ANIMAL MODELS

The LIM-Only Protein Four and a Half LIM Domain Protein 2 Attenuates Development of Psoriatic Arthritis by Blocking Adam17-Mediated Tumor Necrosis Factor Release

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Accepted for publication
July 26, 2017.

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Four and a half LIM domain protein 2 (Fhl2) is an intracellular adaptor molecule with a high protein-protein interaction capacity. It acts as a modulator of several signaling molecules in the cytosol and as a cofactor of transcription in the nucleus. Recent studies suggest the role of Fhl2 in tissue repair and the anti-inflammatory response. Herein, we show that Fhl2-deficient mice develop a more severe psoriatic arthritis disease under induction of the inducible human tumor necrosis factor (*hTNF*) transgene than wild-type mice. The disease was accompanied by increased infiltration of activated macrophages and T regulatory cells in skin and digit joints as well as by increased expression of matrix metalloproteases and bone-specific proteases. The more severe pathogenesis of psoriatic arthritis in *Fhl2* knockout mice coincided with enhanced levels of soluble hTNF cytokine, but surprisingly not with transcription of the *hTNF* transgene. Studying the shedding of cell membrane-bound hTNF by Adam17, a known Fhl2 interacting protein, revealed an enhanced release of TNF in the absence of Fhl2. In summary, our results show that Fhl2 anticipates the emerging inflammation and specifically the development of psoriatic arthritis by impeding the Adam17-mediated release of TNF. (*Am J Pathol* 2017, 187: 2388–2398; <http://dx.doi.org/10.1016/j.ajpath.2017.07.015>)

Autoimmune diseases, like psoriasis or arthritis, are driven by an interplay of many biological processes, the particular activity of which is forced by differential gene expression. One of the most eminent genes is tumor necrosis factor- α (*TNF*), the pathological up-regulation of which results in severe immune effects.¹ *TNF*, in turn, affects, either positively or negatively, the activity of other genes that regulate both proinflammatory and anti-inflammatory events. Recently, we showed that prolonged stimulation of synovial fibroblasts with *TNF* down-regulates the four and a half LIM domain protein 2 (*Fhl2*),^{2,3} thereby facilitating expression of matrix metalloproteases (MMPs) and enhanced tissue destruction.⁴ This work indicated that *Fhl2* has a distinct anti-inflammatory activity. Other reports showed that *Fhl2* attenuates lung inflammation during fibrosis or asthma^{5,6} and

supports wound healing after skin, liver, or intestine injury.^{7–9} Furthermore, deletion of the *Fhl2* gene impairs inflammatory reaction and scar formation after cardiac ischemia¹⁰ and attenuates neovascularization after corneal injury.¹¹ These observations, along with data showing that *Fhl2* regulates the assembly of extracellular matrix and migration of fibroblasts and dendritic cells,^{9,12,13} prompted us to study the impact of *Fhl2* in the development of autoimmune diseases. Thus, we induced a proinflammatory reaction in *Fhl2*-deficient mice using our recently established

Supported by Coordination for the Improvement of Higher Education Personnel Brazil grant 99999.013234/2013-07 (R.L.D.) and the German Research Foundation grants SFB 492 A17 (V.W.) and SFB 1009 B08 (S.L.).
Disclosures: None declared.

doxycycline (Dox)—inducible human *TNF*-transgenic mouse line that develops a psoriatic arthritis—like phenotype under Dox stimulation,^{14,15} and then studied the inflammatory response in skin and joint tissues. Our data support the assumption that Fhl2 is an anti-inflammatory protein and further demonstrate that Fhl2 prevents the development of psoriatic arthritis by blocking the Adam17-mediated cleavage of membrane-bound TNF- α .

Materials and Methods

Animals

All animal experiments were approved by the local ethics committee and performed in strict accordance with the German regulations of the Society for Laboratory Animal Science and the European Health Law of the Federation of Laboratory Animal Science Associations. The protocols were approved by the North Rhine-Westphalia State Agency for Nature, Environment, and Consumer Protection (LANUV-NRW; Recklinghausen, Germany).

