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Research Paper

IL-4 Receptor Alpha Signaling through Macrophages Differentially Regulates Liver Fibrosis Progression and Reversal

Shih-Yen Wenga,b, Xiaoyu Wanga,b, Santosh Vijayan a,b, Yilang Tang a,b,c, Yong Ook Kim a,b, Kornelius Padberg a,b, Tommy Regen b,c, Olena Molokanovaa,b, Tao Chen a,b, Tobias Bopp b,d, Hansjörg Schildd b,d, Frank Brombacher e, Jeff R. Crosby, Michael L. McCalebf, Ari Waismanb,c, Ernesto Bockampa,b,g, Detlef Schuppan a,b,g, Tobias Bopp d,e, Hansjörg Schild b,d, Frank Brombacher e, Detlef Schuppan a,b,g.

a Institute of Translational Immunology, University Medical Center, Johannes Gutenberg University, Mainz, Germany
b Research Center for Immunotherapy (FZI), University Medical Center, Johannes Gutenberg University, Mainz, Germany
c Institute for Molecular Medicine, University Medical Center, Johannes Gutenberg University, Mainz, Germany
d Institute for Immunology, University Medical Center, Johannes Gutenberg University, Mainz, Germany
e International Center for Genetic Engineering and Biotechnology, Institute of Infectious Disease and Molecular Medicine, South African Medical Research Council, Cape Town, South Africa
f Ionis Pharmaceuticals, Carlsbad, CA, United States
g Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, United States

Abstract

Chronic hepatitis leads to liver fibrosis and cirrhosis. Cirrhosis is a major cause of worldwide morbidity and mortality. Macrophages play a key role in fibrosis progression and reversal. However, the signals that determine fibrogenic vs fibrolytic macrophage function remain ill defined. We studied the role of interleukin-4 receptor α (IL-4Rα), a potential central switch of macrophage polarization, in liver fibrosis progression and reversal. We demonstrate that inflammatory monocyte infiltration and liver fibrogenesis were suppressed in general IL-4Rα−/− as well as in macrophage-specific IL-4Rα−/− (IL-4RαmMice). Moreover, with deletion of IL-4RαΔLysM spontaneous fibrosis reversal was retarded. Results were replicated by pharmacological intervention using IL-4Rα-specific antisense oligonucleotides. Retarded resolution was linked to the loss of M2-type resident macrophages, which secreted MMP-12 through IL-4 and IL-13-mediated phospho-STAT6 activation. We conclude that IL-4Rα signaling regulates macrophage functional polarization in a context-dependent manner. Pharmacological targeting of macrophage polarization therefore requires disease stage-specific treatment strategies.

Research in Context:

1. Introduction

Chronic liver diseases can progress to cirrhosis, which is characterized by excessive accumulation of scar tissue (extracellular matrix, ECM) that is mainly composed of fibrillar collagens, glycoproteins and proteoglycans and that leads to severe distortion of the liver vascular architecture (Schuppan and Afshar, 2008). While activated myofibroblasts and hepatic stellate cells (HSC) are the major producers of the fibrotic scar, their fibrogenic activation and proliferation depends on a complex interplay with other resident or recruited cells and their secreted factors. Here, immune cells, which promote or attenuate fibrogenesis, have become targets of antifibrotic treatments (Friedman et al., 2013; Schuppan and Kim, 2013; Trautwein et al., 2015). Importantly, recent studies of highly...
effective antiviral therapies in hepatitis B or C patients proved cirrhosis to be a reversible condition, but the detailed mechanism underlying reversal remain unclear (Chang et al., 2010; D’Ambrosio et al., 2012; Marcellin et al., 2013).

As precursors of macrophages and dendritic cells, blood monocytes are recruited to sites of injury by cytokines such as CCL2, CCL3 and CCL6, which are abundant in patients with chronic liver diseases and cirrhosis (Karlmark et al., 2009; Marra et al., 1998; Shimizu et al., 2001). In advanced CCl4-induced liver fibrosis, there is a massive recruitment of CCL2/CCR2-guided monocytes that develop into CD11b+ F4/80+ Ly-6C° mononcytic macrophages (Karlmark et al., 2009). These infiltrating cells produce cytokines and chemokines with potent pro-inflammatory or direct fibrogenic effects on HSC and (myo)fibroblasts, such as IL-1β, CCL2, CCL5, CCL7, CCL8, CXCL4, TNFα, TGFβ, and PDGF-BB (Barron and Wynn, 2011; Mehal and Schuppan, 2015; Ramachandran et al., 2012). With cessation of the damaging stimulus and by yet ill-defined mechanisms, these cells can undergo a shift towards an anti-inflammatory and fibrolytic resident macrophage phenotype.

Resident macrophages may originate from Ly-6C° CCR2− monocytids and have the potential to transform into classical (M1) or alternatively (M2) polarized macrophages in vivo (Eagawa et al., 2013). M1- and M2-type polarization may be induced in vitro by cytokines that also favor T helper (Th)1 vs Th2 cell polarization, i.e., by IFNγ and IL-12 vs IL-4 and IL-13, respectively (Jagun et al., 2013). M1-type macrophages are considered pro-inflammatory and potentially antifibrotic, while the role of M2-type macrophages, which can be subdivided into at least 5 subtypes, in inflammation and fibrosis is less well defined (Gundra et al., 2017). The various M2-subtypes may exhibit either pro- or anti-fibrotic activity (Borthwick et al., 2016; Gordon and Martinez, 2010; Murray et al., 2014; Sica et al., 2014). This and other observations suggest that the overall functional relevance of the classical M1 and M2 paradigm is limited (Murray et al., 2014). Therefore, also the utility of therapeutic approaches using classical Th1/M1 vs Th2/M2 polarizing cytokines (Barron and Wynn, 2011; Duffield et al., 2013) remains unclear.

