human disease cohorts.[46]

We acknowledge some limitations in this study. While the use of human-derived DCs provides a powerful ex vivo model, it restricts the range of laboratory manipulations and techniques that can be applied. Therefore, we had to use an immortalized mouse cell model to perform additional experiments, which might introduce variability in the system. Nevertheless, we demonstrated that the 2 models are comparable, supporting the robustness and reliability of our conclusions.

Collectively, these data establish YAP1 as a key modulator of DC responses to hypoxic and inflammatory signals. Beyond its well-characterized roles in tissue homeostasis and tumorigenesis, YAP1 emerges here as a critical node in innate immune regulation. Dysregulated YAP1 activity—whether suppressed or hyperactivated—may contribute to aberrant DC activation and chronic inflammation. Our study extends the understanding of YAP1 beyond its well-established roles in organ size control and tumorigenesis, highlighting its importance in innate immunity. The interplay between YAP1, hypoxia, and TLR-4 signaling may be particularly relevant in inflamed tissues, where DCs encounter simultaneous hypoxic stress and microbial products.[47] Dysregulation of this axis could contribute to chronic inflammation or impaired immune responses, suggesting that YAP1 may represent a novel therapeutic target to modulate DC-driven immunity.

Author contributions

All authors contributed to the study conceptualization and design. Formal analysis was performed by S.M. Data collection and analysis were performed by S.M., F.Co., I.F., and J.B. under the project administration of S.M. and A.N. C.A., G.Fr., and G.Fa. generated the CRISPR-Cas9 cell line. S.M., F.Ca., and A.N. wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

Supplementary material

The original uncropped blot images and unedited confocal images have been submitted together with the paper to the editorial board.

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Conflicts of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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