The details of inducible *ihTNFtg* mouse generation were published recently.^{14,15} These mice contained two transgenes: the modified reverse tet transactivator *rtTA2S-M2*, published by Katsantoni et al,¹⁶ and the full-length human *TNFalpha* cDNA (<https://www.ncbi.nlm.nih.gov/nucleotide/395132451>; accession number NM_000594), cloned behind the Tet-responsive P-tight promoter in the plasmid pTRE-Tight (BD Biosciences Clontech, Heidelberg, Germany). The 3'-untranslated region of *TNF* was replaced by the SV40 Poly A site, to avoid post-transcriptional regulation. The mice expressed the human TNF cytokine only after doxycycline administration. By crossing them with *Fhl2*^{-/-} mice, the *ihTNFtg/Fhl2*^{KO} animals homozygous for *rtTA2S-M2*, *hTNF* transgenes and for mutated *Fhl2* locus were obtained. To induce the expression of the *hTNFalpha* transgene, mice were provided drinking water containing 1 mg/mL doxycycline (Sigma, Taufkirchen, Germany) for 21 days. The major source of human TNF (hTNF) in these mice is keratinocytes and, to a lesser extent, macrophages and dendritic cells, but not granulocytes or lymphocytes.¹⁴

Histology and Disease Assessment

Skin and forepaw specimens were fixed in 4% paraformaldehyde for 2 hours and overnight, respectively, and after dehydration were embedded in paraffin. Paws were decalcified with 0.5 mol/L EDTA solution for approximately 3 weeks before embedding. Tissue sections (4 μ m thick) were analyzed. For X-Gal staining, paws of mice were fixed in acetone overnight at 4°C and incubated in X-Gal solution for 8 hours at 37°C. After overnight incubation in 4% paraformaldehyde, they were rapidly decalcified with 0.1 mol/L EDTA during 6 days at 42°C, dehydrated, and embedded in paraffin.¹⁷ To allow a better penetration of the X-Gal solution into the tissue, the skin of digits was cut.

Using a grading scale of 0 to 7, the psoriasis area and severity index (PASI) was determined separately for five different areas: head, neck, back, throat, and abdominal areas. Then, the severity of the affected area, including hair loss, erythema, scaling, and thickening, was considered. The sum of the five area scores (scale, 0 to 35) represented the final psoriasis area and severity index per mouse.¹⁴ Epidermal thickness was evaluated using hematoxylin and eosin-stained skin samples and AxioVision software version 4.8.2.0 (Carl Zeiss AG, Oberkochen, Germany). A total length of 1000 to 3000 μ m of each skin specimen was scored, and mean values of measurements that have been performed each 50 μ m were taken.

The extent of inflammation and cartilage damage in the paws was determined by morphometry, as described elsewhere.¹⁵ Usually, four different sections per mouse sample and five to seven mice per case were quantified. The inflamed area was defined as the infiltrated tissue area and was assessed in relation to the total tissue area [in toes, the area from the apex of a toe to the proximal tendon basis of the distal interphalangeal (DIP) joints was defined as the total area].

Disease assessments were performed in a blinded manner by R.L.D., T.S., and V.W.

Analysis of Skin and Paw Immune Microenvironment

Single-cell suspensions of skin and paws were obtained by enzymatic digestion with 3.2 mg/mL collagenase type I, 0.417 mg/mL liberase TL, and 1 mg/mL DNase I. Enzymes were added to Dulbecco's modified Eagle's medium plus 5% fetal calf serum, and tissue samples were digested at 37°C for 1.25 and 1 hour. Samples were passed through a filter cartridge to remove large pieces of tissue and washed with phosphate-buffered saline plus 5% fetal calf serum. The most antibodies used in flow cytometry analysis were from eBioscience (Frankfurt, Germany) and were routinely used at concentrations of 0.5 to 1.5 μ g/mL, as previously described.¹⁴ After cell fixation and permeabilization, intracellular staining for Foxp3 was performed using the Foxp3/Transcription Factor Staining Kit (eBioscience), according to the manufacturer's instructions.

During generation of *Fhl2*^{KO} mice, part of exon 2, including the translation initiation codon ATG, of the *Fhl2* allele was replaced with a LacZ reporter gene and a neo resistance cassette.¹⁸ Thus, in some experiments, the presence of the LacZ determining the β -galactosidase enzyme was used by us for determining FHL2-positive immune cells. Freshly isolated skin cells from *ihTNFtg/Fhl2*^{KO} mice were kept at 37°C, and 100 μ L of cell suspension in phosphate-buffered saline plus 5% fetal calf serum was mixed with 100 μ L of prewarmed 1 mmol/L fluorescein di- β -D-galactopyranoside (Sigma-Aldrich, St. Louis, MO) and incubated for 10 minutes. The number of fluorescein isothiocyanate-positive cells was verified by flow cytometry. Fluorescein di- β -D-galactopyranoside is one of the most sensitive substrates

for β -galactosidase and is hydrolyzed by the enzyme to the fluorescence product fluorescein isothiocyanate.