IL-4Rα that responds to both IL-4 and IL-13 should represent a central switch for M1- towards M2-type macrophage polarization. Since its cell specific function in inflammation and fibrosis has not been clearly investigated, we extensively characterized its role during liver fibrosis progression and regression, including its myeloid cell-specific deletion and therapeutic inhibition. We show that macrophage IL-4Rα regulates fibrosis progression and reversal discordantly through modulation of macrophage subsets, indicating that antifibrotic therapies targeting IL-4Rα require disease stage-specific pharmacological intervention.

2. Materials and Methods

2.1. Mice

Il4ra−/−, Il4/Il13 double knockout (DKO), Il4rafl/fl and LysMCre mice were on a Balb/c background (Herbert et al., 2004; McKenzie et al., 1999; Mohrs et al., 1999). Balb/c wild type mice were obtained from Janvier (Saint Berthevin Cedex, France). For the generation of Il4raΔCD4 mice (CD4CreIl4rafl/fl) with T cell-specific deletion of IL-4Rα, CD4Cre mice were mated with Il4rafl/fl mice. To generate Il4raΔmyelM mice (LysMCreIl4rafl/fl−) with myeloid-specific deletion of IL-4Rα, LysMCre, Il4rafl/fl−, mice were bred with Il4rafl/fl mice. Littermates of LysMCreIl4rafl/fl− and Il4rafl/fl were used as controls in all related studies. All mice were housed under specific pathogen-free conditions. All experiments were performed with female 8- to 16-week-old mice and approved by the ethical committee of the Government of Rhineland Palatinate under the reference number 2317707/G12-1-007.

2.2. Fibrosis Models

Escalating CCL4 doses (0.875 mL/kg; week 1–3, 1.75 mL/kg; week 4–6, 2.5 mL/kg) were given via oral gavage three times per week during 6 weeks as described (Popov et al., 2011), to induce a primarily parenchymal liver fibrosis. Fibrosis reversal was examined at 1, 2 or 4 weeks after the last CCL4 treatment. For therapeutic intervention a 3 week CCL4-regimen was chosen. Diet-induced liver fibrosis, resembling human nonalcoholic steatohepatitis, was induced in 8-week old male mice that were fed a methionine and choline deficient (MCD) diet for 8 weeks. Mice on a methionine and choline sufficient diet (MCS) served as controls (Wang et al., 2018).

2.3. In Vivo and In Vitro Application of Antisense Oligonucleotides (ASOs)

All 2nd-generation chimeric antisense nucleotides (ASOs) were produced by Ionis Pharmaceuticals (Calsbad, CA) with base modifications as described (Seth et al., 2008). To enhance binding affinity and avoid degradation by nucleases, the phosphorothioate backbone nucleotides were synthesized with 2′-O-methoxymethyl (MOE) or locked 2′-O-((S)-ethylenylidene) (cEt) modifications in the ribose backbone (3′–5′ bases at both ends), while the internal 10 nucleotides remained unchanged. ASOs were administered 3 times per week (40 mg/kg in a volume of 10 μL PBS per g BW) by intraperitoneal injection. In short-term progression experiments (CCL4 for 3 weeks) mice were treated with ASOs during the 3 weeks. Alternatively, ASO treatment was started off CCL4 for one week to assess efficacy during fibrosis regression. ASO sequences were as follows: Control ASO (Ionis 141923): 5′-CCTTCCCTGAAGGTTCCTCC-3′; IL-4Rα ASO1 (Ionis 629123): 5′-AGTAGGTAGGACAACCA-3′; IL-4Rα ASO2 (Ionis 231894): 5′-CCGGTCTTCACAGGTGACAT-3′.

2.4. Hydroxyproline (Hyp) Determination

300 mg liver tissue (150 mg from the right and 150 mg from the left liver lobe) was lyzed in 5 mL HCl (6 N) at 110 °C for 16 h as previously described (Popov et al., 2005). Briefly, the 5 μL of hydrogenated sediment and Hyp standards (5 points of serial dilutions) were incubated with 50 μL citrate acetae buffer (5% citric acid, 7.24% sodium acetate, 3.4% NaOH and 1.2% glacial acetic acid, pH 6.0) and 100 μL of chloramine-T solution (14.1 mg/mL chloramine T hydrate, 10% n-propanol, 80% citrate acetae buffer) for 30 min at room temperature. Then 100 μL of Ehrlich’s reagent (0.17 g/mL 4-dimethylaminobenzaldehyde dissolved in 20.7% perchloric acid and 70.5% n-propanol) were added to the mixture and incubated at 65 °C for 30 min. The reagents were measured for absorbance at 550 nm. Hyp concentrations in liver tissue were determined by their absorbance relative to the standards after subtraction of the reaction after and before Ehrlich’s reagent incubation.

