

Dual blockade of interleukin-17A and interleukin-17F as a therapeutic strategy for liver fibrosis: Investigating the potential effect and mechanism of brodalumab

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ABSTRACT

Liver fibrosis is a terminal manifestation of various chronic liver diseases. There are no drugs that can reverse the condition. Recently, the importance of interleukin-17 (IL17) in the pathophysiology has been revealed and has attracted attention as a therapeutic target. We aimed to reveal the roles of IL17A and IL17F in liver fibrosis, and to validate the potential of their dual blockade as therapeutic strategy. First, we retrospectively reviewed the longitudinal change of FIB-4 index, a clinical indicator of liver fibrosis, among psoriasis patients treated by brodalumab, which blocks IL17 receptor A (IL17RA). Next, we examined anti-fibrotic efficacy of anti-IL17RA antibody (Ab) in two murine liver fibrosis models by histopathological investigation and real-time reverse transcription polymerase chain reaction (RT-PCR). Finally, we analyzed the effect of IL17A and IL17F upon human hepatic stellate cells with RNA sequencing, real-time RT-PCR, western blotting, chromatin immunoprecipitation, and flow cytometry. Clinical data showed that FIB-4 index significantly decreased among psoriasis patients treated by brodalumab. *In vivo* studies additionally demonstrated that anti-IL17RA Ab ameliorates liver fibrosis induced by tetrachloride and methionine-choline deficient diet. Furthermore, *in vitro* experiments revealed that both IL17A and IL17F enhance cell-surface expression of transforming growth factor- β receptor II and promote pro-fibrotic gene expression via the JUN pathway in human hepatic stellate cells. Our insights suggest that IL17A and IL17F share their pro-fibrotic function in the context of liver fibrosis, and moreover, dual blockade of IL17A and IL17F by anti-IL17RA Ab would be a promising strategy for the management of liver fibrosis.

1. Introduction

Liver fibrosis is a progressive condition characterized by the replacement of liver tissue with extracellular matrices. It commonly arises as a consequence of chronic viral hepatitis, alcohol abuse, and non-alcoholic fatty liver disease (NAFLD) [1]. If left untreated, liver fibrosis can advance to cirrhosis, leading to liver dysfunction and potentially fatal conditions such as hepatocellular carcinoma. Unfortunately, there are currently no effective drugs available to reverse fibrosis and cirrhosis. In advanced stages, a liver transplant becomes the only option, but the procedure is complex, expensive, and hindered by limited donor organs. Consequently, there is a significant unmet medical need for managing this condition [2].

Psoriasis is a chronic inflammatory cutaneous condition

characterized by erythematous and scaly plaques. It primarily affects the skin, but it has also been recognized as a systemic disease that can affect multiple organ systems [3]. Patients with psoriasis have an increased risk of developing cardiovascular diseases such as myocardial infarction and stroke [4]. Psoriasis has also been linked to metabolic syndrome, which is a cluster of conditions including obesity, insulin resistance, high blood pressure, and abnormal cholesterol levels [5]. NAFLD, a hepatic manifestation of metabolic syndrome, is another condition associated with psoriasis [6]. The exact relationship between psoriasis and these conditions is not fully understood, but it is believed to be related to systemic inflammation and shared risk factors. The coexistence of these conditions in individuals with psoriasis can accumulate the risk of complications and mortality. Therefore, it is important for individuals with psoriasis to work closely with their healthcare

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providers to manage not only their skin symptoms but also to monitor and address any potential systemic complications [3].

In recent years, research on the effects of biologics, specifically targeting the interleukin-17 (IL17) pathway, has gained interest as a potential treatment avenue for psoriasis and its associated comorbidities [7]. IL17 is a pro-inflammatory cytokine produced by T helper 17 (Th17) cells, playing a vital role in the immune response to pathogens and the development of autoimmune diseases [8–10]. The IL17 family comprises six members: IL17A to IL17F, with IL17A being the most extensively studied [11]. IL17F, having a close amino acid sequence to IL17A, is believed to possess similar biological activities [12]. Both IL17A and IL17F bind to the heterodimeric complex of IL17 receptor A (IL17RA) and IL17 receptor C (IL17RC) on target cells, triggering various intracellular signaling pathways [13–14].

Among them, the JUN amino terminal kinase (JNK), an upstream regulator of JUN, has been proposed as a key player in the pro-fibrotic response in the liver [15–16]. The involvement of the JUN pathway in the development of liver fibrosis has been explored in human hepatic stellate cells (HSCs), a significant source of extracellular matrix proteins including collagens and tissue inhibitors of metalloproteinases (TIMPs), thereby contributing to the remodeling of liver architecture [17]. In response to liver injury, HSCs undergo a phenotypic transformation into activated myofibroblast-like cells, primarily mediated by transforming growth factor- β (TGF β), followed by the activation of the downstream Smad signaling pathway [18]. A previous study has demonstrated that the JUN pathway upregulates the expression of pro-fibrotic genes, such as collagen type I alpha 2 chain (COL1A2) and TIMP1 in human hepatic stellate cells (HSCs) by enhancing the cell surface expression of transforming growth factor- β (TGF β) receptor II (TGF β RII), which enhances the sensitivity of HSCs to TGF β [19].

Among the IL17-targeting agents, brodalumab (BRO) stands out as a biologic that blocks IL17RA, effectively inhibiting signaling transduction mediated by both IL17A and IL17F. Its efficacy and safety have been favored in several clinical trials for the treatment of psoriasis and ankylosing spondylitis [20–21]. Especially in psoriasis, a network meta-analysis has shown that BRO was more powerful in the ability of skin rash improvement compared to other biologics targeting IL17A alone, [22] which suggested the importance of blocking both IL17A and IL17F to achieve better treatment outcomes, or to manage cases or conditions refractory to conventional therapies. Its potential has also been observed in a phase 3 trial for systemic sclerosis, a challenging autoimmune disease [23–24].

In this study, we aim to validate the effectiveness of the dual blockade of IL17A and IL17F through anti-IL17RA antibodies such as BRO in both *in vivo* and *in vitro* models. First, we will present longitudinal improvement of the FIB-4 index, [25] a clinical indicator of liver fibrosis, in psoriasis patients receiving BRO, which serves as motivation for our investigation. Next, we will demonstrate the amelioration of liver fibrosis through the administration of anti-IL17RA antibodies in two distinct murine models. Finally, we will explore the molecular mechanism by which IL17A and IL17F induce pro-fibrotic gene expression in HSCs. These findings will provide new insights into the pathophysiology of liver fibrosis involving IL17 families and underscore the potential of IL17RA-targeting agents such as BRO, in addressing the unmet medical needs associated with liver fibrosis management.

2. Materials and methods

2.1. Clinical data

We consecutively collected all the psoriasis patients who first arrived at our clinic since April 2019 to March 2020. Of these, patients who were treated with BRO (anti-IL17RA Ab) or secukinumab (anti-IL17A Ab) for longer than 6 months were recruited. Those who were treated only by topical agents were selected as biologics-naïve controls. Clinical data were collected from the electric medical records. We gathered basic

patient information, symptoms, medications, and clinical outcomes. Basic patient information encompassed sex, age, and comorbidities at the initial visit. Their skin symptoms were evaluated by psoriasis area and severity index (PASI) score. Laboratory examinations included measuring aspartate transaminase (AST), alanine transaminase (ALT), and peripheral blood platelet counts (Plt). FIB-4 index at the baseline and 6 months later were calculated as previously described [25]. Only patients whose FIB-4 index at the baseline was more than 1.659, which has been reported as a threshold predictive of liver fibrosis, [26] were enrolled into our analysis. Overall, 20 patients were recruited into our study, whose features are described in Table 1. This study has been approved by The University of Tokyo Ethical Committee (Approval number 0695). Written informed consent has been obtained from all the participants.