Quantitative Real-Time RT-PCR

Skin or forepaw samples were minced and immediately transferred into RNAlater solution. After overnight incubation at 4°C, they were homogenized and the RNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany). RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany), and the RNA was reverse transcribed into cDNA using the high-capacity cDNA reverse transcription kit from Applied Biosystems (Darmstadt, Germany). The relative levels of mRNA were determined by TaqMan quantitative real-time PCR using the LightCycler 480 II (Roche Diagnostics, Mannheim, Germany). Each cDNA sample was analyzed in triplicate, and specific signals were scored in relation to the signals of two reference transcripts (*Gapdh* and *Cycs*). The results from different experiments were normalized to the expression of a calibrator sample, which was applied as a positive control in each experiment. The primers used were assigned using the Universal ProbeLibrary Assay Design Center of Roche Diagnostics.

Determination of Soluble Cytokines

The amount of human or mouse TNF cytokines and mouse S100A8/A9 proteins in the serum of mice was analyzed by conventional enzyme-linked immunosorbent assay, as described previously.¹⁵ The concentration of IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, interferon- γ , TNF- α , and granulocyte-macrophage colony-stimulating factor in sera was measured by LUNARISTM Mouse 12-Plex Cytokine Kit384 and LUNARISTM Analysis Suite software version 1.0.1 from AYOXXA Biosystems (Cologne, Germany; catalog numbers LMC-20121S and LAS-001, respectively), and the concentration of chemokine (C-C motif) ligand (CCL) 2 was measured by LEGENDplex flow-based 13-plex mouse inflammation panel kit from Biolegend (Fell, Germany; catalog number 740150).

Inhibition of Adam17

The role of Fhl2 in ADAM17-mediated shedding of surface hTNF was studied in primary keratinocytes, as these cells express the highest amount of recombinant hTNF. Keratinocytes were isolated from untreated control *ihTNFtg* or *ihTNFtg/Fhl2^{KO}* mice. Skin specimens were incubated in 0.25% trypsin-EDTA solution as floating tissue pieces at 4°C for 11 hours. Afterward, the epidermis was separated from the dermis, cut into small pieces, and suspended in KBM-Gold medium (Lonza, Cologne, Germany; catalog number 195130). After passing through a cell strainer (70 μ m), cells were pelleted at 300 $\times$ g for 7 minutes and resuspended in Eagle's minimal essential medium (Lonza; catalog number BE06-174G). Obtained cells were incubated in 24-well plates

in 0.5 mL tissue culture medium plus 5% fetal calf serum with 10⁶ cells per well for 4 hours. To induce human TNF expression, cells were stimulated during these 4-hour incubations with 20 μ g/mL Dox. To prevent the shedding of newly synthesized TNF, keratinocytes were cultured in the presence of different concentrations of ADAM17 inhibitor TMI-1 (Tocris Bioscience, Wiesbaden-Nordenstadt, Germany; catalog number 5960).¹⁹ Subsequently, cells were resuspended, stained for surface-expressed human TNF and cytosolic pan-keratin, and analyzed by flow cytometry.

Statistical Analysis

Data are expressed as the means $\pm$ SEM. Statistical analysis was performed using either the *U*-test or the one-way analysis of variance, followed by the Newman-Keuls test. GraphPad Prism software version 7.0 (GraphPad Inc., San Diego, CA) was used.

Results

Fhl2 Deficiency Exacerbates the TNF-Mediated Psoriatic Arthritis

To elucidate the potential anti-inflammatory activity of Fhl2, homozygous *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mice were stimulated for 3 weeks with Dox to induce psoriatic arthritis. On Dox stimulation, transgenic *ihTNFtg* mice expressed high levels of human TNF, causing inflammation of the skin and toes. Particularly strongly affected tissues were the skin of the head and neck and DIP joints.^{14,15} Scoring Fhl2-deficient and wild-type mice for body weight loss during Dox stimulation (Supplemental Figure S1) or skin lesions according to psoriasis area and severity index (Figure 1A) showed significantly more enhanced disease in *ihTNFtg/Fhl2^{KO}* than in *ihTNFtg* mice. Also, histological analyses of skin (Figure 1, A and C) or DIP joint (Figure 1, B and D) sections at day 21 of Dox stimulation confirmed the more severe pathology of both skin and finger joints. The epidermal thickness of the skin and cartilage alteration of DIP joints were significantly enhanced in *ihTNFtg/Fhl2^{KO}* mice compared with *ihTNFtg* mice. The same was true for inflamed areas and formation of pannus-like tissues in DIP joint structures (Figure 1, B and D, and Supplemental Figure S2).

