Cutaneous neurofibroma cells with active YAP promotes proliferation of macrophages resulting in increased accumulation of macrophages by modulating CCL5 and TGF-β1

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Abstract. Cutaneous neurofibromas (cNFs) are present in the majority of patients with neurofibromatosis type 1 (NF1), and results in disfigurements of the body, which is associated with psychological distress. A hallmark feature of cNF is the infiltration of inflammatory cells, among which macrophages are an important component of the microenvironment. Loss of neurofibromin (Nf1) expression results in activation of the PI3K and MAPK signaling pathways; however, the therapeutic effects of specific inhibitors targeting these pathways are not satisfactory. The present study showed increased macrophage infiltration accompanied by activation of effectors of the Hippo signaling pathway. Additionally, it was shown that XMU-MP-1 enhanced macrophage accumulation, *in vivo* and *in vitro*, by elevating the levels of C-C motif chemokine ligand 5 (CCL5) and transforming growth factor (TGF)-β1 expression. However, neither CCL5 nor TGF-β1 ablation alone were able to effectively reverse the XMU-MP-1-induced upregulation of macrophage accumulation, whereas concurrent ablation of these two genes significantly decreased macrophage accumulation. EdU staining and flow cytometry suggested that activated Yes-associated protein 1 promoted proliferation rather than inhibiting apoptosis in macrophage cells, and this may underlie the increase in the accumulation of macrophages. Both CCL5/C-C motif chemokine receptor 5 and TGF-β1/TGFβ1 receptor served crucial roles in modulating macrophage proliferation, which ultimately contributed to macrophage accumulation. The function of the Hippo pathway in the development of cNF development and its potency as a therapeutic target merit further investigation.

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Introduction

Cutaneous neurofibromas (cNF) are present in a large number of individuals with neurofibromatosis type 1 (NF1), a tumor susceptibility syndrome with autosomal dominant inheritance (1). As a hallmark symptom of patients with NF1, cNFs typically develop at puberty and rarely progress to malignancy. However, these benign tumors are may be a substantial burden to the individual due to the consequent disfigurement of the body, which is associated with significant psychological distress (2). Unfortunately, the available treatment options for cNF are limited to surgical excision, which is not feasible with larger tumors or with the presence of multiple tumors. Therefore, determining novel methods for treating cNF is required; however, current efforts have thus far yielded insufficient success (3).

cNFs commonly consist of Schwann cells, endothelial cells, fibroblasts and inflammatory cells (4). Mast cells are the most abundantly present inflammatory cells (5). Efforts to target mast cells in order to shrink neurofibromas have presented difficulties, as certain patients do not exhibit a response to the inhibition of mast cell activity (6), which suggests that there may be compensatory factors facilitating neurofibroma progression. Furthermore, altered macrophage activity has been detected in cNF tissues (7,8), nerve injury with coincident macrophage invasion promotes neurofibroma formation (9) and macrophage depletion impairs tumor maintenance in mice (10). All these results highlight the possibility that macrophages may infiltrate into the neurofibroma microenvironment and contribute to tumor progression. However, the detailed mechanism underlying macrophage accumulation requires further clarification, and this may result in the identification of potentially viable therapeutic targets for treating neurofibromas.

Nf1 encodes neurofibromin, which serves as the 'off' signal for RAS, a GTPase activating protein which activates various receptor tyrosine kinases, such as cytokine receptors (11). Schwann cells, the primary pathogenic cells, are capable of secreting multiple types of cytokines which mediate macrophage recruitment (12,13). Although active RAS primarily increases PI3K and RAF-MEK-ERK mediated signaling in neurofibroma cells, the Hippo signaling pathway has been validated as an important modifier in cNF tumorigenesis (14). Yes-associated protein 1 (YAP) and tafazzin (TAZ) are key effectors of Hippo signaling (15). MST1 and 2 are kinases involved in the Hippo signaling pathway which modulates the protein expression levels and localization of YAP by upregulating YAP degradation and phosphorylation (16). Activated MST1 and 2 promotes the maintenance of YAP in the cytoplasm, and repressing MST1 and 2 activity enhances the nuclear translocation of YAP, where it interacts with T-domain transcription factor (TEAD) to activate a panel of genes (15-17). The Hippo pathway contributes to a conducive environment for tumor growth (18) and may modulate the tumor microenvironment (19). Regarding the role of macrophages in neurofibromas, the association between the Hippo pathway and macrophage accumulation requires further study.

In the present study, it was hypothesized that Hippo pathway effectors participated in macrophage accumulation induced by cNF cells to contribute to the development of cNF, and that the Hippo pathway may modulate macrophage accumulation through cytokine secretion.

Materials and methods

Macrophage polarization. The macrophage polarization model was generated using the human acute monocytic leukemia cell clone THP-1 (American Type Culture Collection). A total of $3x10^6$ THP-1 cells were seeded in a 10 cm dish containing RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences), and 300 nmol/l phorbol 12-myristate 13-acetate was added to the cells for 48 h, followed by exposure for 24 h to lipopolysaccharide (20 ng/ml) and interferon (IFN)-γ (20 ng/ml) for M1-polarization. Subsequently, the cells were propagated in Human Endothelial Serum-Free Medium (Thermo Fisher Scientific, Inc.) with lipopolysaccharide and IFN-γ at the same concentrations for an additional 24 h.

Cell culture and stable RNA interference. SW10 (murine Schwann cells) and Hs 53.T cells (human skin fibroblasts from neurofibroma patients) were grown in DMEM/F12 (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.). THP-1 cells were grown in RPMI-1640 medium with 10% FBS.

Cells were maintained in a humidified atmosphere with 5% CO_2 at 37˚C. Two reconstructed replication-defective lentiviruses containing specific short hairpin RNA (shRNA) sequences targeting Nf1, CCL5 or TGF-β1, or an shRNA control were used for SW10 and Hs 53.T cell infection; infected cells were identified as shNf, shCCL5, shTGF-β1 and shNC cells, respectively. shRNA sequences targeting CCR5 or TGFβ1R, or the shRNA control were used in THP-1 cells; infected cells were termed shCCR5, shTGFβ1R or shNC cells. The lentivirus was purchased from Shanghai Genechem Co. Ltd. and used to transfect cells with a viral titer $>1x10^8$ particles/ml and 0.8 μl Hitrans G A (Shanghai Genechem Co., Ltd.). The transfected cells with a confluence of 70-80% were considered the first generation. The efficiency of gene knockdown was determined by western blot analysis 3 days after transfection. The transfected cells were used for 10 passages following transfection. To obtain conditioned medium, the cell culture supernatant was collected by centrifugation (167 x g, 5 min at 37˚C) and filtration (0.22 μm), followed by immediate use or storage until use at -80˚C.

Antibodies and reagents. GAPDH (cat. no. 6c5) antibody was purchased from Santa Cruz Biotechnology, Inc. Phospho-(p-)YAP (Ser127; cat. no. 13008S), YAP (cat. no. 14074), PARP/cleaved-PARP (cat. no. 9532), Caspase 3/cleaved-Caspase 3 (cat. no. 9662), proliferating cell nuclear antigen (PCNA; cat. no. 13110), Ki67 (cat. no. 2586), p21 (cat. no. 2947), p27 (cat. no. 3686), CDK4 (cat. no. 12790), CDK6 (cat. no. 13331), cyclin D1 (cat. no. 2978) and TGFβ receptor (TGFβR; cat. no. 5544) antibodies were all purchased from Cell Signaling Technology, Inc. C-C motif chemokine receptor 5 (CCR5; cat. no. ab65850), CCL5 (cat. no. ab10394), TGF-β1 (cat. no. ab64715), HLA-DR (cat. no. ab92511) and TAZ (cat. no. ab84927) antibodies were purchased from Abcam. CD68 (cat. no. M087601-2) antibody was purchased from Dako; Agilent Technologies, Inc. XMU-MP-1 and Verteporfin (VP) were purchased from Selleck Chemicals. The Click-iT™ plus EdU Alexa Fluor™ 488 Imaging kit (cat. no. C10637) was purchased from Thermo Fisher Scientific, Inc. The TGF-β1 ELISA kits (cat. no. ab119557 for SW10 cells; cat. no. ab100647 for Hs 53.T cells) and CCL5 ELISA kits (cat. no. ab215537 for SW10 cells; cat. no. ab174446 for Hs 53.T cells) were purchased from Abcam.

Macrophage recruitment assay. Collected conditioned medium (1 ml) was added to the lower chambers of 24-well Transwell plates with 5-μm pore polycarbonate membrane inserts. A total of $1x10^5$ THP-1 cells in 0.5 ml medium were seeded into the upper chamber. After 20 h of incubation, the cells which had migrated to the lower surface of the membrane were washed with PBS three times, fixed with 4% paraformaldehyde for 20 min at room temperature, and stained with 0.1% crystal violet for 15 min at room temperature. Subsequently the cells were washed with PBS three times at room temperature and counted. The number of cells which had migrated were counted in five randomly chosen fields using light microscopy (magnification, x40; Olympus IX50-S8F2; Olympus Corporation).