2.5. Quantitative PCR

Liver samples (100 mg; 50 mg from the right and 50 mg from the left liver lobe) were lysed in Trizol (Invitrogen, Carlsbad, CA) and 500 ng total RNA was used for reverse transcription (Quanta Bio, Gaithersburg, MD). cDNAs were subjected to qPCR using either the Taqman or SYBR green technology (Applied Biosystems, Foster City, CA) and run on a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA). Gene expression was normalized to expression of Gapdh. Primers and probes are specified in the Supplemental Information.

2.6. Histology and Immunohistochemistry

Liver tissue was fixed in 4% Roth-Histofix (Carl Roth, Karlsruhe, Germany), dehydrated, embedded in paraffin, sectioned and rehydrated as described (Popov et al., 2005). For Sirius Red staining, rehydrated sections were exposed to 0.1% Direct Red (Sigma-Aldrich, St Louis, MO) in saturated picric acid for 30 min. Slides were examined under the microscope using a 100× magnification and in each case 10 random fields
were analyzed using the ImageJ software package (National Institute of Health, Bethesda, MD).

For immunohistochemistry, rehydrated sections were boiled in 10 mM sodium citrate buffer for 10 min. Endogenous peroxidase was quenched using 3% H2O2 in PBS and sections were blocked with 5% goat or horse serum for 1 h, incubated for 1 h with the primary antibody at 4 °C and finally exposed to either a biotinylated or a fluorochrome-conjugated secondary antibody for 1 h at room temperature. The biotin signal was amplified with the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA). Sections were developed with dianino-benzidine, counterstained with Hematoxylin and mounted (Roti-Histokit II, Carl Roth, Karlsruhe, Germany). Fluorescent sections were treated with DAPI (Sigma-Aldrich, St Louis, MO) and mounted using Fluoroshield (Sigma-Aldrich, MO). Primary antibodies were directed to α-SMA (Abcam, Cambridge, UK), Ym1 (Stemcell Technologies, Vancouver, Canada), CD68 (Biorad, Hercules, CA), CD206 (Biolegend, San Diego, CA) and Ly-6G (BD Bioscience, San Jose, CA). Secondary antibodies were either biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) or Alexa Fluor 488 donkey anti-goat antibodies (Invitrogen, Carlsbad, CA).

2.7. Isolation of Hepatic Non-parenchymal Cells

Livers were minced and cells treated with collagenase buffer (0.4% collagenase type IV, Sigma-Aldrich, St Louis, MO), 154 mM NaCl, 5.6 mM KCl, 5.5 mM glucose, 20.1 mM HEPES, 25 mM NaHCO3, 2 mM CaCl2, 2 mM MgCl2, 1.6 nM DNase I (Applichem, Darmstadt, Germany) (pH 7.4) and dispensed with a gentleMACS dissociator (Miltenyi Biotec, Bergisch-Gladbach, Germany). Homogenates were incubated at 37 °C for 30 min, passed through a 100 μm cell strainer (BD Bioscience, San Jose, CA) and centrifuged at 21 × g for 4 min in ice cold PEB buffer (PBS, 2 mM EDTA, 0.5% BSA). Supernatants were centrifuged at 300 × g for 10 min and cell pellets were resuspended in PEB buffer. Red blood cells were lysed by adding 10 volumes of 150 mM NH4Cl, 10 mM KHCO3, 1 mM EDTA-2Na. Non-lysed immune cells were washed twice and suspended in PEB buffer.

2.8. Isolation of Splenic CD4 T Cells

Spleens were harvested, passed through a 70 μm cell strainer (BD Bioscience, San Jose, CA) and centrifuged at 300 × g for 10 min in ice cold PEB buffer. Pellets were resuspended in PEB and incubated with CD4 microbeads at 4 °C for 15 min. Labelled cells were washed, centrifuged, resuspended in PEB and separated using MACS columns (Miltenyi Biotec, Bergisch-Gladbach, Germany).

2.9. Flow Cytometry

Non-parenchymal liver cells were blocked with the 2.4G2 anti-Fc receptor antibody (BD Bioscience, San Jose, CA) following by staining with antibodies recognizing CD4, CD8, CD11b, CD11c, CD19, CD45, CD86, CD90.2, F4/80, IL-4Rα, Ly-6C, Ly-6G, MHCIId (BD Bioscience, San Jose, CA; Biolegend, San Diego, CA; eBioscience, San Diego, CA). For intracellular staining, cells were fixed in Fix/Perm buffer (BD Bioscience, San Jose, CA), washed in PBS containing 2% serum and incubated in Perm/Wash buffer (BD Bioscience, San Jose, CA) with an anti-CD206 antibody serving as M2 macrophage marker (eBioscience, San Diego, CA). For intracellular detection of IL-4 and IL-13, cells were stimulated with phorbol myristate acetate (PMA, 50 ng/mL), ionomycin (500 ng/mL) and brefeldin (1 μg/mL) at 37 °C for 3 h, fixed, permeabilized as above and subsequently stained with IL-4 and IL-13 antibodies (eBioscience, San Diego, CA). For the determination of phospho-STAT6 or phospho-STAT3, cells were either stimulated with IL-4 (20 ng/mL), IL-13 (20 ng/mL) or IL-6 (100 ng/mL) at 37 °C for 30 min, fixed in 2% formaldehyde for 10 min and permeabilized with cold methanol. After permeabilization, cells were stained with anti-phospho-STAT6 or -STAT3 (BD Bioscience, San Jose, CA). Cell acquisition was performed on a FACS Canto II (BD Bioscience, San Jose, CA) and analyzed with the FLOWJO software (TreeStar, Ashland, OR). Gating Strategy is demonstrated in Fig. S2. Detailed information on all antibodies is listed in Supplemental Information.