2.2. Mice and experimental protocols

Pathogen free male C57BL/6 mice were purchased from Charles River Japan. The mice were housed under controlled temperature (21 $\pm$ 1°C) and relative humidity (60 $\pm$ 10 %) with a 12/12 h dark/light cycle in an air-filtered clean room. To induce CCl₄-induced liver fibrosis, we administered 0.5 g/kg CCl₄ solved in corn oil to the six-week-old mice twice a week for 6 weeks [27]. During the latter 4 weeks, the mice received weekly intraperitoneal injections of anti-IL17RA Ab (R&D Biosystems) 125 μ g solved in 250 μ L PBS or PBS 250 μ L alone (n = 7 respectively; Fig. 2a). In another experiment, nine-week-old mice were fed either a control diet or a methionine-choline deficient (MCD) diet (FUJIFILM Wako Pure Chemical Corporation) for 8 weeks [28]. During the latter 4 weeks, the mice received weekly intraperitoneal injection of anti-IL17RA Ab (R&D Biosystems) 125 μ g solved in 250 μ L PBS or PBS 250 μ L alone (n = 7 respectively; Fig. 3a). The procedures for the handling of animals were approved by the Animal Experiment Committee, The University of Tokyo.

2.3. Histological analysis

The mice were sacrificed after being anesthetized by isoflurane inhalation. The liver tissue was collected and cut into small pieces. Each piece was fixed by Mildform (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for histopathological analysis, or stored in RNAlater (Thermo Fisher Scientific, Waltham, MA) at –20°C for RNA extraction. The fixed liver tissue was embedded in paraffin, cut in thin slices, deparaffinized in xylene, and rehydrated in graded ethanol solutions. Hematoxylin and eosin (HE) staining and Masson’s trichrome (MT) staining were performed according to standard techniques. TdT-

Table 1
Demographic features of psoriasis patients.

	Anti-IL17RA (N = 5)	Anti-IL17A (N = 7)	Naïve (N = 8)	P values
Male/Female, N	5/0	4/3	4/4	0.18
Age, years (median)	71	76	67.5	0.40
PASI (median)	3.6	5.9	1.6	0.50
PsA	40.0	28.6	25.0	1.00
Hypertension	40.0	71.4	25.0	0.27
Dyslipidemia	20.0	57.1	25.0	0.43
Diabetes mellitus	20.0	14.3	25.0	1.00
AST, U/L	33	30	27	0.57
ALT, U/L	25	23	20.5	0.97
Plt, 10 ⁹ /mm ³	13.0	20.4	21.0	0.10
Δ FIB-4 index	–0.9	0.5	0.2	0.02

The values are number of percentages otherwise noted. P values were calculated by Kruskal-Wallis test for continuous variables, and Fisher’s exact test for categorical variables. IL17: interleukin-17, N: number of patients, PASI: psoriasis activity and severity index, PsA: psoriatic arthritis, AST: aspartate transferase, ALT: alanine transferase, Plt: peripheral blood platelet counts. Δ FIB-4 index: longitudinal change of FIB-4 index from the baseline.

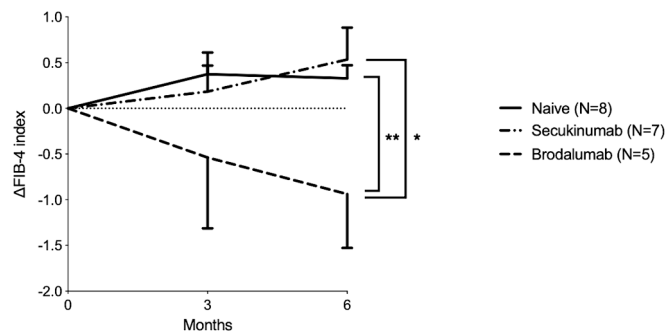


Fig. 1. Longitudinal change of FIB-4 index (Δ FIB-4 index) in patients with psoriasis. FIB-4 index at the baseline and after a 6-month period in three groups of patients with psoriasis: those receiving brodalumab, those receiving secukinumab, and biologics-naïve patients. *P* values are calculated by Mann Whitney's *U* test. *: $P < 0.05$, **: $P < 0.01$.

mediated UTP nick-end labeling (TUNEL) assay was performed using TACS2 TdT *in situ* Apoptosis Detection DAB Kit (R&D systems) according to the manufacturer's protocol. Histologic images were gained by a

microscope. The fibrotic area, framed as the blue region on MT staining, was extracted and quantified using ImageJ software [29].

2.4. Cell culture

Human HSC line LX-2 cells (Merck, Darmstadt, Germany) were cultured in Dulbecco's Modified Eagle's Medium (DMEM)-high glucose (Sigma-Aldrich, St-Louis, MO) supplemented with 2 % fetal bovine serum and 1 % L-Glutamine (Sigma-Aldrich) [30]. When the cells reached 70 % confluency, they were serum-starved in DMEM-high glucose supplemented with 1 % L-Glutamine for 24 h. Subsequently, the cells were stimulated for 24 h by recombinant human IL17A (10 ng/mL; R&D Systems, Minneapolis, MN), IL17F (10 ng/mL; R&D Systems), TGF β (1 pg/mL; R&D Systems), SP600125 (10 μ M; Sigma Aldrich), BRO (1 μ g/mL; Kyowa Kirin), and/or Hizentra (1 μ g/mL; CSL Behring) as control IgG added to DMEM-high glucose supplemented with 1 % L-Glutamine and used for further studies.