RNA extraction and reverse transcription-quantitative PCR (RT-q)PCR). Total RNA from cells was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.) and 1 μg total RNA was reverse transcribed using the PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.) with the following temperature protocol: 37˚C for 15 min followed by 85˚C for 5 sec and held at 4˚C until use. qPCRwas performed using a CFX96 real-time PCR system (Bio-Rad Laboratories, Inc.) with SYBR Green PCR Master mix (Takara Biotechnology Co., Ltd.). The themorcycling conditions were: 95˚C for 3 min; followed by 39 cycles of 95˚C for 10 sec and 55˚C for 30 sec.

The expression levels of the genes of interest were quantified using the $2^{-\Delta\Delta Cq}$ method (20) and normalized to the mRNA levels of GAPDH. The sequences of the primers used are presented in Table SI.

Western blot analysis. The cells were washed three times in cold PBS and then lysed using RIPA buffer containing Tris (50 mM, pH 8.0), NaCl (150 mM), SDS (0.1%), NP40 (1%), sodium deoxycholate (0.5%) and proteinase inhibitors [1% cocktail and 1 mM PMSF (Sigma-Aldrich; Merck KGaA)]. Equivalent quantities of protein for each sample (30-35 μg) were loaded on a 12% SDS-gel and resolved using SDS-PAGE, before transferring to nitrocellulose membranes. Skimmed milk (5%) was used to block the non-specific binding sites on the membrane prior to incubation with the specific primary antibodies (1:1,000 for antibodies purchased from Cell Signaling Technology and 1:2,000 for antibodies purchased from Abcam). The membranes were incubated with the primary antibodies overnight (>10 h) at 4˚C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies; anti-mouse IgG (cat. no. 7076) or anti-rabbit IgG (cat. no. 7074) both used at 1:200 (both from Cell Signaling Technology, Inc.) for 1 h at room temperature. The blots were treated with luminol and hydrogen peroxide (ECL Super Sensitive kit; DiNing), followed by visualization using the Molecular Imagery ChemiDoc XRS System (Bio-Rad Laboratories, Inc.).

Clinical specimens and immunohistochemistry. To investigate macrophage infiltration in neurofibroma tissues, 50 dermal neurofibroma samples and adjacent tissue samples were collected from patients who underwent tumor resection at the First Affiliated Hospital of Xi'an Jiaotong University, between June 2010 and October 2017. Patients diagnosed as NF1, with a cNF who received surgery were included. Patients diagnosed as NF1 without a cNF were excluded. Approval was obtained from the institutional review board of the First Affiliated Hospital of Xi'an Jiaotong University prior to the collection of samples, and consent was also obtained from the patients. The characteristics of the recruited patients are presented in Table SII. The EnVision™ system (Dako; Agilent Technologies, Inc.) was used for immunohistochemistry (IHC) staining, according to the manufacturer's protocol. Tumor sections were deparaffinized (60-65˚C for 4 h), washed with xylene 3 times, rehydrated (100% alcohol 3 times, 5 min each; 95% alcohol 2 times, 5 min each; 80% alcohol 5 min; 70% alcohol 5 min, and 50% alcohol 5 min) and subjected to heat-induced antigen retrieval. Methanol with 3% H_2O_2 was used to block endogenous peroxidase and alkaline phosphatase activity. Subsequently, the slides were incubated with primary antibodies (1:150) at 4˚C overnight, washed three times and sequentially incubated with the EnVision secondary antibody (cat. no. GK600505; Gene Tech Biotechnology Co., Ltd.) for 30 min at room temperature. Proteins of interest were detected using diaminobenzidine buffer and subsequently counterstained with hematoxylin for 3 min at room temperature. Slides were imaged and analyzed using light microscopy (magnification, x40; Olympus BX51; Olympus Corporation). IHC staining was analyzed using a scoring system, in which the staining score was calculated by multiplying the intensity score by the percentage score. The intensity score was defined as follows: 0, 0%; 1, <25%; 2, ≥25% and <50%; 3, ≥50% and <75%; and 4, ≥75%. The percentage score was graded as follows: 0, no staining; 1, slight staining; 2, moderate staining; and 3, strong staining.

Cell Counting Kit-8 (CCK-8) assay. The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. Cells ($5x10^3$ for THP-1 cells, and $1x10^3$ for SW10 and Hs 53.T cells) were seeded in 96-well culture plates. After different treatments for the indicated time, the cells were washed, followed by incubation with serum-free medium (150 μl) containing 10% CCK-8 at 37˚C for 3 h. The absorbance was measured at a wavelength of 450 nm using a Microplate Autoreader (Bio-Tek Instruments Inc.). Independent experiments were repeated in triplicate.

Colony formation assay. A total of $1x10^3$ cells with the indicated gene knocked down were seeded in 6-well plates. After ~2 weeks of treatment with XMU-MP-1 (5 μM) or VP (5 μM), cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min, at room temperature and stained with 0.1% crystal violet for 15 min at room temperature. The number of colonies (≥50 cells) in each well were counted. The colony number for each sample was defined as the average colony number of four wells and the experiments were performed four times.

Cell cycle analysis. Cells with the indicated treatments were washed with cold PBS, followed by resuspension in cold 70% ethanol and stored at -20˚C for >24 h. The ethanol was removed, and the cell pellets were washed twice with cold PBS. Prior to flow cytometry, the cells were resuspended in PBS containing RNAse A (0.5 μg/ml) and propidium iodide (50 μg/ml) and incubated in the dark for 30 min at room temperature. Flow cytometry was performed using a BD FACSCalibur Flow Cytometer (BD Biosciences) and flow data was analyzed using ModFit LT™ version 3.3 (BD Biosciences).

In vivo macrophage recruitment assay and EdU staining. A total of $3x10^6$ shNf1-SW10 cells were mixed with Matrigel 1 (1:8; BD Biosciences) and subcutaneously injected into the right thigh region of 4-week-old BALB/c nude mice (18 mice in total, 9 male and 9 female; purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.). The mice were raised in the animal laboratory center of Xi'an Jiaotong University in specific-pathogen free conditions. After 1 week, the xenografts formed a tumor with a volume of ~0.5 cm^3, and the mice were randomly divided into three groups (6 mice/group). The mice were injected intraperitoneally with XMU-MP-1 (1 mg/kg), VP (10 mg/kg) or 10% DMSO as a control. After 2 weeks treatment, $5x10^6$ red fluorescent protein (RFP)-labeled THP-1 cells were injected via the tail vein and the treatments were continued. The RFP-labeled THP-1 cells were generated by transfecting the cells with an RFP plasmid (Shanghai Genechem, Co. Ltd.; 2.5 μg/$5x10^5$ cells) using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 1 week, the mice were sacrificed, and the tumors were obtained. For SW10 cells with the indicated gene knocked down, $3x10^6$ shNf1-SW10 cells were injected subcutaneously

to form the tumors. The RFP-labeled THP-1 cells ($5x10^6$) were injected 3 weeks after the subcutaneous injection of SW10 cells, and the tumors were obtained after 1 week. Frozen sections of tumor tissues were prepared to analyze the THP-1 cells using fluorescence microscopy (Olympus, IX50-S8F2; magnification, x40). For EdU staining, after deparaffinization, Alexa-488-azide (10 μM; cat. no. A10266; Thermo Fisher Scientific, Inc.) was used to stain the sections on glass slides for 10-30 min, followed by washing with PBS in Coplin jars. Subsequently, the EdU^+ cells were analyzed by fluorescence microscopy (magnification, x40). The animal experiments were approved by the institutional review board of the First Affiliated Hospital of Xi'an Jiaotong University.