Immunological Status of the Inflamed Skin and Paws

To gain a deeper insight into the process of ongoing inflammation in the skin and DIP joints of *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mice and to explore whether the pattern of inflammatory infiltrates in the two organs is similar or not, single-cell suspensions were isolated from skin and paws by enzymatic digestion and analyzed for different sets of immune cells by flow cytometry. Disease-affected mice had received Dox for 21 days before analysis. Non-treated mice were used as controls. The obtained results (Figure 2) are

Figure 1 Deficiency in Fhl2 exacerbates the tumor necrosis factor (TNF)–mediated psoriatic arthritis. Six-month-old *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* animals were left untreated (cntr) or received 1 mg/mL doxycycline (Dox) with drinking water for 3 weeks, and the severity of developed psoriasis (Skin) and arthritis of distal interphalangeal (DIP) joints were scored. **A:** The psoriasis area and severity index (PASI; left panel) and the mean epidermal thickness (right panel) of individual mice. **B:** Inflammation (left panel) and cartilage destaining (right panel) values of individual mice. Mean values of four phalanges for each mouse are shown. **C:** Hematoxylin and eosin images of cntr and Dox-treated mouse skin. **D:** Toluidine blue images of distal interphalangeal joints. Note the invasion of pannus-like tissue into bone (asterisks) and cartilage destaining (arrowheads). Mean values per group are shown (red lines). Statistical analysis was performed using one-way analysis of variance, followed by the Newman-Keuls test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: 200 μm (C); 100 μm (D).

particularly interesting in two respects. First, both skin and DIP joints of *ihTNFtg/Fhl2^{KO}* mice always showed a significantly stronger infiltration with activated macrophages and T regulatory cells (Tregs) than *ihTNFtg* mice. Second, the pattern of immune cells infiltrating into affected skin and into DIP joints in mice with psoriatic arthritis was similar, independently of the mouse line analyzed. Generally, the fact that *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mice responded to Dox-mediated TNF stimulation with increased infiltration of macrophages and Tregs is in accordance with our previous data showing that TNF-mediated psoriasis is mainly determined by macrophages and that their proinflammatory activity is concurrently limited by TNF-induced Tregs.¹⁴ However, in *ihTNFtg/Fhl2^{KO}* mice, an increased ratio of CD4⁺Foxp3⁺ Tregs was always measured (Figure 2). Furthermore, the number of monocytes (CD11b⁺ cells) and macrophages (CD11b⁺F4/80⁺ cells), including activated macrophages (CD11b⁺MHCII⁺), was also increased in the inflamed skin and paws of *ihTNFtg/Fhl2^{KO}* mice when compared with *ihTNFtg* animals. The number of dendritic cells (represented by CD11c⁺ cells) and neutrophils (represented by CD11b⁺Ly6G⁺ cells) was also higher

in *ihTNFtg/Fhl2^{KO}* mice, but the increase was not as prominent as it was observed for the macrophage population. Altogether, these results show that *ihTNFtg/Fhl2^{KO}* mice respond to TNF induction with increased infiltration of both skin and DIP joints with macrophages and Tregs.

Cytokine and Protease Status of the Inflamed Skin and Paws

Inflammatory diseases are accompanied by activation of diverse genes, including genes coding for cytokines and chemokines, but also for proteases that are ultimately responsible for infiltration of affected tissues by immune cells and for tissue damage. The expression profile of a specific set of genes commonly associated with psoriatic diseases was analyzed in the skin and DIP joints after Dox treatment. First, we studied the expression of *Fhl2* itself. Its transcriptional activity, both in the skin and in DIP joints, was significantly down-regulated on TNF induction (Figure 3, A and B). Interestingly, the detection of β-galactosidase enzyme activity of the expressed reporter gene within the targeted *Fhl2* locus, by hydrolysis of either