2.5. RNA isolation and real-time RT-PCR

Total RNA was extracted from LX-2 cells or murine livers utilizing

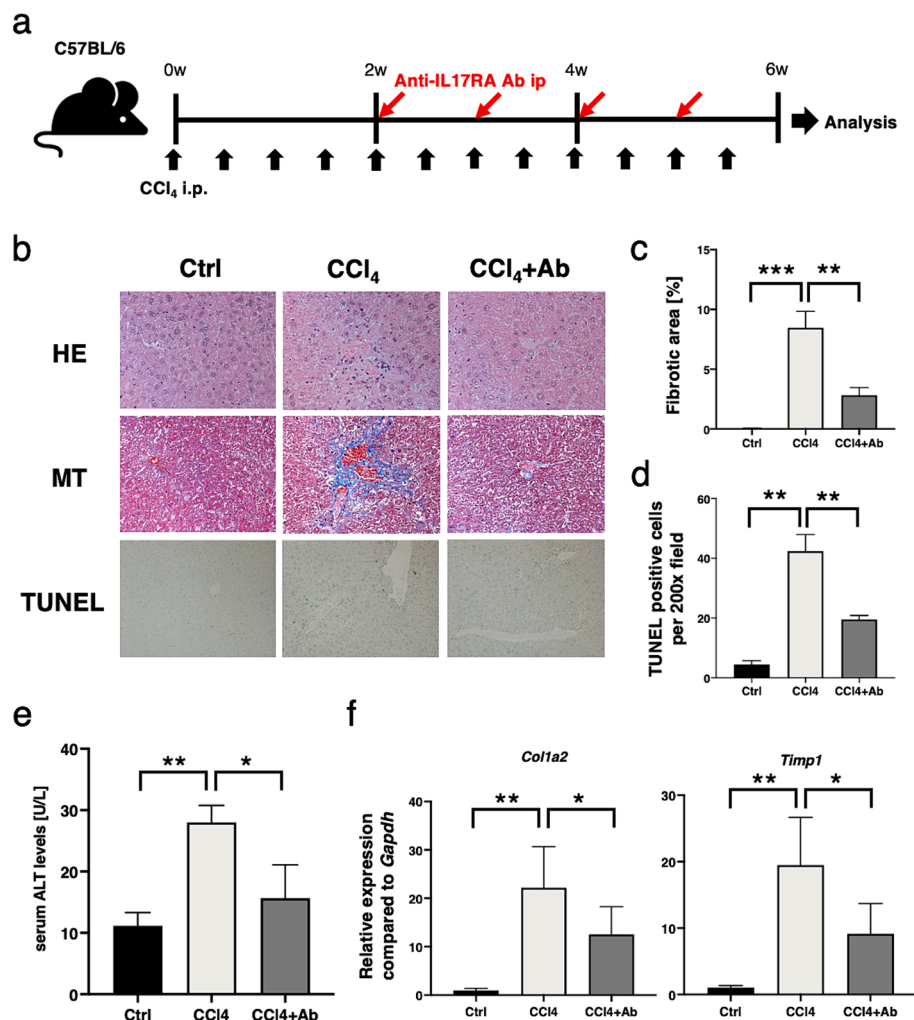


Fig. 2. Effect of anti-IL17RA Abs against CCl₄-induced liver fibrosis in mice. (a) Scheme of the experiment design. Ip: intraperitoneal injection. W: weeks. (b) Sections of paraffin-embedded liver tissue from control mice (Ctrl), mice treated with CCl₄ (CCl₄), and mice treated with both CCl₄ and anti-IL17RA Ab (CCl₄ + Ab) were treated with hematoxylin-eosin (HE) staining, Masson's trichrome (MT) staining, or TdT-mediated UTP nick-end labeling (TUNEL) assay. (c) Fibrotic area detected by MT staining within 200x histologic images from each mouse was quantified by ImageJ. (d) The number of TUNEL positive cells within 200x histologic images from each mouse was counted. (e) Relative expression of pro-fibrotic genes (*Col1a2* and *Timp1*) in the liver was examined by quantitative RT-PCR. Results are shown as fold change compared to Ctrl. *P* values were calculated by Mann Whitney's *U* test. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. Error bars indicate standard error of the mean.

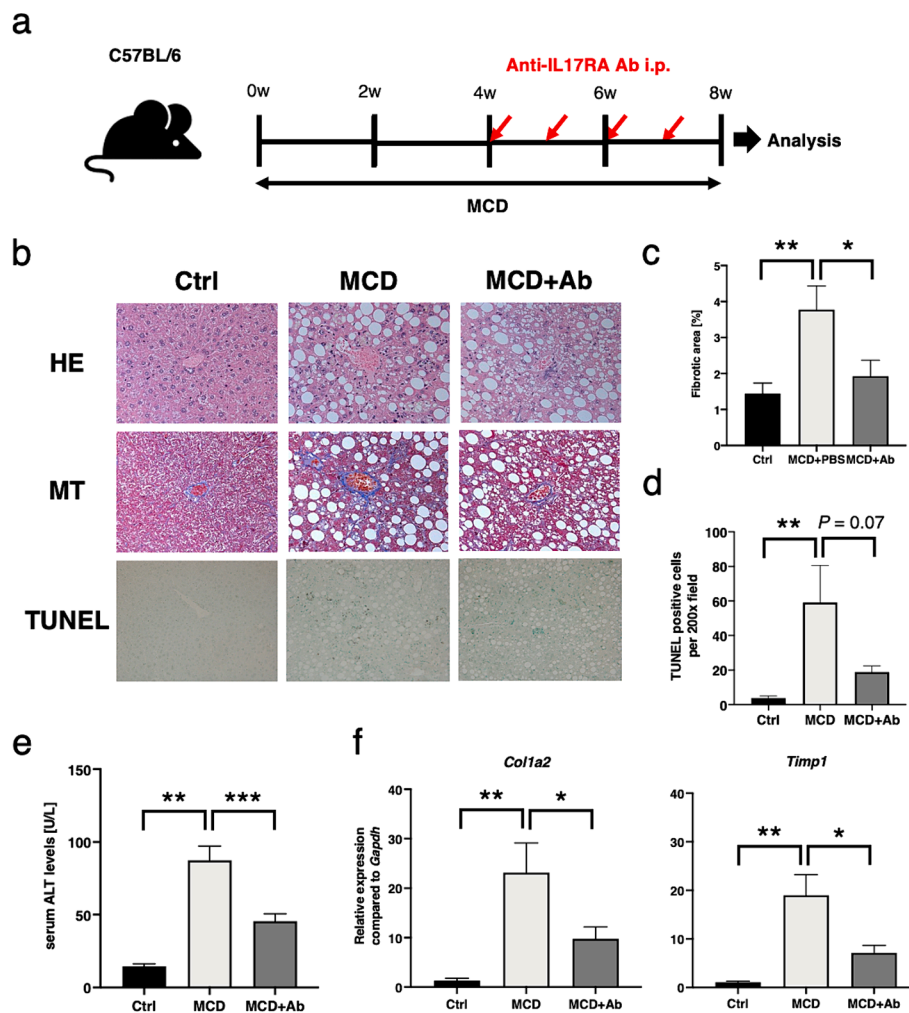


Fig. 3. Efficacy of anti-IL17RA Abs against MCD diet-induced liver fibrosis in mice. (a) Scheme of the experiment design. ip: intraperitoneal injection. w: weeks. (b) Sections of paraffin-embedded liver tissue from control mice (Ctrl), mice fed by MCD diet, and mice fed by MCD diet and received anti-IL17RA Ab ip were treated with hematoxylin-eosin (HE) staining, Masson's trichrome (MT) staining, or TdT-mediated UTP nick-end labeling (TUNEL) assay. (c) The fibrotic area detected by MT staining within 200x histologic images from each mouse was quantified by ImageJ. (d) The number of TUNEL positive cells within 200x histologic images from each mouse was counted. (e) Relative expression of pro-fibrotic genes (*Col1a2* and *Timp1*) in the liver was examined by quantitative RT-PCR. Results are shown as fold change compared to Ctrl. *P* values were calculated by Mann Whitney's *U* test. *: *P* < 0.05, **: *P* < 0.01, ***: *P* < 0.001. Error bars indicate standard error of the mean.

the RNeasy Mini Kit (Qiagen, Hilden, Germany) and RNase-Free DNase Set (QIAGEN) according to the manufacturer's protocol. Complementary DNA was generated using the ReverTraAce qPCR RT Master Mix (Toyobo, Osaka, Japan). Relative expression of target genes was measured and normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*/*Gapdh*) expression. The sequence of primers used for real-time RT-PCR is shown in **Supplementary Table 1**.