Statistical analysis. A Student's t-test was used for comparisons between two groups, using GraphPad Prism version 5.0 (GraphPad Software, Inc.). A one-way ANOVA with a Fisher's Least Significant Difference t-test was used to compare ≥3 groups, using SPSS Version 10 (SPSS, Inc.). For correlation analysis, a Spearman's correlation test was performed using SPSS. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Pharmacologically-activated Hippo pathway effectors upregulate cell proliferation and macrophage accumulation in cNF. IHC analyses was performed using anti-CD68 (specific marker of macrophages) (21), and anti-YAP and anti-TAZ (core effectors of the Hippo pathway) in 50 neurofibroma tissues and adjacent normal tissues. YAP and TAZ expression was notably increased, concurrent with increased numbers of infiltrating macrophages in cNF tissues (Fig. 1A). Further analysis of the IHC staining showed a positive association between macrophage density and YAP or TAZ expression in cNF tissues (Fig. S1A). Schwann cells are generally considered to be the precursor cells of neurofibromas, although there is some controversy regarding this (22). In addition, fibroblasts are an important component of cNF, and ~50% of the dry weight of human cNF is collagen (22). Thus, the present study established a cell model of cNF by knocking down Nf1 in murine SW10 cells and Hs 53.T cells, termed shNf1-SW10 and shNf1-Hs 53.T (Fig. S1B). Expression of YAP and TAZ was increased and phosphorylation of YAP was decreased in the Nf1-ablated cells, suggesting that Nf1 knockdown overactivated the core effectors of the Hippo pathway in shNf1-SW10 and shNf1-Hs 53.T cells (Fig. S1C). Furthermore, treatment with XMU-MP-1 enhanced the activity of YAP and TAZ in SW10 and Hs 53.T cells, irrespective of Nf1 ablation (Fig. S1C). Preliminary studies discovered both M1-and M2-macrophages in neurofibroma tissue, but very few CD163+ macrophages were identified (23). Thus, M1-polarized macrophages were induced in the present study (Fig. S2). To investigate the potential association between macrophage infiltration and Hippo pathway activity, an *in vitro* macrophage recruitment assay was used, with conditioned medium added to the lower chamber. Nf1-ablation in the SW10 and Hs 53.T cells resulted in improved macrophage penetration. Furthermore, the conditioned medium collected from XMU-MP-1-treated shNC-SW10 and shNC-Hs 53.T cells facilitated THP-1 infiltration, whereas conditioned medium from VP (a YAP inhibitor that acts by disturbing the YAP-TEAD interaction)-treated shNC-SW10 and shNC-Hs 53.T cells impaired THP-1 infiltration (Fig. 1B). This suggests that activated YAP induced by XMU-MP-1 treatment or Nf1 ablation may promote THP-1 infiltration, and attenuation of YAP activity reduced THP-1 infiltration. For further confirmation, a subcutaneous tumor xenograft model was generated in mice to examine the *in vivo* effects. When the volume of tumors reached 0.5 cm^3, the mice were treated with intraperitoneal injections of XMU-MP-1 (1 mg/kg) or VP (10 mg/kg) or 10% DMSO (as a control) daily. At 1 week prior to sacrifice, $5x10^6$ RFP-labeled THP-1 cells were injected via the tail vein. The results showed that XMU-MP-1 treatment significantly increased tumor weight, THP-1 infiltration and YAP levels, which were decreased in the VP-treated mouse xenograft tumors (Fig. 1C and D). No difference in the body weight of mice was observed among the three groups (Fig. S3). Furthermore, both the colony formation assay and CCK-8 assay confirmed the positive association between cell viability and YAP activity in SW10 and Hs 53.T cells, as VP impaired the increase in cell viability induced by Nf1 knockdown and XMU-MP-1 accelerated the cell viability of Nf1-expressing cells (Fig. 1E and S1D).

Taken together, the results from Fig. 1 suggest that activation of Hippo pathway effectors facilitates SW10 and Hs 53.T cell proliferation, and their ability to recruit macrophages *in vivo* and *in vitro*.

The Hippo pathway modulates CCL5 and TGF-β1 expression in cNF cells. As cell increasing viability of the YAP-activated cNF cells, the involvement of chemokines in affecting the Hippo pathway to mediate macrophage infiltration was investigated. Thus, the expression of 11 chemokines were detected to investigate altered macrophage accumulation. The results showed that CCL5 and TGF-β1 may be involved in Hippo pathway-regulated macrophage recruitment in Nf1-ablated SW10 and Hs 53.T cells (Fig. 2A and B). Western blotting, RT-qPCR and ELISA confirmed that the expression and secretion of CCL5 and TGF-β1 were upregulated when treated with XMU-MP-1 and downregulated when treated with VP. The protein expression levels of YAP and p-YAP, and the mRNA expression levels of YAP were not altered by VP, as VP serves its inhibitory role by disturbing the interaction between YAP and TEAD (Fig. 2C-E). IHC with anti-CCL5 and anti-TGF-β1 validated the robust levels of CCL5 and TGF-β1 expression in the cNF tissues (Fig. 2F). Furthermore, the staining scores in cNF tissues showed that both CD68 and YAP were positively associated with CCL5 and TGF-β1 (Fig. S4).

Taken together, Fig. 2 suggests that CCL5 and TGF-β1 may serve an important role in altered Hippo pathway modulation of macrophage infiltration.

Knockdown of either CCL5 or TGF-β1 alone fails to significantly reduce macrophage accumulation. As CCL5 was involved in recruiting macrophages and the CCL5 expression was altered with altered Hippo pathway activity, it was hypothesized that the production of CCL5 by cNF cells may recruit macrophages into the tumor microenvironment. Therefore, shRNA targeting CCL5 was transfected into shNf1-SW10 and shNf1-Hs 53.T cells (Fig. 3A) prior to collecting the conditioned medium for the