Figure 2 Immunological status of *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mouse skin (A) and distal interphalangeal (DIP) tissues (B). Six-month-old *ihTNFtg* or *ihTNFtg/Fhl2^{KO}* mice were left untreated (cntr) or stimulated with 1 mg/mL doxycycline (Dox) for 3 weeks, after which immune cells present in the skin and forepaws were analyzed by flow cytometry. Representative dot blot images emphasizing the gating strategy for quantification of CD4⁺Foxp3⁺, CD11b⁺Ly6G⁺, CD11b⁺F4/80⁺, and CD11b⁺MHCII⁺ skin (C) and DIP joints (D) cells are shown. Statistical analysis was performed using the *U*-test. Data are expressed as means ± SEM (A and B). *n* = 5 to 6 mice per group (A and B). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

fluorescein di-β-D-galactopyranoside in isolated *Fhl2^{-/-}* macrophages or X-Gal in *Fhl2^{-/-}* chondrocytes of finger tissue sections revealed a decreased β-galactosidase activity in these cells under sustained TNF induction (Figure 3, C and D). The results indicate that *Fhl2* expression is reduced in response to the TNF stimulus, which is in full agreement with our previous data showing that proinflammatory cytokines suppress *Fhl2* expression⁴ and with the concept that *Fhl2* counteracts inflammatory reactions.

Several cytokines that are commonly linked to psoriasis and arthritis, like IL-1 (*Il1b*), IL-6 (*Il6*), IL-23 (*Il23a*), or interferon-γ (*Ifng*), were strongly and significantly up-regulated after Dox stimulation in *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mice, but not regulated between the genotypes (Figure 3, A and B). Consistent with increased numbers of Tregs and macrophages, an augmented expression of Foxp3 (*Foxp3*) and especially of IL-10 (*Il10*) and CCL2 (*Ccl2*) cytokines was observed in skin and joints of *ihTNFtg/Fhl2^{KO}*

Figure 3 **A** and **B**: Cytokine and chemokine status of *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mouse skin (**A**) and distal interphalangeal (DIP) tissues (**B**). Six-month-old *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mice received either drinking water only (cntr) or drinking water containing 1 mg/mL doxycycline (Dox) for 3 weeks, and gene expression in skin (**A**) or forepaw (**B**) samples was analyzed by TaqMan quantitative real-time RT-PCR. The amount of mRNA in control *ihTNFtg* mice was assigned a value of 1. Statistical analysis was performed using one-way analysis of variance, followed by the Newman-Keuls test. **C**: Flow cytometry analysis of freshly isolated skin single-cell suspension for macrophage marker (CD11b and F4/80) and Fhl2/β-Gal expression. Cells were incubated with fluorescein di-β-D-galactopyranoside (FDG), and the number of fluorescein isothiocyanate (FITC)-positive cells was measured. The **top left panel** represents the percentage of FDG-FITC-positive macrophages, and the **top right panel** represents the GeoMean values of FDG-FITC. The **bottom panel** shows a flow cytometric analysis of FDG-FITC intensity in macrophages from a representative Cntr or Dox-stimulated *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mouse. **D**: DIP joints were stained with X-Gal to mark the Fhl2/β-Gal protein (light blue) and counterstained with hematoxylin and eosin (H&E; dark blue and red). Note the strong staining of chondrocytes for Fhl2/β-Gal in Cntr and the weak staining in Dox-treated *ihTNFtg/Fhl2^{KO}* mice (**left panel**). Asterisks mark the pannus-like tissue, and arrowheads mark the Fhl2/β-Gal-positive chondrocytes. The **right panel** shows the relative intensity of Fhl2/β-Gal staining in chondrocytes of Cntr and Dox *ihTNFtg/Fhl2^{KO}* mice. The light blue area was always assessed to the total cartilage tissues area of the joint. Mean values (red lines) of four phalanges for each mouse are shown. Slides without H&E counterstaining were used herein. Data are expressed as means ± SEM (A–C). $n = 6$ to 10 animals per group (**A** and **B**); $n = 5$ animals per group (**C**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bar = 100 μm (**D**).

mice. Hence, cytokines activating and attracting Tregs and macrophages were much more strongly expressed in *ihTNFtg/Fhl2^{KO}* mice after Dox stimulation (Figure 3). In accordance with a stronger psoriatic arthritis phenotype in *ihTNFtg/Fhl2^{KO}* mice, there was a significantly higher expression of MMPs in skin and joints of *Fhl2*-deficient mice compared to *ihTNFtg* animals. Proteases responsible for bone destruction, like tartrate-resistant acid phosphatase (*Acp5*) and cathepsin K (*Ctsk*), were also much more strongly expressed in joints of Dox-stimulated *ihTNFtg/Fhl2^{KO}* mice. Interestingly, variations in MMP expression were seen between the affected skin and joints. Although MMP13 (*Mmp13*) showed the highest up-regulation under induction of TNF in the skin, MMP3 (*Mmp3*) was the most abundant MMP in DIP joints. Remarkably, some MMPs [eg, MMP3 (*Mmp3*), MMP9 (*Mmp9*), or MMP13 (*Mmp13*)] already showed a significantly higher expression in untreated control joints of *ihTNFtg/Fhl2^{KO}* mice compared to *Fhl2*-containing *ihTNFtg* mice (Figure 3B). These results, together with increased infiltration of TNF-affected tissues in *Fhl2^{KO}* mice and the fact that sustained high levels of TNF down-regulate *Fhl2* expression in diverse cell types, altogether highlight *Fhl2* as a critical regulator of autoimmune diseases.