2.6. RNA sequencing

RNA sequencing libraries were generated from the total RNA isolated from LX-2 cells treated with IL17A (*n* = 2), IL17F (*n* = 2), or mock (*n* = 2), by messenger RNA purification using poly-T oligo-attached magnetic beads, fragmentation, reverse transcription, end repair, A-tailing, adapter ligation, size selection, amplification, and purification. After quality control, sequencing was performed on the Illumina NovaSeq6000 PE150 platform (Illumina, California, USA). An average of 48,794,762 reads were obtained across the samples. Sequencing data were aligned to the human reference genome (assembly GRCh38, BioProject accession PRJNA31257) using the HiSAT2 (version 2.1.0), [31] and gene counts were calculated from the alignment data using

StringTie (version 2.2.1) in Mac, [32] on the Subio Platform (version 1.24.5853).

2.7. Differential gene expression analysis

Differential expression analysis was carried out on the data with the DESeq2 analysis, [33] using the Graphical User Interface for TCC package with default settings applied [34–35]. Genes which showed statistically significant (false discovery rate < 0.05) changes in expression following IL17A or IL17F stimulation were defined as differentially expressed genes (DEGs). Over-representation analyses targeting the list of DEGs were performed for tissue-specificity analysis and transcriptional regulatory network analysis utilizing the Metascape, [36] the PaGenBase database, [37] and the TRRUST database [38].

2.8. Western blotting

Proteins were extracted from LX-2 cells using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or Nuclear Extraction Kit (RayBiotech, Norcross, GA, USA) according to the manufacturer's manual. Extracted proteins were separated by SDS-PAGE and

transferred to PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5 % non-fat milk or 5 % BSA in TBS/0.05 % Tween 20 (Cell Signaling Technology, MA, USA) for 30 min at room temperature and incubated with primary antibodies against JNK (Cell Signaling Technology), phosphorylated JNK (p-JNK; Cell Signaling Technology), COL1A2 (GeneTex, CA, USA), TIMP-1 (Cell Signaling Technology), SMAD2/3 (Cell Signaling Technology), phosphorylated SMAD2/3 (Cell Signaling Technology), TATA-binding protein (TBP), and β -actin (Cell Signaling Technology) overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies (Cell Signaling Technology) for one hour at room temperature. Proteins were visualized using Chemi-Lumi One Ultra (Nacalai Tesque, Kyoto, Japan) and FUSION SOLO.7S.EDGE (Vilber Bio Imaging, Collégien, France.) Densitometry analysis was conducted utilizing Evolution Capt software (Vilber Bio Imaging.).

2.9. Flow cytometry

LX-2 cells were detached with cell scrapers, washed twice in the PBS (Nacalai Tesque) supplemented with 1 % FBS, and incubated for 30 min with phycoerythrin-conjugated antibodies against TGF β RII (R&D Systems, MN, USA). The cells were then washed again in the Dulbecco's PBS supplemented with 1 % FBS, fixed by incubation with CytoPerm (BD Biosciences, CA, USA) for 15 min, and examined by a BD-LSRII (BD Biosciences) using FACSuite software (BD Biosciences, NJ, USA). Data analysis was performed on Kaluza software (Beckman Coulter, CA, USA).

2.10. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed using ChromaFlash High-Sensitivity ChIP kit (Epigentek, NY, USA) according to the manufacturer's instruction. Briefly, LX-2 cells were cross-linked with 1 % formaldehyde (Sigma Aldrich, MZ, USA) and the cross-linked chromatin was sonicated to an average size of 300–500 base pairs. Anti-SMAD2/3 antibody (Cell Signaling Technology) was used for immunoprecipitation with non-immune IgG as the negative control. The immunoprecipitated chromatin was analyzed by agarose gel electrophoresis and quantitative real-time RT-PCR using primers specific for the CAGACA motifs in the promoter regions of *COL1A2* and *TIMP1* [39]. The sequence of the primers is described in **Supplementary Table 1**.

2.11. Statistical analysis

Statistical analyses were performed by Mann-Whitney's *U* test for two-group comparison and Kruskal-Wallis test for multi-group comparison regarding continuous variables, and Fisher's exact test for categorical variables. All the analyses were performed using Prism 9 (GraphPad Software, CA, USA).

3. Results

3.1. Blockade of IL17RA decreases FIB-4 index in psoriasis patients

We recruited 5 patients on BRO (a humanized monoclonal anti-IL17RA Ab), 7 patients on secukinumab (a humanized monoclonal anti-IL17A Ab), and 8 patients who were treated only by topical agents into our retrospective observation. Background features including sex, age, PASI score, comorbidities, and laboratory data were balanced among the three groups (**Table 1**). In the BRO group, FIB-4 index significantly decreased 6 months later compared to the biologics naïve group ($P < 0.01$) or even compared to the secukinumab group ($P < 0.05$; **Fig. 1**).

3.2. Anti-IL17RA Ab administration ameliorates liver fibrosis in murine models

We subsequently validated the therapeutic effect of anti-IL17RA Ab in two murine models of liver fibrosis. In the CCl₄-induced murine model (**Fig. 2a**), hepatocyte ballooning and perisinusoidal fibrosis, which are common in NAFLD, were induced (**Fig. 2b**). The area of fibrosis revealed by MT staining and the number of TUNEL positive cells significantly decreased by administration of anti-IL17RA Ab (**Fig. 2c–d**). Serum levels of ALT also reduced by anti-IL17RA Ab treatment with statistical significance (**Fig. 2e**). Consistently, the expression levels of fibrosis-promoting factors *Col1a2* and *Timp1* were significantly suppressed by anti-IL17RA Ab in the liver (**Fig. 2f**). Likewise, we examined the anti-fibrotic effect of anti-IL17RA Ab in the MCD diet-induced murine model (**Fig. 3a**). Anti-IL17RA ameliorated liver fibrosis on a histologic level (**Fig. 3b–d**). Serum levels of ALT were also reduced (**Fig. 3e**). Real-time RT-PCR additionally revealed that expression levels of *Col1a2* and *Timp1* in the liver decreased by administering anti-IL17RA Ab (**Fig. 3f**).

3.3. Both IL17A and IL17F induce JUN signaling pathway in HSCs

Next, we conducted RNA-seq to find the major signaling pathway in the response of LX-2 to IL17A/F. RNA-seq detected 34,657 transcripts in LX-2 cells treated with IL17A or IL17F (**Fig. 4a**), which revealed 1,703 DEGs in total (**Supplementary Table 2**). While most of the DEGs were induced only by IL17A, 40 genes were common between IL17A and IL17F. Of note, there were 28 genes modulated only by IL17F (**Fig. 4b**). Enrichment analysis in PaGenBase revealed that genes specific to the smooth muscle were enriched within both IL17A- and IL17F-associated DEGs (**Fig. 4c and Supplementary Table 3**), suggesting myofibroblastic transformation of HSCs with IL17A/F stimulation. In addition, transcriptional network analysis by TRRUST demonstrated that genes regulated by JUN were primarily enriched within both IL17A- and IL17F-induced DEGs (**Fig. 4d and Supplementary Table 4**). We also conducted western blotting to certify activation of JUN pathway in LX-2 stimulated with IL17A or IL17F, which demonstrated phosphorylation of JNK, an upstream regulator of JUN, in a time-dependent manner (**Fig. 4e**). We also showed that phosphorylation of JNK induced by IL17A/F is reduced by BRO (**Fig. 4f**), which certified modulation of JUN pathway by IL17A/F via IL17RA.