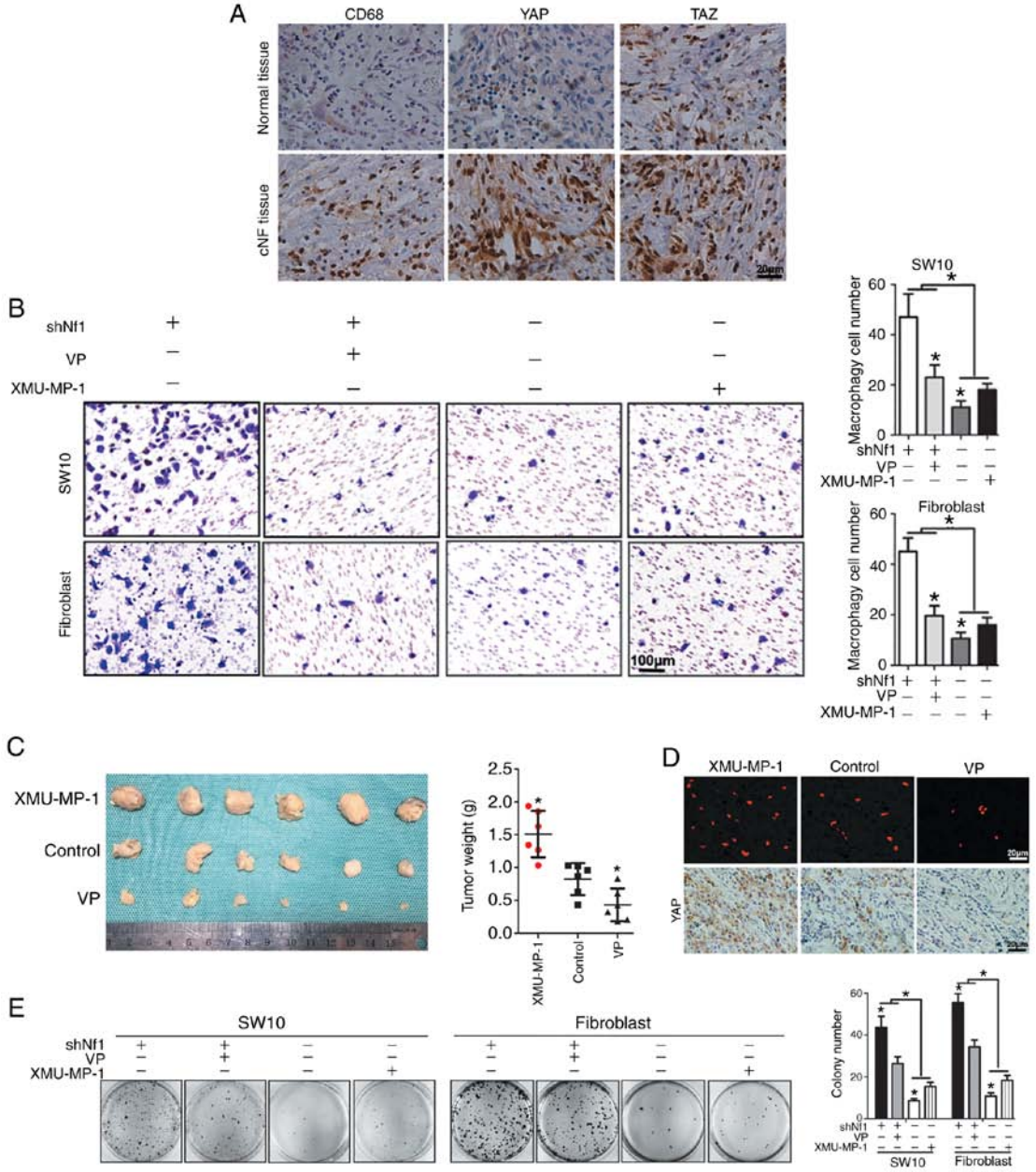

Figure 1. Hippo pathway regulates cNF growth and macrophage infiltration. (A) IHC staining for CD68, a marker of macrophages, and YAP and TAZ, core effectors of the Hippo pathway, in human cNF tissues and adjacent non-tumor tissues. Magnification, x400. (B) VP was used to inhibit YAP activity in Nf1-ablated SW10 and Hs 53.T cells, and XMU-MP-1 was used to enhance YAP activity in the Nf1-NC cells. Subsequently, conditioned media was collected and used for the macrophage recruitment assay. Left, representative images of migrated THP-1 cells. Right, quantitative analysis of the recruited macrophages. Magnification, x200. (C) Nude mice were injected with shNf1-SW10 cells subcutaneously. When the volume of the tumors reached 0.5 cm3, the mice were treated with XMU-MP-1, VP or DMSO intraperitoneally. A total of 1 week prior to harvesting the tumors, RFP-labeled THP-1 cells were injected via the tail vein and tumor weights were analyzed. *P<0.05. (D) THP-1 cells were identified by fluorescence microscopy in sections of SW10 xenograft tumor samples, and IHC staining for YAP expression was performed in paraffin-embedded tumor tissues. Magnification, x400. (E) In the colony formation assay, shNf1-transfected or shNC-transfected SW10 and Hs 53.T cells were treated with VP and XMU-MP-1 for 2 weeks. *P<0.05. IHC, immunohistochemistry; VP, Verteporfin; cNF, cutaneous neurofibroma; sh, short hairpin; RFP, red fluorescent protein; NC, negative control; Nf1, neurofibromin 1; YAP, Yes-associated protein 1; TAZ, tafazzin.

subsequent macrophage recruitment assays. There was no significant difference observed in treatment with conditioned media from the CCL5-ablated cNF cells with or without XMU-MP-1 treatment (Fig. 3B). The failure to abolish macrophage accumulation by CCL5 knockdown may be due to the compensatory efficiency of TGF-β1. Therefore, CCL5-ablated cells were transfected with shTGF-β1 to knock down TGF-β1 (Fig. 3C). Neither conditioned media from CCL5-knockdown cells nor from TGF-β1-knockdown cells could attenuate the macrophage accumulation. However, conditioned media from cells with both CCL5 and TGF-β1 knocked down significantly reduced macrophage accumulation (Fig. 3D). Furthermore, subcutaneous tumor xenografts were generated in mice using shNf1-SW10 cells with ablation of CCL5, TGF-β1 or both. A total of 3 weeks later, $5x10^6$ RFP-labeled THP-1 cells were injected via the tail vein 1 week before the mice were sacrificed. The number of recruited THP-1 cells only decreased in xenograft tumors composed of cells with ablation of both CCL5 and TGF-β1 (Fig. S5).

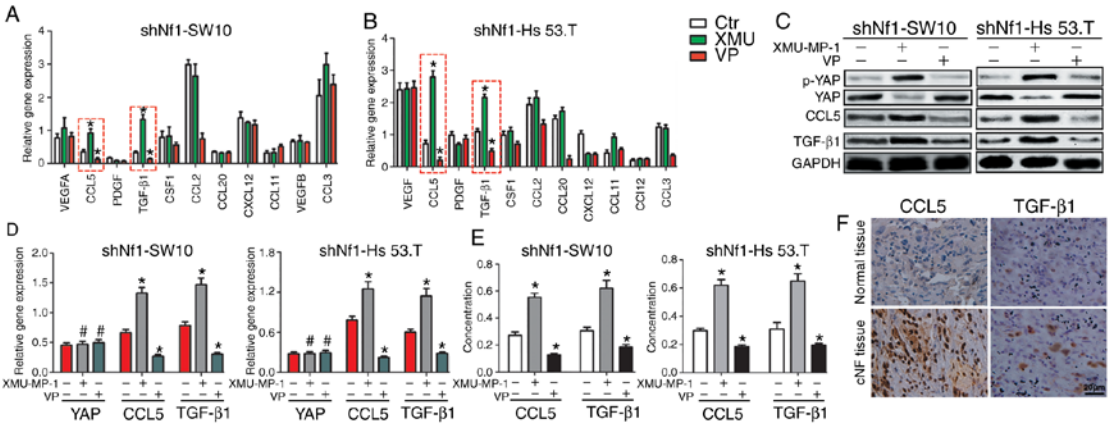

Figure 2. The Hippo pathway regulates the expression of CCL5 and TGF-β. (A and B) Reverse transcription-quantitative PCR was used to profile the mRNA levels of cytokines associated with macrophage recruitment in shNf1-SW10 and shNf1-Hs 53.T cells treated with VP, XMU-MP-1 or DMSO. (C) CCL5 and TGF-β1 were upregulated when YAP was activated and downregulated when YAP activity was decreased at the protein level. (D) Expression of YAP, CCL5 and TGF-β1 in shNf1-SW10 cells and shNf1-Hs 53.T cells treated with XMU-MP-1, VP or DMSO. *P<0.05, XMU-MP-1/VP vs. DMSO. (E) Concentration of CCL5 and TGF-β1 in the medium of shNf1-SW10 and shNf1-Hs 53.T cells with the indicated treatments. *P<0.05, XMU-MP-1/VP vs. DMSO. (F) Expression of CCL5 and TGF-β1 in the patient cNF tissues and adjacent tissues. Magnification, x200). *P<0.05. VP, Verteporfin; sh, short hairpin; CCL5, C-C motif chemokine ligand 5; TGF-β1, transforming growth factor-β1; cNF, cutaneous neurofibroma; Nf1, neurofibromin 1; ctr, control.

Taken together, Fig. 3 suggests that knockdown of CCL5 or TGF-β1 alone failed to significantly attenuate YAP-induced macrophage accumulation. Therefore, it was hypothesized that the upregulated levels of CCL5 and TGF-β1 may exhibit additional effects on macrophages apart from facilitating their infiltration.

Conditioned media from YAP activated cNF cells promotes macrophage proliferation. A previous study revealed that the number of resident macrophages increased in injured peripheral nerves where neurofibromas had formed (24). Therefore, a CCK-8 assay was performed, which showed that viability of THP-1 cells was increased when grown in conditioned media from XMU-MP-1-treated shNf1-SW10 and shNf1-Hs 53.T cells, and viability was decreased when grown in conditioned media from VP-treated cells (Fig. 4A). Additionally, there was a significant increase in the protein expression levels of PCNA induced by conditioned media from XMU-MP-1-treated cNF cells; whereas the levels were decreased when cells were grown in conditioned media from VP-treated cNF cells, further confirming the increase in THP-1 cell proliferation (Fig. 4B). Conditioned media from both VP- and XMU-MP-1-treated cNF cells did not significantly affect the apoptotic rates of THP-1 cells (Fig. 4C). For cell cycle analysis, flow cytometry was performed, and it was determined that the proportion of macrophages which entered S phase and G2/M phase were increased when grown in conditioned medium from XMU-MP-1-treated shNf1-SW10 and shNf1-Hs 53.T cell; whereas conditioned medium from VP-treated shNf1-SW10 and shNf1-Hs 53.T cells resulted in arrest of macrophages in the G1 phase (Figs. 4D and S6). Similarly, EdU detection in subcutaneous tumor xenografts showed that the number of proliferative cells (EdU^+ cells) were increased in XMU-MP-1-treated tumor sections and attenuated in VP-treated tumor sections. Overlaying the images showed co-localized EdU^+ cells and red fluorescent cells, suggesting a positive association between macrophage cell proliferation and YAP activity. The IHC assays for Ki67 further confirmed the increased cell proliferation in XMU-MP-1-treated tumors and impaired cell proliferation in VP-treated tumors (Fig. 4E).

Taken together, Fig. 4 suggests that shNf1-SW10 and shNf1-Hs 53.T cells with activated YAP facilitated THP-1 cell proliferation in an indirect manner, which eventually contributed to macrophage accumulation in cNFs.