2.10. Cell Culture and Transfection

RAW264.7 murine macrophages (ATCC TIB-71) were seeded in 12-well plates (105 per well). After 6 h, cells were transfected with IL-4Rx-specific or control ASOs using the jetPEI-Macrophage DNA transfection reagent (Polyplus, Illkirch, France). Sixteen hours after transfection, cells were harvested, lysed in Trizol (Invitrogen, Carlsbad, CA) and subjected to qPCR analysis. For the isolation of peritoneal macrophages, mice were euthanized and i.p. injected with 10 mL DMEM/F12 medium. After a short soft massage of the abdominal area, a small incision was made to recover the injected medium. Cells were isolated by centrifugation and plated in DMEM medium (supplemented with 10% (v/v) fetal bovine serum, 10 mM l-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin). Nonadherent cells were removed 24 h later, and cells were used for further experiments. For the isolation of bone marrow-derived macrophages, mice were euthanized, and femurs and tibias removed. A small cut was made on both ends of the bones, and a 25-G needle attached to a syringe was inserted into the cavity to flush out the bone marrow. The bone marrow cells were centrifuged and then plated in differentiation RPMI-1640 medium (supplemented with 10% (v/v) fetal bovine serum, 10 mM l-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin) containing M-CSF (25 ng/mL). Medium was changed every 2–3 days, and cells were well differentiated after one week. For in vitro M1 polarization, cells were treated with lipopolysaccharide (LPS, 1 μg/mL, Sigma-Aldrich, St Louis, MO) and IFN-γ (20 ng/mL, BD Bioscience, San Jose, CA) for 24 h; for M2 polarization, IL-4 (20 ng/mL, BD Bioscience, San Jose, CA) and IL-13 (20 ng/mL, BD Bioscience, San Jose, CA) were applied for 24 h. Human monocyte-derived U937 cells were activated by PMA, 100 ng/mL for 48 h, to generate nonpolarized (Mo) macrophages and left untreated or polarized towards M1 using 100 ng/mL LPS and 20 ng/mL IL-13, or towards M2 using hIL-4 20 ng/mL and 20 ng/mL hIL-13 (PeproTech, Rocky Hill, NJ). RNA was extracted, reverse transcribed and analyzed by qPCR.

2.11. Hydrodynamic Transfection

MMP-12 CDNA was cloned into the pcDNA3 vector to generate pcDNA3-MMP12. The plasmids were purified using the QIAGEN Plasmid Maxi Kit (Qiagen, Hilden, Germany). Expression of MMP-12 was assessed by SDS-PAGE and Western blotting (not shown). Mice were treated for 6 weeks with CCl4. At day 3 after the last CCl4 injection wildtype mice were i.e. injected once with plasmid DNA (pcDNA3 empty vector or pcDNA3-MMP12, 100μg/20 g body weight) mixed with TransIT-QR Hydrodynamic Delivery Solution (Mirus, Madison, WI). Groups of three mice were sacrificed and analyzed either 48 h or 11 days after hydrodynamic injection, for assessment of in vivo transfection efficacy and for fibrosis assessment, respectively. Proteins were extracted from 100 mg liver using RIPA buffer (Cell Signaling, Danvers, MA) and cleared lysates resolved in a 4–10% polyacrylamide gel (Bio-Tris, Thermo Fischer, Waltham, MA), transferred to a PVDF membrane and detected with primary followed by secondary antibody (anti-MMP-12 1:1000, anti-tubulin 1:5000, goat and mouse secondary antibody 1:5000, all Santa Cruz Biotechnology, CA) followed by the Amersham ECL luminescence kit (GE, Chicago, IL).

2.12. Statistics

Data are shown as means ± SEM. Statistical differences were analyzed either with the 2-tailed unpaired Student’s t-test (For comparison between two groups) or one-way analysis of variance (one-way ANOVA with Newman–Keuls’ post-tests among multiple groups) using GraphPad Prism 5.0 (GraphPad software, La Jolla, CA).
3. Results

3.1. IL-4Rα Deficiency Retards Progression of Liver Fibrosis

Advanced liver fibrosis was induced in wildtype (WT) and IL-4Rα deficient (Il4ra−/−) BALB/c mice by escalating doses of oral CCl4 for 6 weeks. Mice receiving vehicle served as controls (Fig. S1A). In WT animals hepatic Il4ra transcripts increased >2-fold during progression (Fig. S1B). Morphometrical quantification of deposited collagen showed that after 6 weeks of progression Il4ra−/− mice developed significantly less fibrosis than their WT littermates (Fig. 1A). These results were confirmed by morphometrical quantification of alpha smooth muscle actin (α-SMA), by biochemical collagen (hydroxyproline, HYP) quantification and hepatic transcript levels of Col1a1 (Fig. 1B–C; Fig. S1C). Serum ALT, a surrogate marker of liver inflammation, remained near normal during progression in Il4ra−/− mice as compared to a 3-fold increase in their WT controls (Fig. 1D). Collectively, these data provide in vivo evidence for an active role of overall IL-4Rα signaling in promoting liver inflammation and liver fibrogenesis during progression.