3.4. IL17A/F enhances pro-fibrotic gene expression with a TGF β -dependent manner in HSCs

We performed flow cytometry to analyze the effect of IL17A/F upon the cell-surface expression levels of TGF β RII in LX-2 cells (**Fig. 5a–b**). As a result, both IL17A and IL17F enhanced the expression level of TGF β RII with statistical significance ($P < 0.05$). Moreover, treatment with SP600125, a JNK inhibitor, diminished the induction of TGF β RII by IL17A and IL17F ($P < 0.05$). Real-time RT-PCR also showed that co-stimulation of IL17A or IL17F with TGF β induced gene expression of *COL1A2* and *TIMP1* in LX-2 cells with a JNK-dependent fashion, whereas IL17A, IL17F, or TGF β alone did not enhance expression of those genes (**Fig. 5c**). The results of western blotting were consistent with the findings by real-time RT-PCR (**Fig. 5d**), and additionally showed that both IL17A and IL17F increase the amount of SMAD2/3, transcriptions induced by TGF β in the nucleus (**Fig. 5e**). ChIP assay confirmed that SMAD2/3 were recruited in the promoter regions of *COL1A2* and *TIMP1* costimulated by IL17A/F and TGF β (**Fig. 5f**). These results suggest that IL-17A and IL-17F each enhance the SMAD2/3-mediated fibrosis-promoting effects of TGF β by increasing the expression of TGF β RII in LX-2 cells, and at the same time, either IL-17A or IL-17F is required for TGF β to exert its fibrosis-promoting effects.

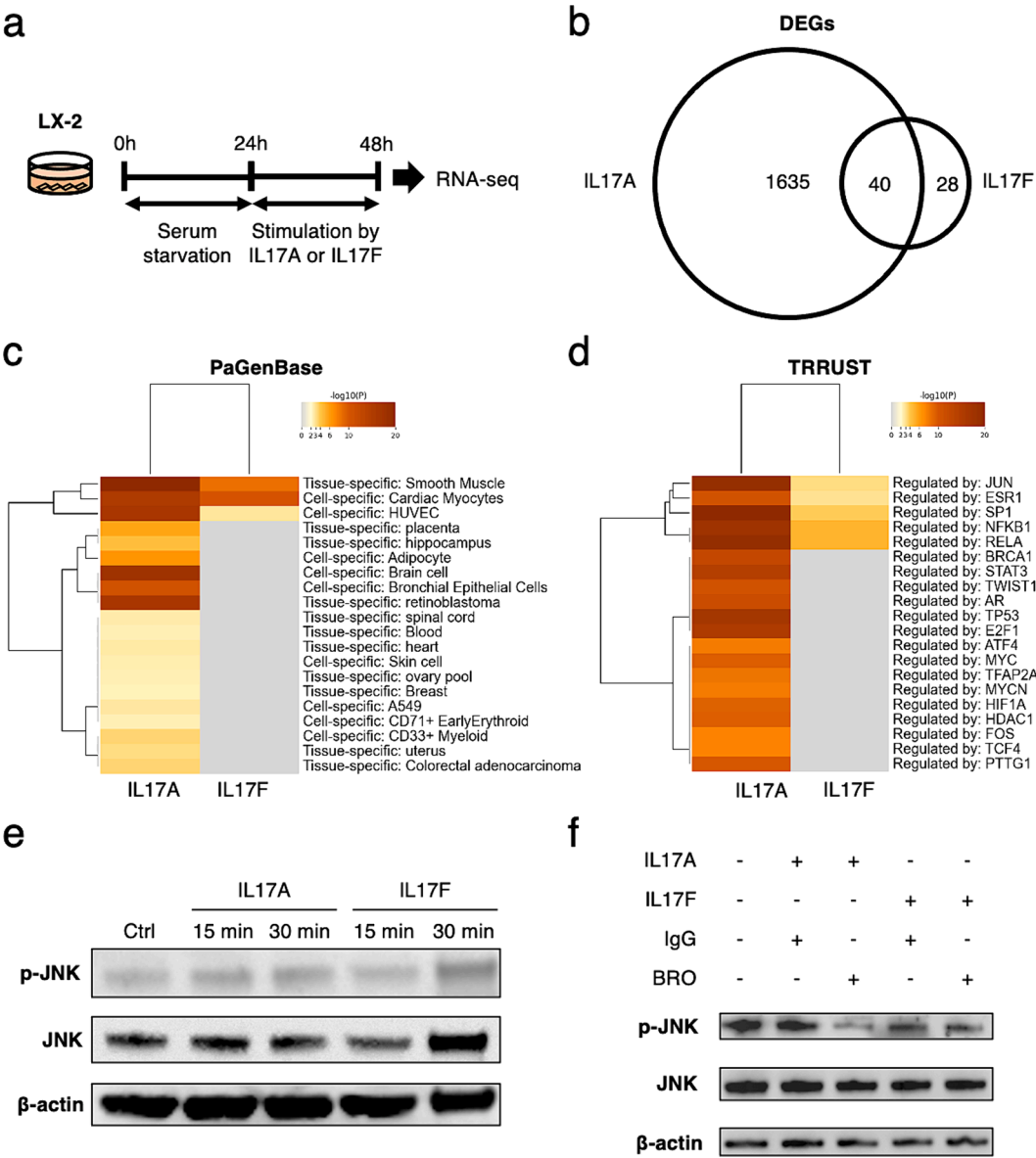


Fig. 4. Enrichment of the JUN pathway in human hepatic stellate cells treated with IL17A and IL17F. (a) Scheme of the experiment design. (b) A Venn diagram showing the inclusion relationship between IL17A- and IL17F-modulated differentially expressed genes (DEGs). (c) A heatmap representing the result of the disease association analysis according to the PaGenBase database for IL17A- and IL17F-modulated DEGs. (d) A heatmap illustrating the result of the transcriptional network analysis based on the TRRUST database for IL17A- and IL17F-modulated DEGs. (e) Western blots showing IL17A or IL17F-induced phosphorylation of JUN in LX-2 cells with a time-dependent fashion. (f) Western blots demonstrating the canceling of JUN phosphorylation induced by IL17A or IL17F with Brodalumab (BRO) but not with control IgG.

3.5. Blockade of IL17RA reverses activation of pro-fibrotic gene expression

Finally, we treated LX-2 cells stimulated by IL17A or IL17F by BRO, to investigate the mechanism of its anti-fibrotic effect. Flow cytometry demonstrated that BRO reduces the expression level of TGFβRII on the cell surface of LX-2 induced by IL17A or IL17F ($P < 0.05$; Fig. 6a-b). In addition, real-time RT-PCR and western blotting showed that enhanced expression of COL1A2 and TIMP1 mRNA and production of COL1A2 and TIMP1 in LX-2 by co-stimulation of IL17A or IL17F with TGFβ was cancelled by BRO treatment (Fig. 6c-d). Thus, amelioration of liver fibrosis by BRO seemed to be attributable to inhibition of TGFβ-dependent pro-fibrotic process by down-regulation of cell surface expression of TGFβRII in HSCs.