Both CCL5/CCR5 and TGF-β1/TGFβ1R suppress p21 and p27 expression. To examine the detailed mechanism involved in THP-1 proliferation with stimulation from cNF cells with altered YAP activity, the protein levels of cell cycle-related molecules were measured. The results showed that altered YAP activity regulated the expression of p21 and p27, but not that of CDK4, CDK6 or cyclin D1, in shNf1-SW10 and shNf1-Hs 53.T cells (Fig. 5A). As p21 and p27 are generally dephosphorylated prior to entering the nucleus, where they inhibit the cell cycle, the cytoplasmic protein and nucleoprotein levels were assessed separately. It was demonstrated that p21 and p27 levels were higher in the cytoplasm when treated with conditioned media from XMU-MP-1-treated shNf1-SW10 and shNf1-Hs 53.T cells, and translocated into the nucleus when treated with conditioned media from VP-treated shNf1-SW10 and shNf1-Hs 53.T cells (Fig. 5B). To investigate whether CCL5 and TGF-β1 were responsible for THP-1 proliferation, THP-1 cells were treated with conditioned media from the treated cNF cells. The results showed that p21 and p27 levels were upregulated and PCNA levels were downregulated following treatment with conditioned media from CCL5- or TGF-β1-ablated shNf1-SW10 and shNf1-Hs 53.T cells. There was a more notable alteration in the levels of p21, p27 and PCNA in THP-1 cells stimulated with conditioned media from both CCL5- and TGF-β1-ablated cNF cells. XMU-MP-1 activated YAP and reduced p-YAP levels, as well as downregulating p21 and p27 levels, and upregulating PCNA levels in the cNF cells (Figs. 5C, and S7A and B). For further confirmation, specific shRNAs were transfected to knock down CCR5 or TGFβ1R in THP-1 cells (Fig. 5D). Accordingly, p21 and p27 levels were increased and PCNA levels were decreased in both

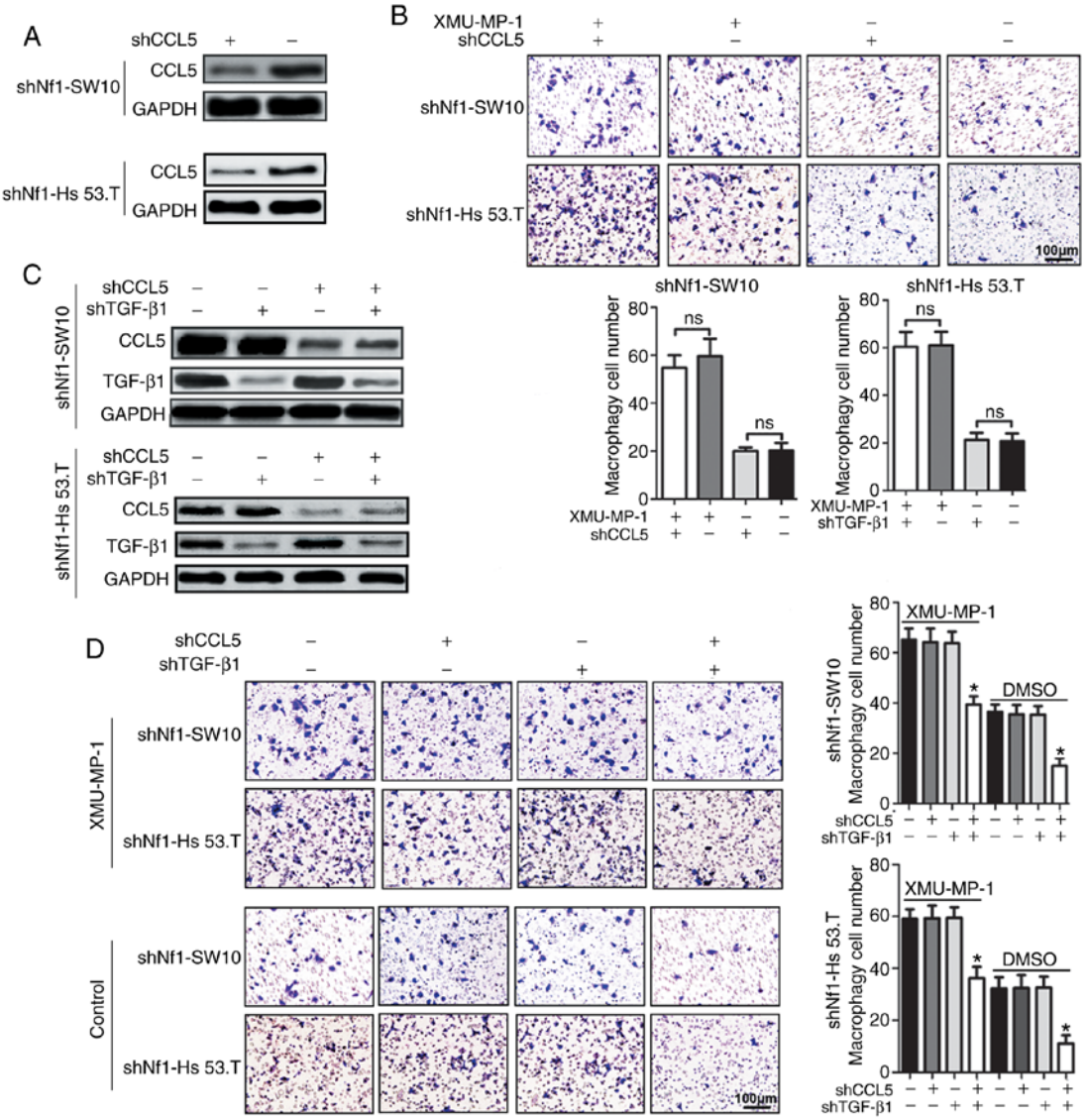

Figure 3. CCL5 and TGF-β1 knockdown does not reduce macrophage infiltration induced by upregulated YAP activity. (A) Efficiency of CCL5 knockdown in shNf1-SW10 cells and shNf1-Hs 53.T cells. (B) CCL5-ablated shNf1-SW10 and shNf1-Hs 53.T cells were treated with XMU-MP-1 or DMSO for 24 h, and the conditioned media was collected. Conditioned media was added to the lower chamber of the Transwell insert, and THP-1 cells passing through the membrane were stained and analyzed. Magnification, x200. (C) Protein expression levels of CCL5 and TGF-β1 in shNf1-SW10 and shNf1-Hs 53.T cells transfected with shCCL5, shTGF-β1, or both combined. (D) Conditioned media was collected from cNF cells with the indicated gene knocked down following treatment with XMU-MP-1 or DMSO for 24 h. Subsequently, conditioned media was added to the lower chamber of the Transwell insert and THP-1 cells passing through the membrane were stained and analyzed. Magnification, x200. *P<0.05, shCCL5 + shTGF-β1 vs. shNC + shNC. shRNA, short hairpin RNA; CCL5, C-C motif chemokine ligand 5; TGF-β1, transforming growth factor-β1; Nf1, neurofibromin 1; ns, not significant.

CCR5-ablated and TGFβ1R-ablated THP-1 cells when treated with conditioned media from shNf1-SW10 and shNf1-Hs 53.T cells with or without XMU-MP-1 treatment (Fig. 5E).

CCL5 and TGF-β1 were overexpressed in shNf1-SW10 and shNf1-Hs 53.T cells, and the conditioned media were collected for subsequent experiments (Fig. 5F). As shown in Fig. 5G, conditioned media from cells overexpressing CCL5 and/or TGF-β1 in the presence or absence of VP treatment reduced p21 and p27 levels, and increased PCNA levels in THP-1 cells. No notable alterations in YAP or p-YAP levels were observed in these cNF cells (Figs. S7C and D), consistent with the results in Fig. 2D. Subsequently, CCR5 and TGFβ1R were overexpressed in THP-1 cells (Fig. 5H). Similarly, the levels of p21 and p27 were increased and the levels of PCNA were decreased in the CCR5- or TGFβ1R-overexpressing THP-1 cells treated with conditioned media from VP-treated or DMSO-treated shNf1-SW10 and shNf1-Hs 53.T cells (Fig. 5I).

Taken together, these results suggest that CCL5/CCR5 and TGF-β1/TGFβ1R may modulate the expression of p21 and p27 individually and synergistically in THP-1 cells.