3.2. IL-4 and IL-13 Cytokines Are Important Drivers of Liver Fibrosis Progression

Both IL-4 and IL-13 can activate IL-4Rα signaling. To investigate whether loss of IL-4 and IL-13 will affect liver fibrosis progression, Il4/Il13 double knockout (DKO) mice were subject to a 6-week course of CCl4 treatment. Histological analysis revealed reduced liver fibrosis in the Il4/Il13 DKO mice as demonstrated by Sirius Red and α-SMA staining (Fig. 1E; Fig. S1D), and by HYP determination (Fig. S1E). In line with this finding, compared to their WT controls, CCl4-treated Il4/Il13 DKO mice demonstrated a significant downregulation of serum ALT and of Col1a1, matrix metalloproteinase (Mmp-8, -12 and -13) and tissue inhibitor of MMPs (Timp-1) transcripts (Fig. S1F; Fig. 1F–G). In Il4ra−/− mice numbers of IL-4- and IL-13-producing T cells were significantly reduced (Fig. S2A, B), proving that constitutive IL-4Rα deficiency reduces absolute T cells.

3.3. IL-4Rα Loss of Function Changes Liver Macrophage Populations During Liver Fibrosis Progression

In WT mice fibrosis induced by CCl4 lead to an increase of hepatic but not splenic CD45+ immune cells in wildtype as well as Il4ra−/− mice (Fig. 2A, B). However, differences between WT and Il4ra−/− immune cells were found in the progression and early recovery phase of CCl4-induced fibrosis, and evident in the hepatic B and CD4 T cell but not the CD8 T cell population (Fig. 2C).

Since monocyte-macrophages have been implicated as important regulatory cells in liver fibrosis progression and reversal, we focused on the effect of IL-4Rα signaling in monocyteic (CD11b+ F4/80int) and resident (CD11bint F4/80hi) macrophages. FACS analysis demonstrated that IL-4Rα deletion lead to a significant decrease of resident macrophages in the vehicle treated control group (Fig. 2D). Moreover, upon CCl4 treatment the increase of pro-inflammatory Ly-6Ch+ monocyteic
macrophages was significantly attenuated in livers of Il4ra−/− mice (Fig. 2D; Fig. S3A–G).

3.4. IL-4Rα Signaling Does Not Contribute to Fibrosis in a Model of Severe Non-alcoholic Steatohepatitis

Severe hepatocyte lipoapoptosis was induced in mice receiving the MCD diet for 8 weeks. Liver collagen was increased 4-fold in wild type mice, but fibrosis was not attenuated in Il4ra−/− mice, different from mice with CCl4-induced liver fibrosis (Fig. S4A, B). Notably, there was a downregulation of Il4ra in wildtype mice on the MCD vs the control MCS diet, in contrast to mice with CCl4-induced liver fibrosis, and as expected its complete absence in the Il4ra−/− mice (Fig. S4C). Moreover, IL-4Rα deletion did not affect macrophage CD68 expression, as in the CCl4 as above model, indicating a less important role of this receptor in fibrosis progression in this model (Fig. S4C).
3.5. IL-4Rx as a Therapeutic Target for Treating Liver Fibrosis In Vivo

We used optimized antisense oligonucleotides (ASOs) to the Il4ra gene with high hepatic delivery (Kurreck et al., 2002; Lee et al., 2013) that were first tested in vitro on RAW murine macrophages to demonstrate a robust target knockdown compared to a negative control ASO (Fig. S5A). For in vivo studies we chose ASO2 for its stronger inhibition of IL-4Rx expression (Fig. S5B). ASOs were applied during CCl4-induced fibrogenesis for 3 weeks (Fig. 3A). During progression, therapy with ASO2 strongly inhibited hepatic collagen deposition (Fig. 3B) which was accompanied by a significant suppression of Col1a1, α-SMA and Tgfb-1 transcripts (Fig. 3D). Moreover, ASO2 significantly reduced the number of liver CD68+ macrophages and levels of Cd68, iNOS and Mrc1 transcripts (Fig. 3C and E). Taken together, IL-4Rx appears instrumental in increasing the influx of monocyte-macrophages.

3.6. Loss of IL-4Rx Signaling in T Cells Does Not Alleviate Toxin-induced Liver Fibrosis

IL-4Rx signaling and the associated Th2 T cell response are drivers of portal liver fibrosis induced by Schistosomal parasites (Chiaramonte et al., 1999; Pesce et al., 2009). To study the role of T cell IL-4Rx in our model of parenchymal fibrosis progression and reversal, mice with a T cell-specific IL-4Rx deletion (Il4raΔCD4) were generated and analyzed in the progression model. Quantitative PCR and flow cytometry demonstrated a significant reduction of IL-4Rx in CD4+ T cells of Il4raΔCD4 mice (Fig. S6A–B), whereas several fibrosis related readouts showed no

![Figure 3](image-url)

Fig. 3. Inhibition of IL-4Rx expression by specific antisense oligonucleotides decreases fibrosis progression. (A) Mice received 9 doses of either control oligonucleotide (C) or IL-4Rx-ASO2 during three weeks of CCl4 treatment. (B) Sirius Red and (C) CD68 (macrophage) staining in livers of mice with fibrosis progression. Bar: 200 and 50 μm, respectively (n = 4–5). (D) Relative hepatic transcript levels of key fibrosis-related genes in CCl4-treated mice receiving either the control oligonucleotide or the IL-4Rx-specific ASO2. (E) Transcript levels for macrophage marker genes (Cd68, Mrc1 and iNOS) for control oligonucleotide- and IL-4Rx-specific ASO2-treated livers during fibrosis progression (n = 5). *p < 0.05, **p < 0.01 (unpaired Student’s t-test). All data are expressed as means ± SEM. Data are representative of three independent experiments.
demonstrate that the abrogation of IL-4R was highly expression of IL-4R during progression, the number of myeloid lineage cells and their ex-
mean percentages of stained areas from 10 microscopic
5). (D) Hepatic means ± SEM. Results are representative of two independent experiments.