Serum Levels of Soluble Cytokines in *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* Mice after Dox Stimulation

To determine whether the development of psoriatic arthritis in these mice causes a systemic up-regulation of proinflammatory cytokines, the levels of cytokines in serum of Dox-stimulated *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mice were compared to non-stimulated control mice using enzyme-linked immunosorbent assay style binding experiments. Fifteen different cytokines linked to inflammation, including those that showed a strong Dox-dependent up-regulation by quantitative real-time RT-PCR (IL-1, IL-23, and interferon- γ), were analyzed. Among those, only IL-6, IL-10, and CCL2 showed increased levels in serum after Dox stimulation and were higher in *ihTNFtg/Fhl2^{KO}* compared with *ihTNFtg* mice. However, the differences were not statistically significant, probably because of the low number of independently tested animals and the variations in the values (Figure 4A). Nevertheless, increased values of IL-10 and CCL2 cytokines in the serum of *ihTNFtg/Fhl2^{KO}* mice match well with increased numbers of Tregs and macrophages in the skin and digits. Surprisingly, soluble human TNF, being strongly increased after Dox stimulation in both mouse lines, showed significantly higher levels in *ihTNFtg/Fhl2^{KO}* mice than in *ihTNFtg* mice (Figure 4A). Even more, control *ihTNFtg/Fhl2^{KO}* animals also displayed higher levels of hTNF in serum. Consistent with stronger psoriatic arthritis in *ihTNFtg/Fhl2^{KO}* mice and higher levels of hTNF in their serum, these animals also produced greater amounts of S100A8/A9 alarmins. S100A8/A9 heterodimers are usually expressed and secreted by activated keratinocytes and monocytes as well

Figure 4 Inflammatory cytokines in serum of *ihTNFtg* (closed triangles) and *ihTNFtg/Fhl2^{KO}* (open triangles) mice after doxycycline stimulation. **A:** Six-month-old *ihTNFtg* or *ihTNFtg/Fhl2^{KO}* mice were left untreated (cntr) or stimulated with 1 mg/mL doxycycline (Dox) for 3 weeks, after which the amount of cytokines in sera was measured by either classic enzyme-linked immunosorbent assay [human tumor necrosis factor (hTNF), murine tumor necrosis factor (mTNF), and S100A8/A9] or the AYOXXA (IL-6 and IL-10) or Legendplex [chemokine (C-C motif) ligand (CCL2)] method. **B:** Gene expression in skin or forepaw samples from mice shown in **A** was analyzed by TaqMan quantitative real-time RT-PCR. Mean values (red lines) per group are shown. Statistical analysis was performed using the *U*-test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

as neutrophils at sites of inflammation and TNF, and S100A8/A9 are mutually up-regulated in a functional feedback loop.²⁰

To assess whether increased levels of soluble hTNF in *ihTNFtg/Fhl2^{KO}* mice resulted from enhanced transcription of the human *TNF* transgene, the amount of *hTNF* mRNA in skin and digit samples of *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mice before and after Dox stimulation were compared. The transcription of the *hTNF* transgene was strongly enhanced in both tissue types after Dox stimulation, but surprisingly no differences between *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mice could be measured (Figure 4B). Hence, the increased hTNF serum level in *ihTNFtg/Fhl2^{KO}* mice is most probably driven by post-transcriptional active mechanisms.