Both CCL5/CCR5 and TGF-β1/TGFβ1R promote macrophage proliferation. CCL5-ablated, TGF-β1-ablated and CCL5 + TGF-β1-ablated shNf1-SW10 and shNf1-Hs 53.T cells were treated with XMU-MP-1 or DMSO for 24 h, and the conditioned media was subsequently collected. THP-1 cells were treated with the prepared conditioned media, which validated the finding that CCL5 ablation and/or TGF-β1 ablation could reduce the increased cell viability of THP-1 cells induced by shNf1-SW10 and shNf1-Hs 53.T (Fig. 6A). The reduced cell

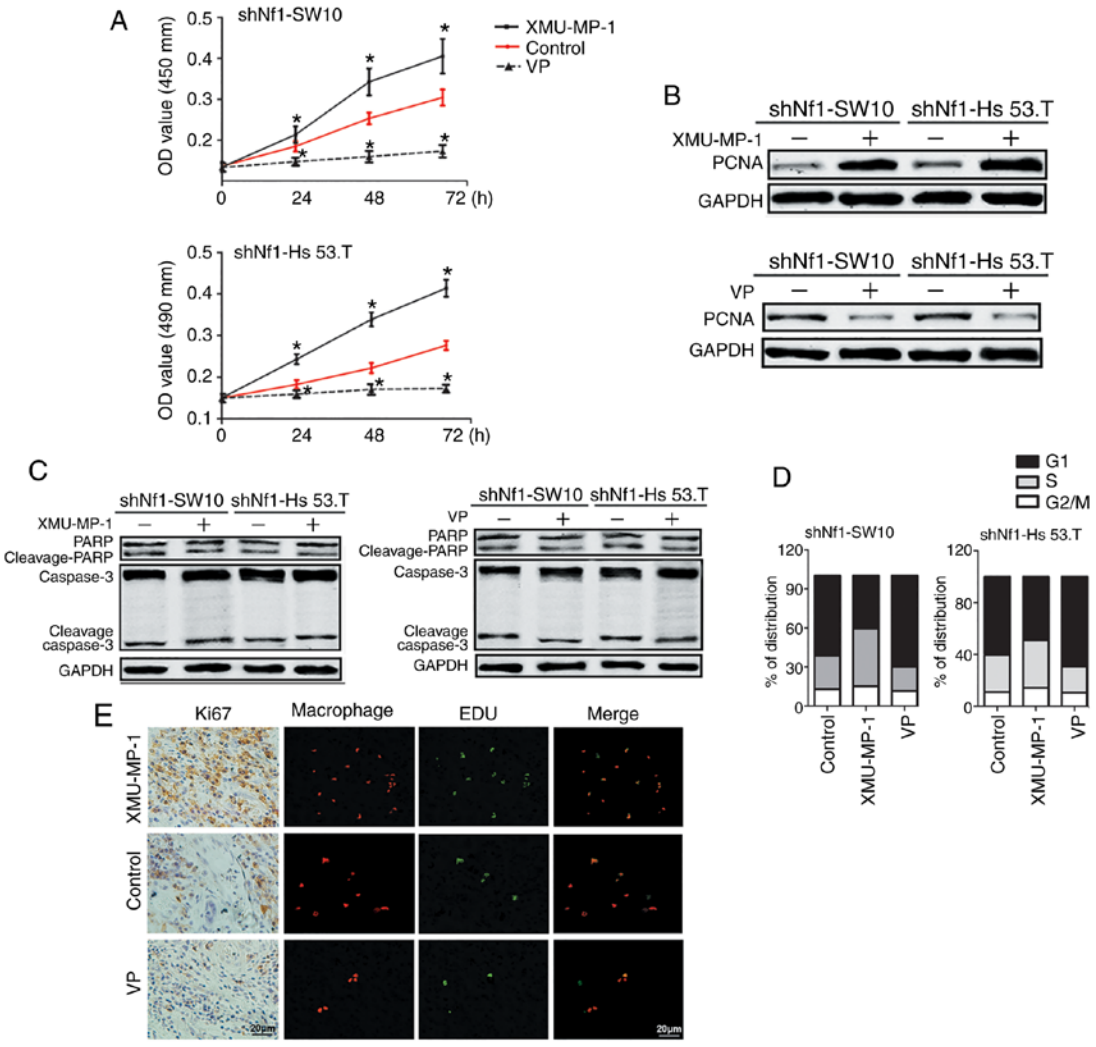

Figure 4. Conditioned media from cNF cells with activated YAP promotes THP-1 cell proliferation. (A) Conditioned media was collected from XMU-MP-1-, VP- or DMSO-treated Nf1-ablated shNf1-SW10 and shNf1-Hs 53.T cells. The conditioned media was used to treat THP-1 cells for different periods of times, and a Cell Counting Kit-8 assay was used to assess the cell viability of THP-1 cells. (B) Conditioned media from XMU-MP-1- or VP-treated shNf1-SW10 or shNf1-Hs 53.T cells were collected to treat THP-1 cells. Expression of PCNA, a marker of proliferation, was assessed. (C) Conditioned media from XMU-MP-1 or VP treated shNf1-SW10 cells and shNf1-Hs 53.T cells were collected to treat THP-1 cells. The protein levels of PARP, cleaved-PARP, Caspase 3 and Cleaved-Caspase 3, all markers of apoptosis, were detected in the THP-1 cells. (D) Cell cycle distribution of these clones was detected using flow cytometry. (E) Representative images of Ki67, RFP-labeled THP-1 cells and EdU triple staining in subcutaneous tumors from mice treated with XMU-MP-1, VP or DMSO. Magnification, x200. *P<0.05. VP, Verteporfin; sh, short hairpin; cNF, cutaneous neurofibromas; PCNA, proliferating cell nuclear antigen; medium; Nf1, neurofibromin 1; RFP, red fluorescent protein.

viability of CCR5-ablated and TGFβ1R-ablated THP-1 cells when treated with conditioned media from XMU-MP-1-treated or DMSO-treated cNF cells further indicated the importance of CCL5/CCR5 and TGF-β1/TGFβ1R in promoting THP-1 proliferation (Fig. 6B). CCL5 or TGF-β1 knockdown prevented the YAP-activated shNf1-SW10- and shNf1-Hs 53.T cell-induced THP-1 cells from entering into the S and G2/M phases (Figs. 6C, and S8A and B). Furthermore, both CCR5 and TGFβ1R knockdown arrested THP-1 cells in the G1 phase when treated with conditioned media from shNf1-SW10 and shNf1-Hs 53.T cells, with or without XMU-MP-1 treatment (Figs. 6D, S8C and D).

Similarly, VP-treated shNf1-SW10 and shNf1-Hs 53.T cells exhibited a decreased capacity to promote proliferation of THP-1 cells. CCL5 and/or TGF-β1 overexpression reduced this suppression (Fig. 6E). Increased cell viability was also observed in the CCR5- or TGFβ1R-overexpressing THP-1 cells treated with CM from VP- or DMSO-treated shNf1-SW10 and shNf1-Hs 53.T cells (Fig. 6F). Subsequently, cell cycle distribution was assessed, and it was found that the proportion of cells transitioning to the S and G2/M phases was increased when treated with conditioned media from CCL5- and/or TGF-β1-overexpressing shNf1-SW10 and shNf1-Hs 53.T cells, in the presence or absence of VP (Figs. 6G, S9A and B). Accordingly, overexpressing CCR5 or TGFβ1R also promoted THP-1 cell progression to the S and G2/M phases, when treated with conditioned media from VP- or DMSO-treated cNF cells (Figs. 6H, S9C and D). The results in Fig. 6E-H suggested that YAP reduced THP-1 cell proliferation, whereas overexpressing CCL5/CCR5 or TGF-β1/TGFβ1R, separately or concomitantly, reversed the YAP-mediated reduction.

Taken together, Fig. 6 suggests that either CCL5/CCR5 or TGF-β1/TGFβ1R mediated cell cycle progression of THP-1 cells and regulated THP-1 cell proliferation synergistically.