3.7. Myeloid Cell-Specific Deletion of IL-4Rx Attenuates Fibrosis Progression

To address the role of macrophage IL-4Rx in liver fibrosis progression, we generated mice with myeloid-specific IL-4Rx deletion (Il4ra\(^{\text{ΔLysM}}\)). When these mice were subject to 6 weeks of CCl\(_4\), total hepatic IL4Ra expression was significantly suppressed compared to control mice, with analysis shortly after the last dose of CCl\(_4\) (Fig. S6F). Comparable to our findings in general Il4ra\(^{-/-}\) mice, Sirius Red and α-SMA staining, HYP content and Coll1a1 expression showed attenuated liver fibrogenesis and decreased ALT levels in Il4ra\(^{\text{ΔLysM}}\) compared to Il4ra\(^{+/+}\) control mice (Fig. 4A–D). These results indicate that IL-4Rx signaling through myeloid but no other hepatic cells plays an important regulatory role in liver fibrosis progression.

3.8. IL-4Rx Is Specifically Deleted in Resident Macrophages in Il4ra\(^{\text{ΔLysM}}\) Mice during CCl4-induced Liver Fibrosis

A prior study showed that deletion of IL-4Rx in Il4ra\(^{\text{ΔLysM}}\) in a Schistosomiasis model was incomplete (Vannella et al., 2014). To unambiguously define the cell type that drives IL-4Rx-mediated liver fibrogenesis during progression, the number of myeloid lineage cells and their expression of IL-4Rx were investigated (Figs. S6G and S7A, B). We could demonstrate that the abrogation of IL-4Rx, as mediated by LysM-Cre, was highly efficient in resident macrophages (CD11b\(^{\text{hi}}\) F4/80\(^{\text{lo}}\)), but insignificant in neutrophils or Ly-6Ch or Ly-6Clo monocytic macrophages (CD11b\(^{\text{hi}}\) F4/80\(^{\text{hi}}\)) (Fig. S7A, B). Refined FACS analysis showed a decreased expression of MHC class II and CD86 on these resident macrophages in Il4ra\(^{\text{ΔLysM}}\) mice compared to control mice. This suggests that the attenuated liver fibrosis in IL-4Rx deleted mice may be largely due to fewer pro-inflammatory resident macrophages (Fig. S7C), but also to fewer freshly recruited monocyte-macrophages which apparently were only affected by the general IL-4Rx deletion (see Fig. 2D).

In line with these findings, qPCR and immunohistochemical analysis of livers of Il4ra\(^{\text{ΔLysM}}\) mice, with a macrophage specific IL-4Rx deletion, showed a reduction of overall macrophages (CD68\(^{+}\)), and of putative M1-, but to a lesser degree also of M2-type populations, accompanied by a decrease of Tgfb1 (profibrotic), iNos and Il1b (M1), Arg1, Mrc1 and Ym1 (M2) shortly after termination of 6 weeks of CCl\(_4\), when features of both progression and early reversal may coexist (Fig. 5A–B).

We therefore conclude that liver fibrosis progression is mediated by IL-4Rx partly through enhanced pro-inflammatory conversion of resident macrophages. There is also a reduction of M2-type macrophage markers in Il4ra\(^{\text{ΔLysM}}\) mice during progression and early reversal (3 days after cessation of CCl\(_4\)) which likely impacts the fibrogenic over the fibrolytic response.

3.9. Loss of IL-4Rx Reduces M2-type Macrophage Subsets and Retards Spontaneous Liver Fibrosis Regression

To study the role of IL-4Rx in longer-term fibrosis reversal, we used a model of spontaneous fibrosis regression (6 weeks of CCl\(_4\) followed by 2 or 4 weeks without treatment), where fibrolytic macrophages play an important role (Fig. S8A). As shown in Fig. S8B–D, approx. 30% of total liver collagen deposition had disappeared after 2 weeks off CCl\(_4\) in WT mice, whereas fibrosis resolution was retarded in Il4ra\(^{\text{ΔLysM}}\) mice (Fig. 6A). This divergent effect during reversal vs progression was further confirmed in a second experiment showing elevated Col1a1 and α-SMA transcript levels in Il4ra\(^{\text{ΔLysM}}\) vs the IL-4Rx expressing mice (Fig. 6B).

To confirm the translational relevance of these findings, we designed a pharmacological intervention using a specific IL-4Rx ASO to fibrotic WT mice during the reversal phase (Fig. 6C). Here, ASO2 significantly attenuated resolution, as shown by a relative increase of liver collagen and Col1a1 mRNA expression vs mice treated with a scrambled control ASO (Fig. 6D–E). Moreover, ASO2 significantly reduced the protein product of the Mrcl gene (CD206\(^{+}\) M2-type macrophage numbers and suppressed the M2-related genes Mrc1 and Arg1, without affecting M1-related Il1b mRNA (Fig. 6F–G).