Fhl2 Deficiency Boosts the Adam17-Directed Shedding of Surface-Bound hTNF

Newly synthesized TNF molecules are expressed as 26-kDa large transmembrane proteins. The soluble 17-kDa TNF cytokine is released by the membrane-bound protein with disintegrin and metalloprotease activity Adam17, also called TNF- α -converting enzyme.²¹ Fhl2 physically interacts with the cytosolic tail of the transmembrane Adam17²² and was proposed to influence Adam17 function. Hence, we studied whether the increased amount of soluble hTNF in *ihTNFtg/Fhl2^{KO}* mice might be a result of enhanced shedding activity by Adam17 because of the absence of Fhl2 protein. Freshly isolated skin cells from intact *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mice were stimulated with Dox in the presence or absence of Adam17 inhibitor TMI-1.¹⁹ Subsequently, the relative amounts of hTNF molecules on the surface of TNF-expressing cells were measured by flow cytometry. As shown in Figure 5, A and B, non-Dox-stimulated keratinocytes hardly expressed hTNF on their surfaces, but after Dox stimulation, both *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* derived keratinocytes were strongly positive for membrane-bound hTNF. Interestingly, *ihTNFtg/Fhl2^{KO}* cells expressed fewer hTNF molecules on their surface than *ihTNFtg* keratinocytes. However, in the presence of the Adam17 inhibitor TMI-1, the amount of surface-bound hTNF increased. The increase was TMI-1 concentration dependent and began from 10 mmol/L of the inhibitor, but no differences in surface-expressed hTNF molecules were visible anymore between *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* cells (Figure 5C). Thus, the Adam17-mediated shedding of surface TNF was increased in the absence of Fhl2. Notably, the synthesis of *Adam17* transcripts was not altered, neither under Fhl2 deficiency nor on Dox stimulation (Figure 5D), indicating that the sheddase activity of Adam17 is increased in the absence of its binding partner Fhl2.

Discussion

In this work, we demonstrate that Fhl2 most certainly has anti-inflammatory activity. Using a mouse model for psoriatic arthritis, the TNF-inducible *ihTNFtg* mouse, we show that in animals lacking Fhl2, prolonged high induction of TNF induces a much more severe disease phenotype than in wild-type mice expressing Fhl2. We further show that Fhl2 suppresses the sheddase activity of Adam17, thus reducing the release of membrane-bound TNF and declining serum levels of TNF- α , the key inflammatory cytokine. Finally, we illustrate that on TNF-mediated induction of psoriatic arthritis, a similar kind of inflammation develops in affected skin and joint tissues.

On development of psoriatic arthritis, the expression of Fhl2 was significantly down-regulated in both skin and DIP joint tissues, including tissue infiltrating immune cells (Figure 3).⁴ This additionally underlines the major result of this work, which was the finding that in the absence of Fhl2,

Figure 5 Fhl2 deficiency encourages the Adam17 responsive release of surface-bound human tumor necrosis factor (hTNF). **A–C:** Keratinocytes were isolated from control *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mice and cultivated *in vitro* for 4 hours in either Dulbecco's modified Eagle's medium plus 10% fetal calf serum alone or in medium containing 20 μ g/mL doxycycline (Dox) and different amounts of the Adam17 inhibitor TMI-1. Then, cells were stained for surface-bound hTNF and intracellular pan-keratins and analyzed by flow cytometry. **A and B:** The distribution of hTNF-positive cells. Keratinocytes incubated with no Dox and no inhibitor (**left panels**), with Dox only (**middle panels**), and with Dox and 100 mmol/L of TMI-1 inhibitor (**right panels**) are shown as histogram (**A**) and dot-plot (**B**) images. Representative dot blot images emphasize the gating strategy for quantification of hTNF α positive cells (**B**). **C:** The geometric mean fluorescence intensity of surface-expressed hTNF. **D:** Skin and distal interphalangeal (DIP) joint tissues of 6-month-old control (cntr) or 3-week-long Dox-stimulated (Dox) *ihTNFtg* and *ihTNFtg/Fhl2^{KO}* mice were analyzed for *Adam17* transcripts by TaqMan quantitative real-time RT-PCR. Results of single mice with mean values per group are presented (red lines). Data are expressed as means $\pm$ SEM (**C**). $n = 4$ repeated experiments (**C**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (U-test).