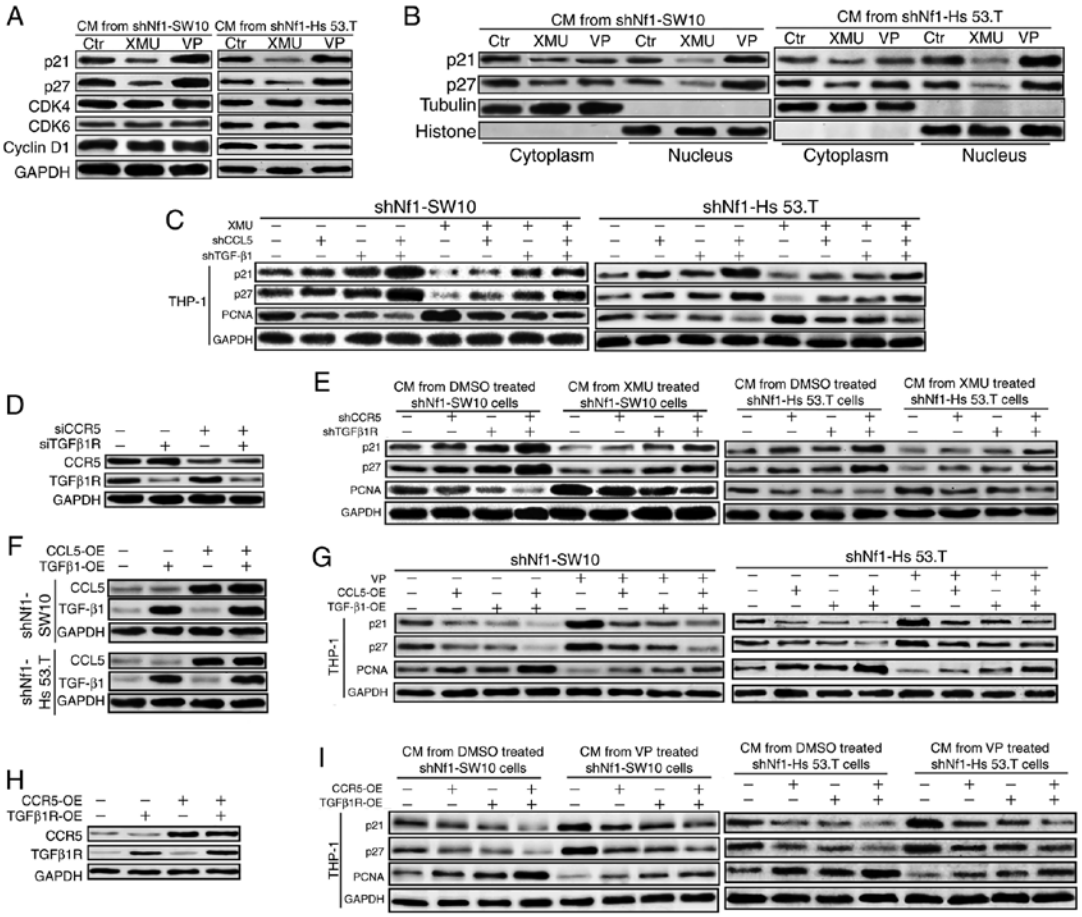

Figure 5. CCL5 and TGF-β1 mediate the expression of p21 and p27. (A) Nf1-ablated shNf1-SW10 and shNf1-Hs 53.T cells were treated with XMU-MP-1, VP or DMSO for 48 h before the conditioned media was collected. Expression of p21, p27, CDK4, CDK6 and Cyclin D1 was detected in the THP-1 cells treated with the prepared conditioned media. (B) Nuclear and cytoplasmic proteins were collected separately for the detection of p21 and p27 by western blotting. (C) XMU-MP-1-treated shNf1-SW10 and shNf1-Hs 53.T cells transfected with shCCL5, shTGF-β1 or both for 48 h prior to collection of conditioned media. The protein levels of p21, p27, Ki67 and PCNA in different conditioned media-treated THP-1 cells was detected. (D) Specific shRNAs were transfected to knock down TGFβ1R and CCR5 in THP-1 cells. (E) Conditioned media collected from XMU-MP-1- or DMSO-treated shNf1-SW10 and shNf1-Hs 53.T cells were used to treat THP-1 cells with knockdown of specific genes, and the expression of p21, p27 and PCNA in the THP-1 cells was detected. (F) Specific shRNAs targeting CCL5 or TGFβ1 were transfected in the shNf1-SW10 and shNf1-Hs 53.T cells and the expression of CCL5 and TGFβ1 was assessed. (G) CCL5- and/or TGFβ1-overexpressing shNf1-SW10 and shNf1-Hs 53.T cells were treated with VP or DMSO, and conditioned media was collected to treat THP-1 cells. The protein levels of p21, p27 and PCNA in the conditioned media-treated THP-1 cells were examined. (H) Specific shRNAs against CCR5 and TGFβ1R were transfected into THP-1 cells. (I) Conditioned media from VP- or DMSO-treated shNf1-SW10 and shNf1-Hs 53.T cutaneous neurofibroma cells was prepared to treat THP-1 cells. The protein levels of p21, p27 and PCNA in the conditioned media-treated THP-1 cells was detected. CDK, cyclin-dependent kinase; VP, Verteporfin; sh, short hairpin; PCNA, proliferating cell nuclear antigen; CCL5, C-C motif chemokine ligand 5; TGF-β1, transforming growth factor-β1; Nf1, neurofibromin 1.

Discussion

At present, treatment of cNF is limited to surgical excision, which is not always curative, as the presence of numerous or large cNF tumors are difficult to completely resect. Thus, improving our understanding of the mechanisms underlying the development of cNF initiation and progression may assist in the identification of novel therapeutic targets. In the present study, it was demonstrated that the number of macrophages was increased in cNF tissues compared with the matching adjacent healthy tissue, and this was accompanied by upregulated expression of YAP and TAZ, effectors of the Hippo pathway. Additionally, it was demonstrated that activation of the Hippo pathway effectors facilitated macrophage accumulation by promoting both macrophage recruitment and macrophage cell proliferation.

Clinically, patients with similar genetic backgrounds, for example patients from the same family, were found to exhibit different types of Cnf (14), which suggests the existence of modifiers during neurofibroma development. A study in Nf1 heterozygous mice showed that the preferential sites for development of neurofibromas are the cervical nerves and the mid-thoracic nerves, flexible sites that are prone to minor but numerous nerve injuries (25). Similarly, the cervical nerve has also been identified as a hot spot for development of neurofibroma formation in patients with NF1 (26). Tissue damage leads to inflammation in order to repair the tissue, during which inflammatory cells produce cytokines and growth factors, stimulating factors of neoplasias (27). Mast cells and macrophages are the two primary types of inflammatory cells in the cNF microenvironment. Infiltrating mast cells in neurofibromas are frequently present in an activated state, as shown by elevated levels of local histamine and circulating serum IgE (28,29). Macrophages in the cNF microenvironment also exhibit an inflammatory status, shown by the