These results consistently showed that IL-4Rx regulates liver fibrosis differently in fibrosis progression vs fibrosis reversal, and that this effect is largely mediated by macrophages and no other IL-4Rx expressing cells such as T cells or hepatocytes.

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**Fig. 4.** Myeloid IL-4Rx deficiency attenuates CCl4-induced liver fibrosis. Il4ra\(^{+/+}\) (control) and myeloid cell IL-4Rx-deleted (Il4ra\(^{\text{ΔLysM}}\)) mice were treated with CCl4 for 6 weeks. Mice were analyzed 3 days after the last dose of CCl4. (A) Representative Sirius Red (upper) and α-SMA (lower) stained sections of CCl4 treated mice. Bar: 200 μm. The diagrams on the right depict mean percentages of stained areas from 10 microscopic fields per liver (n = 5). (B) Hepatic hydroxyproline (Hyp) levels (n = 5). (C) Serum ALT levels of control and Il4ra\(^{\text{ΔLysM}}\) mice (n = 5). (D) Hepatic Coll1a1 and α-SMA transcript levels (n = 5). Transcript levels were normalized to Gapdh mRNA. *p < 0.05, **p < 0.01 (unpaired Student’s t-test). All data are expressed as means ± SEM. Results are representative of two independent experiments.
3.10. IL-4Rα Signals Through STAT6 to Induce Fibrolytic Matrix Metalloproteinase Expression in Macrophages

MMPs are instrumental in ECM remodeling and fibrosis regression. We therefore studied the expression of a panel of MMPs during early fibrosis reversal in control and Il4raΔLysM livers. In early reversal, at 24 or 72 h after CCl4 withdrawal, expression of Mmp12, 14, 16, and 20 mRNA was increased. At 72 h Mmp16 and 20, and especially Mmp12 transcripts were significantly suppressed in Il4raΔLysM compared to control livers (Fig. 7A and see also the Supplemental Information).

To study how far IL-4Rα signaling could directly potentiate the expression of Mmp12 in M2-type macrophages in vitro, peritoneal and bone marrow-derived macrophages (BMDM) from WT and Il4ra−/− mice were isolated and polarized with either IL-4 and/or IL-13 for 24 h. M2 related Mrc1 and Arg1 transcripts were up-regulated in WT but not Il4ra−/− macrophages in response to IL-4 and/or IL-13 (Fig. S9A–B), suggesting that WT but not IL4ra−/− macrophages can be differentiated into M2-type macrophages in vitro. Mmp12 transcripts were prominently induced in both primary WT but not IL4ra−/− macrophages after incubation with IL-4 and/or IL-13 (Fig. 7B–C). Moreover, human MMP-12 expression was dramatically increased in PMA-activated U937 monocytes that were treated with M2 (human IL-4 and IL-13) but not M1 cytokines (LPS and human IFNγ) (Fig. S9C). Moreover, M1-polarized (LPS and IFNγ-treated) WT and IL4ra−/− BMDM did not show significant differences in their low level of Mmp12 expression (Fig. S10A).

Phospho-STAT6 is a major downstream signal transducer for IL-4/IL-13-dependent genes (Lin et al., 1995). In hepatic macrophages lacking IL-4Rα, there was suppressed induction of phospho-STAT6 in response to IL-4 or IL-13 during fibrosis progression (Fig. 7D). In this vein, ASOs targeting the IL-4Rα and STAT6 genes significantly down-regulated Mmp12 mRNA in RAW macrophages (Fig. 7E). No direct connection was found between M1 cytokines and MMP-12 expression and stimulation of macrophages with LPS did not enhance Mmp12 expression, neither in wildtype nor in IL-4Rα deleted cells (Fig. S10A). However, we found decreased pSTAT3-activation in hepatic macrophages isolated from mice with CCl4
fibrosis that lacked IL-4Rα and which were stimulated with IL-6 (Fig. S10B).

To confirm that MMP-12 functions as an inducer of fibrolysis during fibrosis reversal, an empty vector or a vector containing Mmp12 cDNA was delivered into fibrotic livers during the reversal phase using hydrodynamic transfection (Fig. S10C). Sirius Red staining showed that Mmp12 cDNA but not the empty vector induced fibrosis resolution (Fig. 7F). These results identify MMP-12 (and to a lesser degree MMP-16 and -20) as primary bona fide mediator(s) of M2-type macrophage-mediated fibrolysis during the early phase of reversal.

In summary, inhibition of IL-4Rα expression ameliorated the progression but retarded regression of parenchymal liver fibrosis. Therefore, targeting the IL-4Rα appears to be a therapeutic option for patients with progressive liver fibrosis, but may retard resolution when the inflammatory triggers are absent.