4. Discussion

In the current study, clinical observation of psoriatic patients revealed the superiority of IL17RA blockade compared to blocking IL17A alone against liver fibrosis (Table 1 and Fig. 1). We also demonstrated that anti-IL17RA Ab ameliorates liver fibrosis in two distinct murine models (Figs. 2 and 3). Moreover, we showed that IL17F as well as IL17A induces TGFβ-dependent pro-fibrotic gene expression via enhancing cell-surface expression of TGFβRII and nuclear translocation of SMAD2/3 in human HSCs through the JUN pathway, which could be modulated by blocking IL17RA with BRO (Figs. 4, 5 and 6). Collectively, our data suggest that not only IL17A but also IL17F plays a pro-fibrotic role in HSCs via the JUN pathway and TGFβRII-SMAD2/3 axis, and blocking both IL17A and IL17F by anti-IL17RA Ab is a promising strategy for treating liver fibrosis (Fig. 7).

We have demonstrated the therapeutic potential of anti-IL17RA

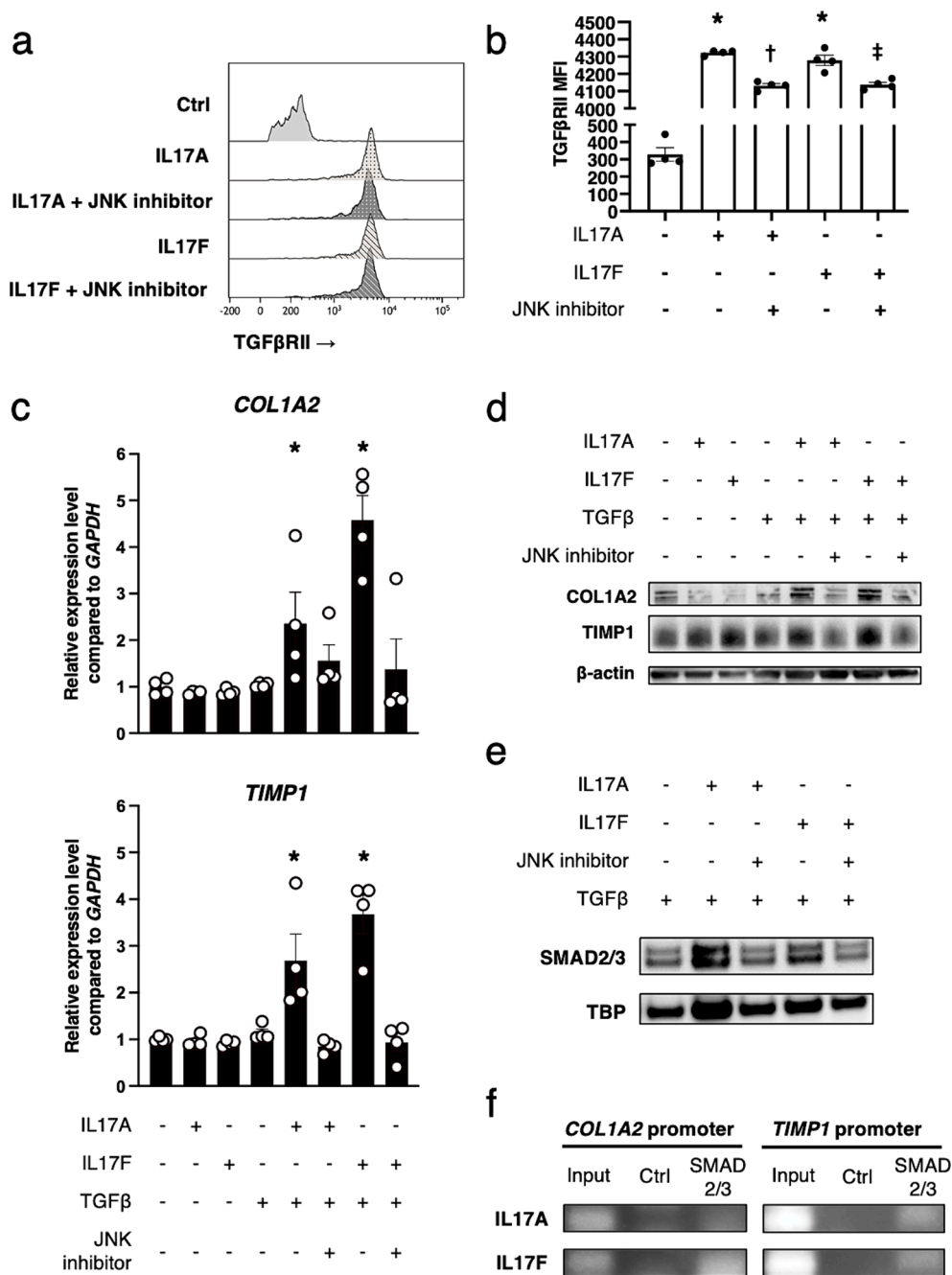


Fig. 5. Enhancement of pro-fibrotic gene expression by IL17A and IL17F in a TGFβ-dependent manner in human hepatic stellate cells. (a) Representative histogram of TGFβRII cell-surface expression induced by IL17A or IL17F. (b) Fold change of mean fluorescent intensity (MFI) compared to the negative control. (c) Fold change of mRNA levels of *COL1A2* and *TIMP1* compared to *GAPDH* measured by real-time RT-PCR. (d) Western blots showing protein levels of *COL1A2* and *TIMP1*. (e) Western blots evaluating the amount of SMAD2/3 in the nucleus. (f) Chromatic immunoprecipitation (ChIP) analysis demonstrating the recruitment of SMAD2/3 in the promoter regions of *COL1A2* and *TIMP1* after IL17A/F stimulation for 24 h followed by TGFβ treatment for 15 min. *P* values are calculated by Mann Whitney's *U* test. *: *P* < 0.01 between non-stimulated controls. †: *P* < 0.01 between samples treated with IL17A and TGFβ. ‡: *P* < 0.01 between samples stimulated by IL17F and TGFβ. Error bars indicate standard error of the mean.

antibody in ameliorating liver fibrosis using two murine models: a chemical-based model utilizing CCL₄ and a diet-based model induced by a MCD diet (Figs. 2 and 3). CCL₄ is widely employed as a hepatotoxin to induce liver fibrosis in mouse models [40]. Its biotransformation by CYP450 enzymes generates reactive metabolites, [41] leading to centrilobular hepatocyte necrosis and response of Kupffer cells, [42] ultimately triggering HSC activation [43]. Meanwhile, the MCD diet is frequently used to study NAFLD in mice. While this model may lack certain key features of human NAFLD, such as obesity and peripheral insulin resistance, it can still induce Kupffer cell response followed by

HSC activation, [44] similar to the CCL₄-induced model. The observed therapeutic effect of anti-IL17RA antibody in both of these distinct models highlights the potential benefits of IL17RA-targeted therapy in addressing fibrotic processes involved in various chronic liver diseases. Our insights are consistent with previous reports that showed mice lacking IL17A, IL17F, or IL17RA were protected from liver fibrosis [45]. The present study added pre-clinical evidence that IL17RA-targeted therapy could be applicable to humans by anti-IL17RA monoclonal antibodies including BRO. This concept was also supported by our retrospective observation of psoriasis patients, in which BRO ameliorated