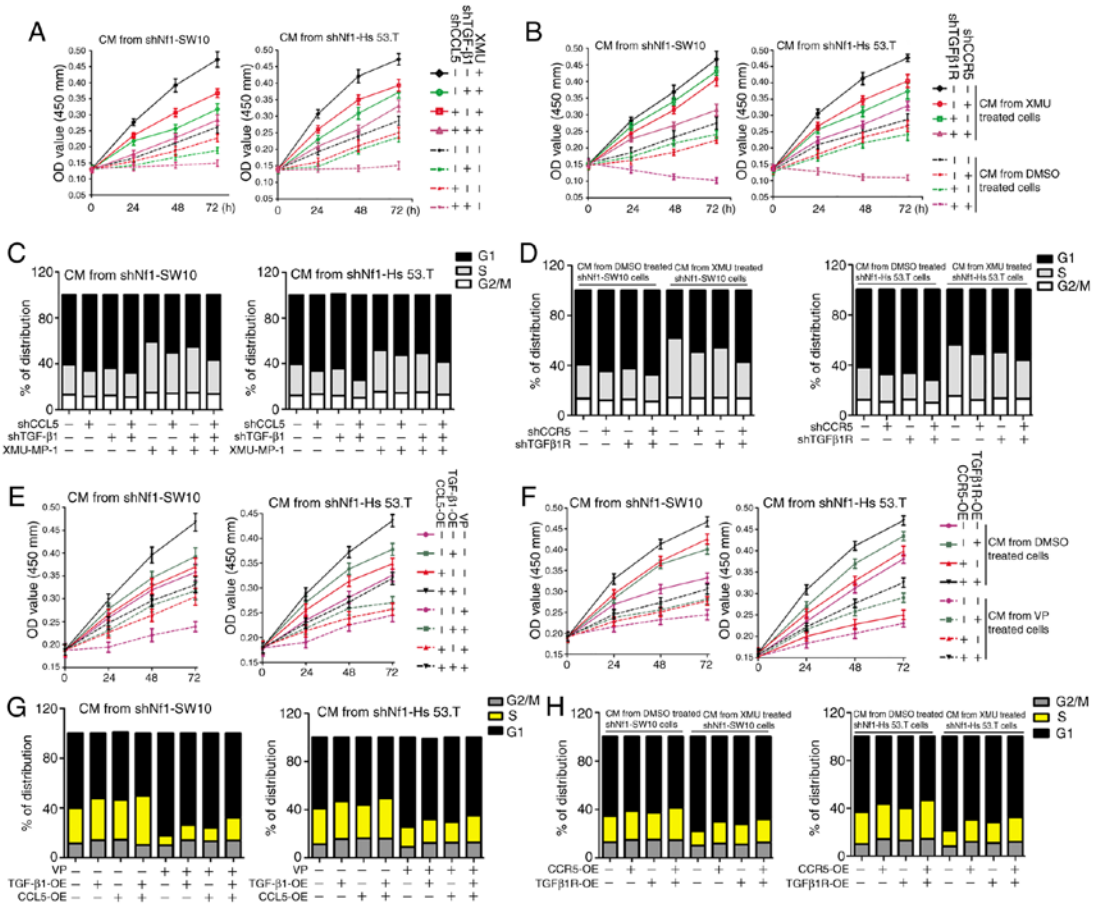

Figure 6. CCL5/CCR5 and TGF-β1/TGFβ1R serve important roles on the effect of cNF cells on proliferation of THP-1 cells. (A) XMU-MP-1-treated cNF cells were transfected with shCCL5, shTGF-β1 or both for 48 h, and conditioned media was subsequently collected. The cell viability of THP-1 cells treated with the conditioned media was analyzed using a CCK-8 assay. (B) Conditioned media was collected from shNf1-SW10 and shNf1-Hs 53.T cells treated with or without XMU-MP-1. THP-1 cells with TGFβ1R, CCR5 or both TGFβ1R + CCR5 knocked down were treated with the prepared conditioned media, and the cell viability was analyzed using a CCK-8 assay. (C) Cell cycle distribution of THP-1 cells following treatment with conditioned media from treated shNf1-SW10 and shNf1-Hs 53.T cells. (D) Cell cycle distribution in the TGFβ1R-, CCR5- or TGFβ1R + CCR5-knockdown THP-1 cells treated with conditioned media from XMU-MP-1-treated shNf1-SW10 and shNf1-Hs 53.T cells. (E) CCL5- or TGF-β1-overexpressing shNf1-SW10 and shNf1-Hs 53.T cells were treated with VP or DMSO and the conditioned media was collected. A CCK-8 assay was used to assess the viability of THP-1 cells following treatment with different conditioned medias. (F) THP-1 cells overexpressing CCR5 or TGFβ1R were treated with conditioned media from VP- or DMSO-treated shNf1-SW10 and shNf1-Hs 53.T cells. The cell viability of THP-1 cells was detected. (G) Cell cycle distribution of THP-1 cells following treatment with conditioned media from CCL5- or TGFβ1-overexpressing cNF cells, treated with or without VP. (H) THP-1 cells overexpressing CCR5 and TGFβ1R prior to the treatment with conditioned media from VP- or DMSO-treated shNf1-SW10 and shNf1-Hs 53.T cells. Cell cycle distribution of these treated THP-1 cells were analyzed. VP, Verteporfin; sh, short hairpin; cNF, cutaneous neurofibromas; PCNA, proliferating cell nuclear antigen; CCL5, C-C motif chemokine ligand 5; TGF-β1, transforming growth factor-β1; Nf1, neurofibromin 1; CCK-8, Cell Counting Kit-8.

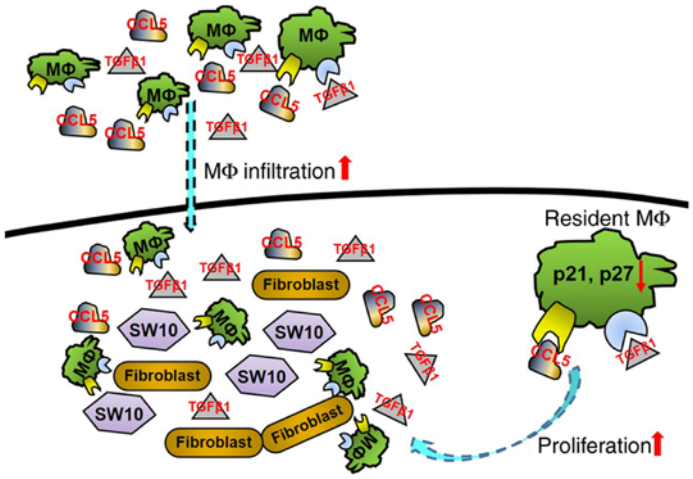

Figure 7. Schematic representation showing that cNF cell-produced TGF-β1 and CCL5 promote both macrophage infiltration and resident macrophage proliferation. cNF, cutaneous neurofibroma; Mf, macrophage; CCL5, C-C motif chemokine ligand 5; TGF-β1, transforming growth factor-β1.

presence of stronger M1 signatures than M2 signatures (23). These findings indicate that continuous inflammation in the microenvironment may contribute to the progression of cNF.

As mast cells are the most abundantly present type of inflammatory cells in the cNF microenvironment (29), efforts to treat cNF by inhibiting mast cells has been attempted. Some success has been achieved in shrinking neurofibromas by targeting mast cells, but there remain a number of patients who do not respond favorably to this treatment (6). Thus, exploring complementary factors that support neurofibroma maintenance may highlight novel potential targets for treatment. Importantly, a clinical trial using ketotifen (3), which possesses antihistamine and anti-inflammatory activities, and mouse models of cNF treated with PLX3397 (10), a dual inhibitor for both mast cells and macrophages, indicated improved efficacy against neurofibromas compared with inhibiting mast cells alone. Nevertheless, there were still instances of unsatisfactory outcomes in the aforementioned studies. Therefore, understanding the detailed mechanism involved in macrophage accumulation may assist in identifying potential therapeutic targets.

The critical role of Nf1 heterozygosity was first noticed in the pioneer mouse plexiform neurofibroma model, which showed that tumors developed in the $Nf1^{f/-}$ mice, but not the $Nf1^{f/f}$ mice (5). However, Nf1 heterozygosity may not always be essential, as some $Nf1^{f/f}$ mice also develop neurofibromas without the microenvironment afforded by a $Nf1^{+/-}$ genotype (30), suggesting the presence of alternative signals mediating neurofibroma development. A recent study showed that that activating Hippo pathway effectors accelerates Nf1-associated cNF development, although altering the Hippo pathway alone did not result in the development of tumors (14). Furthermore, cNF also occurs in patients with neurofibromatosis type 2 who possess mutant neurofibromin 2, a regulator of the Hippo pathway (31). The present study showed that the expression of YAP and TAZ was upregulated in cNF tissues, both of which are core effectors of the Hippo pathway, and demonstrated the positive association between YAP activity and macrophage accumulation. These data suggest that the Hippo pathway may serve as a modifier, although possibly not a driver, in neurofibroma progression and modification of the tumor microenvironment.

A preliminary study showed that the Hippo pathway modulates macrophage recruitment (32). CCL5/CCR5 and TGF-β1/TGFβR can mediate macrophage recruitment (33,34). The present study showed that cNF cells with activated YAP increased macrophage accumulation by upregulating both CCL5 and TGF-β1, and only synergistic ablation of CCL5 and TGF-β1 effectively inhibited macrophage accumulation. The possible reasons for these results may be that other molecules not examined in the present study contributed to macrophage accumulation. Alternatively, cNF cells may also facilitate the proliferation/survival of resident macrophages. The macrophages in the tumor microenvironment included the recruited macrophages and the resident macrophages, and the proliferation of resident macrophages should not be overlooked during the process of eliminating macrophages from the tumor microenvironment. The present study revealed that repression of either CCL5 or TGF-β1 alone significantly reduced THP-1 cell viability, but did not attenuate THP-1 cell accumulation. A possible reason for the above results may be the increased efficiency of enhancing infiltration and promoting proliferation of both CCL5 and TGF-β1. It was also attempted to determine the source of macrophages in tumors, to distinguish between infiltration/recruitment or proliferation, but a suitable method could not be determined.

The results of the present study showed that YAP-active neurofibroma cells regulated cell cycling in macrophages, and this represents just one of the potential mechanisms involved in promoting macrophage proliferation. Other mechanisms participating in macrophage proliferation cannot be excluded. The IHC assay with anti-Ki67 in the xenograft subcutaneous tumors (containing macrophages and shNf1-SW10 cells) showed that XMU-MP-1/VP treatment promoted/suppressed tumor growth. EDU staining in the tumor tissues indicated that macrophage proliferation was also enhanced. These results do not exclude the potential involvement of XMU-MP-1/VP itself in promoting/reducing macrophage proliferation. In the *in vitro* experiments with conditioned media from XMU-MP-1/VP-treating shNf1-SW10/shNf1-Hs 53.T cells confirmed that YAP-activating neurofibroma cells modulated macrophage proliferation. Taken together, these results suggested that neurofibroma cells with activated YAP exhibited upregulated cell growth and promoted macrophage accumulation. If other mechanisms are also involved in neurofibroma cell-mediated increase of macrophage proliferation, these remain to be determined.

In conclusion, the present study demonstrated that activation of YAP promoted proliferation of cNF cells and increased CCL5 and TGF-β1 expression. This resulted in accumulation of macrophages by facilitating both the recruitment and proliferation of macrophages. Reversal of macrophage accumulation caused by treatment with a YAP inhibitor suggested that YAP inhibition may serve as a potential therapeutic avenue for treatment of cNF, via impairment of both tumor cell proliferation and macrophage accumulation.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

MS designed the experiments, performed the data analyses and wrote the manuscript. LH performed the *in vivo* analysis. HBZ and JJ performed the cellular experiments and assisted with the mice experiments. HKZ performed the immunohistochemistry assays. All authors participated in the discussion and revision of the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All procedures performed involving human participants were in accordance with the ethical standards of the Institutional Review Board of The First Affiliated Hospital of the Xi'an Jiaotong University. The present study adhered to the guidelines of the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from adult patients, or from their parents/guardians for patients younger than 18 years old. The animal experiments were approved by the Institutional Review Board of The First Affiliated Hospital of Xi'an Jiaotong University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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