4. Discussion

IL-4 and IL-13 and their common receptor subunit IL-4Rα play a central role in M2-type macrophage and Th2 cell polarization which are held responsible for the promotion of organ fibrosis. Thus an IL-4 neutralizing antibody or the dominant negative IL-13Rα2/IL-13Rα1 antagonist sIL-13Rα2-Fc reduced fibrosis in the mouse model of hepatic Schistosomiasis (Cheever et al., 1994; Chiamonti et al., 1999). Here we demonstrate that, unexpectedly, IL-4Rα on macrophages promotes both hepatic inflammation and fibrosis during progression, while it permits unrestricted fibrosis resolution during spontaneous reversal. Notably, we showed that other cells than macrophages (Bhattacharjee et al., 2013; Stein et al., 1992) or T cells (Cheever et al., 1994; Chiamonti et al., 1999) that can respond to IL-4 and IL-13, such as mast cells (Nilsson and Nilsson, 1995; Ochi et al., 2000), eosinophils (Hao et al., 2000; Sher et al., 1990; Tomkinson et al., 2001), (myo-)fibroblasts (Chandriani et al., 2014), and liver epithelial cells (Richter et al., 2001), did not significantly contribute to liver inflammation or fibrosis, which is strongly supported by our data demonstrating that the macrophage specific IL-4Rα-deletion (Il4ra−/−) yielded similar results as mice with the general IL-4Rα-knockout, with the exception that it did not affect the freshly recruited monocyte-macrophages, as observed in mice with the general IL-4Rα. We also demonstrate that, conversely, macrophage IL-4Rα signaling can also promote liver fibrosis regression via STAT6-dependent transcriptional activation of Mmp12, and that pharmacological inhibition of IL-4Rα recapitulated the functional effects of genetic IL-4Rα deletion.

Schistosomiasis is associated with a prominent infiltration by Th2 cells. While these cells promote fibrogenesis, they also secrete IL-4 and IL-13 that was shown to induce an anti-inflammatory and antifibrotic M2-type macrophage phenotype (Herbert et al., 2004;
Mice in the reversal group were injected with empty pcDNA3 vector (C) or pcDNA3-MMP12 (Mmp12). Bar diagrams show the quantification of phospho-STAT6 in macrophages (n = 4 per group). (B, C) Peritoneal macrophages (pMφ) or bone marrow-derived macrophages (BMDM) were either untreated or treated with IL-4 (20 ng/ml) and/or IL-13 (20 ng/ml) for 24 h. Mmp12 transcript were measured (n = 4 per group). (D) Immune cells from Il4raΔLysM control (C) and Il4raΔLysM (ΔLysM) livers were harvested at day three following CCl4 treatment and stimulated with IL-4 or IL-13 for 30 min for the detection of hepatic C1q transcripts at peak fibrosis and on days 1 and 3 of reversal. Here we could not find significant differences between control and Il4raΔLysM mice.

Our finding of a pro-inflammatory and pro-fibrogenic role of IL-4Rx signaling during fibrosis progression, in contrast to its anti-inflammatory and fibrolytic role during regression, relativates prior results with IL-4, IL-13 or IL-13Rxα in the models of pulmonary (Chandriani et al., 2014), (Schistosomal) liver (Cheever et al., 1994; Chiaramonte et al., 1999; Kaviratne et al., 2004) and intestinal fibrosis (Fichtner-Feigl et al., 2007; Fichtner-Feigl et al., 2006), models that solely represent progression. These settings are also different from human (liver) fibrosis which is characterized by foci or waves of progression and reversal, even in advanced fibrosis (Wanless et al., 2000), features that can only be represented by progression and reversal models as employed by us (Issa et al., 2004; Popov et al., 2011). Finally, parenchymal rather than portal liver fibrosis determines the degree of liver dysfunction and failure (Schuppan and Altilar, 2008). Our data also suggest that IL-4Rx signaling in macrophages may be an important regulator of inflammation and fibrosis in the prevalent cases with parenchymal as opposed to portal liver fibrosis.

Accordingly, therapeutic intervention targeting the IL-4Rx with a specific antisense oligonucleotide that intrinsically addresses the liver preferentially macrophages (Butler et al., 2000) generated the same effects as the macrophage specific (or constitutive) knockout of IL-4Rx in the fibrosis models, as also shown by correlative comparison of the change in fibrosis and the number and subtype of macrophages.

Kaplan et al., 1998; Pesce et al., 2009). This was confirmed by Il4raΔLysM mice with chronic Schistosomiasis that showed increased liver inflammation and fibrosis (Vannella et al., 2014). However, the role of these cytokines and especially of IL-4Rx signaling in non-parasitic hepatic liver disease, which is the prominent cause of liver fibrosis, had not been addressed. While the innate immune response by monocytes/macrophages and neutrophils usually precedes (T cell mediated) adaptive immunity (Campbell et al., 1981; Tacke and Zimmermann, 2014), both the choice of the in vivo model and the timing of organ damage or regeneration may determine how far Th2 cells or M2-type macrophages are primary drivers of inflammation or fibrosis. Th2 T cells are important sources of IL-4, which together with IL-13 is the major ligand for IL-4Rx. A recent paper indicated that IL-13 was produced by type 2 innate lymphoid cells (ILC-2) cells upon exposure to IL-33; however, the deletion of ILC-2 under CCl4 treatment did not completely rule out the participation of Th2 cells (McHedlidze et al., 2013). Thus we generated Il4raΔLysM knockout mouse to exclude a role of Th2 cells in the CCl4 model of parenchymal liver fibrosis, which had not been studied before. In line with the major finding of the paper by McHedlidze, the Il4raΔLysM knockout showed no difference compared to the wildtype control mice under CCl4 treatment. Another recent study showed that IL-4 drives the expression of surfactant protein SP-A in the lung and of complement component C1q in the liver and peritoneal cavity, which amplify IL-4Rx signaling in type-2 infectious models in these organs (Minutti et al., 2017). It should be of interest to study how far C1q may also be involved in our noninfectious liver fibrosis models.
Progression not only reduced the number of hepatic M2-type macrophages, and anti-inflammatory monocytes/macrophages. Free Radic. Biol. Med. 54, 1–16.


Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.01.028.

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