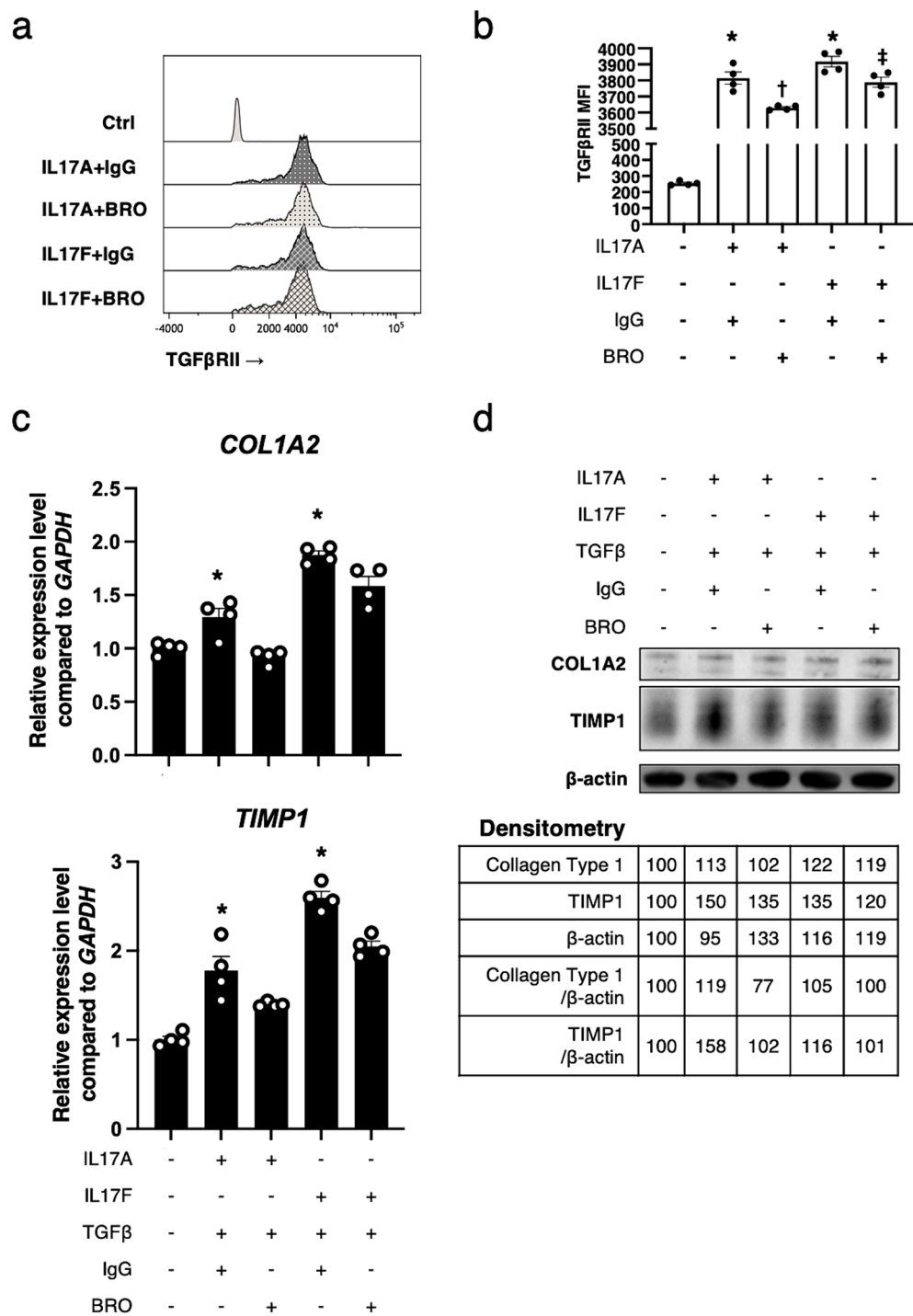


Fig. 6. Effect of Brodalumab (BRO) upon human hepatic stellate cells. (a) Representative histogram of TGFβRII cell-surface expression treated with IL17A or IL17F and with control IgG or BRO. (b) Fold change of mean fluorescent intensity (MFI) compared to the negative control. (c) Fold change of mRNA levels of *COL1A2* and *TIMP1* compared to *GAPDH* measured by real-time RT-PCR. (d) Western blots showing protein levels of *COL1A2* and *TIMP1*. The table below describes the results of densitometry analysis. *P* values are calculated by Mann Whitney's *U* test. *: *P* < 0.01 between non-stimulated controls. †: *P* < 0.01 between samples treated with IL17A and control IgG. ‡: *P* < 0.01 between samples stimulated by IL17F and control IgG. Error bars indicate standard error of the mean.

liver fibrosis compared to treatment without biologics, or secukinumab, which only neutralizes IL17A but not IL17F (Fig. 1). Therefore, dual blockade of IL17A and IL17F would also be a promising therapeutic strategy against cirrhosis.

The contribution of both IL17A and IL17F to liver fibrosis via the JUN pathway was further favored by our molecular biological studies *in vitro* (Figs. 4, 5, and 6). We focused on the JUN pathway for three reasons. First, JNK is one of the major molecules responsible for

intracellular signaling downstream of the IL17A/RC complex. Second, a previous study has shown that IL17A promotes pro-fibrotic gene expression via enhancing cell-surface expression of TGFβRII in human HSCs in a JNK-dependent manner [19]. Third, our transcriptome analysis highlighted activation of the JUN pathway as a major pathway in the cellular response to IL17A or IL17F (Fig. 3). We further endorsed the contribution of both IL17A and IL17F in combination with TGFβ to pro-fibrotic process by demonstrating nuclear translocation of SMAD2/3

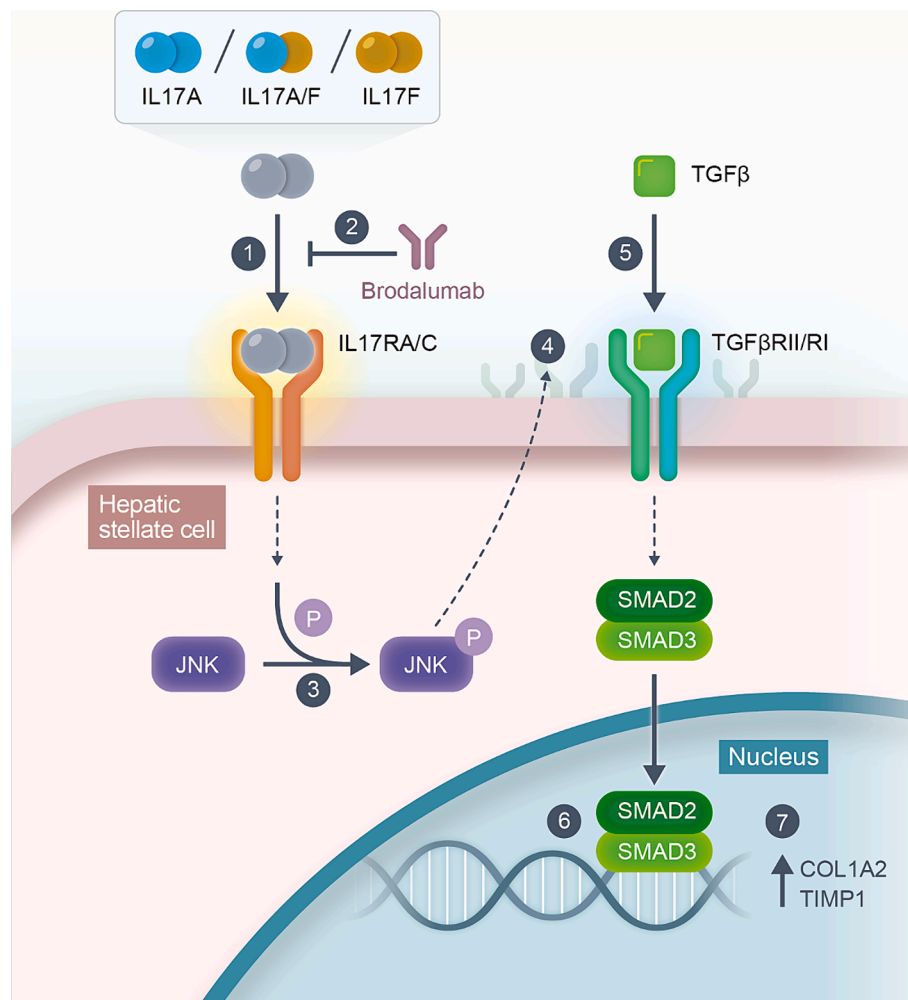


Fig. 7. Molecular mechanism of IL17A and IL17F-induced liver fibrosis. IL17RA: IL17 receptor A. IL17RC: IL17 receptor C. TGFβ: transforming growth factor-β. TGFβRII: TGFβ receptor II. JNK: Jun amino terminal kinase. ① IL17A homodimer, IL17F homodimer and IL17A/F heterodimer bind to IL17RA/RC heterodimer. ② Brodalumab inhibits ①. ③ JNK undergoes phosphorylation downstream of IL17RA. ④ Cell surface expression of TGFβRII increases. ⑤ TGFβ binds to TGFβRII/RI heterodimer. ⑥ SMAD2/3 heterodimers migrate into the nucleus and bind to DNA. ⑦ Gene expression of COL1A2/TIMP1 is induced.

and their recruit to the promoter regions of *COL1A2* and *TIMP1* (Fig. 4), although verification is needed to determine whether TGFβ, IL17A, or IL17F alone can translocate SMAD2/3 into the nucleus in HSCs.

Shared pathophysiologic roles between IL17A and IL17F is reasonable considering that IL17A and IL17F share their cognate receptors; IL17A homodimers, IL17F homodimers, and IL17A/F heterodimers can all bind to the IL17RA/C complex and trigger the same intracellular signaling. Meanwhile, it should be noted that there were a small number of DEGs modulated only by IL17F but not by IL17A (Fig. 4b and Supplementary Table 2), which suggests that IL17A and IL17F have slightly different effects on HSCs and further emphasizes the importance of the dual blockade of IL17A and IL17F. Interestingly, our transcriptional network analysis revealed that JUN pathway was most enriched in IL17A-induced DEGs, while NFκB pathway was more overrepresented in IL17F-modulated DEGs (Fig. 4d and Supplementary Table 4), indicating that the relative activation degree of the JUN pathway and the NFκB pathway is different between IL17A and IL17F. This may be explained by the relative expression levels between IL17RA and IL17RC, [46] or IL17A-independent signaling through homodimeric complex formation of IL17F and IL17RC [47]. Further investigation, including western blotting analysis to investigate the phosphorylation cascades in HSCs when stimulated by IL17A or IL17F, is required to understand the relationship between IL17A and IL17F in the context of liver fibrosis, or other fibrotic disorders such as systemic sclerosis [48].

The limitations of our study are as follows. First, our insights from human subjects were derived from small-scale, single-center, and retrospective cohort involving patients exclusively with psoriasis, and based solely on the FIB4-index, a surrogate marker for liver fibrosis. To address potential biases, it is crucial to conduct larger, multi-center, prospective studies encompassing a wider range of subjects and utilizing more reliable assessment methods such as ultrasound elastography and liver biopsy. In addition, the appropriate sample size for subject patients should be determined through a power analysis, taking into account the estimated effect size of brodalumab obtained from the current study. This will enable a more comprehensive and solid examination of the efficacy of anti-IL17RA antibodies in treating liver fibrosis. Second, our investigation only focused on HSCs, and ignored other cell components involved in the pathogenesis of liver fibrosis and are targeted by IL17, such as the Kupffer cells or the neutrophils [49 50]. Third, we had limited access to clinical specimens of the liver, so the results obtained with the immortalized cell line LX-2 could not be validated in primary human HSCs. Moreover, our current study is limited by the lack of certification *in vivo* regarding the involvement of the JUN pathway in the pathophysiology of liver fibrosis and the efficacy of the anti-IL17RA antibody. Fourth, the molecular mechanism of how the JUN pathway enhances cell surface expression of TGFβRII HSCs remains unclear. One hypothesis is the involvement of Rab proteins, small molecule GTPases, which play roles in maintaining the homeostasis of membranous

proteins including TGF β RII through endocytic recycling [51]. This idea derives from the fact that they interact with IL17RC in an IL17A-dependent manner, [52] and coordinate with JNK-interacting proteins in trafficking intracellular vesicles containing membranous proteins [53]. Investigation of the association between Rab proteins and profibrotic processes including liver fibrosis would be an attractive research subject in future studies.

In conclusion, we demonstrated that IL17A and IL17F share profibrotic effects via the JUN pathway in a TGF β -dependent manner upon human HSCs *in vitro*, and moreover, anti-IL17RA monoclonal antibodies ameliorate liver fibrosis in mice and in humans *in vivo*. These insights suggest IL17A-targeted therapy as a promising strategy against liver fibrosis, one of the difficult-to-treat disorders to date, which would fulfill unmet medical needs if made practical in clinical settings. The molecular mechanism of the JUN pathway-dependent recruitment of TGF β RII, the difference between IL17A and IL17F in their roles in profibrotic processes, and moreover, efficacy and safety of anti-IL17RA monoclonal antibodies should be further investigated in subsequent studies.

CRedit authorship contribution statement

Kazuki M. Matsuda: Writing – original draft, Resources, Project administration, Methodology, Investigation, Data curation. **Hirohito Kotani:** Resources, Data curation. **Teruyoshi Hisamoto:** Resources, Data curation. **Ai Kuzumi:** Resources, Data curation. **Takemichi Fukasawa:** Resources, Data curation. **Asako Yoshizaki-Ogawa:** Resources, Data curation. **Shinichi Sato:** Conceptualization. **Ayumi Yoshizaki:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Kazuki M. Matsuda reports financial support was provided by Okinaka Memorial Institute for Medical Research. If there are other authors, they 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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Data Transparency Statement

RNA sequence data reported are available in the DDBJ Sequenced Read Archive under the accession numbers DRR446388-DRR446393. Other datasets are available from the corresponding authors upon reasonable request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cyto.2024.156587>.

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