TNF-mediated development of psoriatic arthritis is stimulated. Under normal situations *in vivo*, most tissues, except the heart, show only moderate or no expression of Fhl2.^{2,23} However, as an early response gene protein, Fhl2 is rapidly up-regulated in injured tissues during their recovery. Proinflammatory cytokines, such as TGF- β and sphingosine 1-phosphate, induce Fhl2 expression, whereas proinflammatory cytokines, such as IL-1 β , IL-6, or TNF- α , inhibit its expression (this work).^{4,24,25} The precise role of Fhl2 in distinct cell types during TNF-induced inflammation is only partially resolved so far and needs further investigation. Nevertheless, our results are in good agreement with published data and are consistent with the conclusion that Fhl2 is up-regulated during tissue regeneration, but down-regulated during tissue-damaging chronic inflammations.^{4,5,9} Interestingly, the closest Fhl2 homologs, Fhl1 and Fhl3, showed a different behavior in the skin and DIP joints of *ihTNFtg* mice to sustained TNF induction (Supplemental Figure S3), indicating that they may not act in concert with Fhl2 during inflammation. However, this needs to be defined in future experiments.

Fhl2 is a multifunctional adaptor molecule and, as such, is involved in the regulation of different signaling pathways and gene transcription. It interacts with integrins, kinases, structural proteins, and transcription factors, thereby modulating different cellular activities. Depending on interaction partners, Fhl2 may even exert opposite functions,^{2,3} a property characteristic for many adaptor proteins.²⁶ Fhl2 has been reported to be a potent inducer of the epithelial-mesenchymal transition and to contribute significantly to the invasion of tumor cells.^{12,27} Furthermore, Fhl2 deficiency has been shown to lead to impaired wound healing because of defective matrix assembly, cell migration, and contraction of granulation tissue.^{9,28} In lungs, the absence of Fhl2 caused a constantly enhanced inflammation status that was further increased after application of the fibrosis-inducing agent bleomycin.⁵ In liver, Fhl2 deficiency results in delayed tissue regeneration because of the insufficient release of proliferation-supporting cytokines by Kupffer cells.⁷ Finally, Fhl2 plays an important role in bone remodeling by modulating the activity of osteoclasts and osteoblasts.^{29–31} Therefore, Fhl2 seems to be an important component not only in tissue regeneration and in inflammation but also in controlling the expression of matrix metalloproteinases that orchestrate the wound healing and inflammatory functions at various levels. Herein, Fhl2 may modulate the activation of inflammatory mediators via MMP regulation, the recruitment of inflammatory cells, or the processing of extracellular matrix proteins.^{32,33} Uncontrolled high expression of MMPs may also easily run into tissue destruction, and Fhl2 inhibits that by preventing sustained activation of the p38 mitogen-activated protein kinase pathway,⁴ which is crucial for induction of MMPs by proinflammatory cytokines.³⁴

Recently, we showed that Fhl2 inhibits the TNF- α -mediated transcriptional induction of proarthritic MMPs via

modulation of the p38 mitogen-activated protein kinase pathway, playing an essential role in the repair of inflammatory tissue damage during rheumatoid arthritis.⁴ Herein, we describe a new function of Fhl2 in psoriatic arthritis. We show that in addition to suppression of MMP transcription, Fhl2 also inhibits the Adam17-mediated release of membrane-bound TNF. Although TNF is also biologically active as a transmembrane protein, its soluble form has a much greater stimulatory outcome, as it can reach and bind to cells of almost all tissues. Although increased levels of the TNF cytokine lead to many irregularities in the organism, the most prominent disorders are arthritis and psoriasis.¹ Adam17 is recognized for a while as the major TNF sheddase.²¹ However, whether Fhl2 regulates Adam17-mediated surface release of TNF is unknown so far, although the interaction between Fhl2 and Adam17 was described.²² Herein, we show that the absence of Fhl2 leads to enhanced proteolytic ectodomain shedding of the cell surface TNF cytokine, which finally results in an increased psoriatic arthritis disorder.

Given that the absence of Fhl2 results in higher levels of soluble hTNF, the increased infiltration of skin and DIP joints by activated macrophages and Tregs is not so surprising. Indeed, psoriatic arthritis in *ihTNFtg* mice is predominantly a TNF-mediated disorder, which leads to infiltration of affected tissues by macrophages, but not by T helper cell 17 or $\gamma\delta$ T lymphocytes, despite high induction of IL-23.¹⁴ Furthermore, the predominantly TNF-mediated immune disease concurrently induces activation and attraction to inflamed sites of Tregs, most likely to compensate the tissue-destructive effect of macrophages.³⁵ These data are consistent with low or absent induction of lymphocyte-activating transcription factors T-bet (*Tbx21*), GATA4 (*Gata3*), or ROR γ t (*Rorc*) (Figure 3).¹⁴ The emergent high levels of soluble TNF in *Fhl2* knockout mice induce, in turn, increased transcription of MMPs, the induction of which is further favored by the absence of the p38 mitogen-activated protein kinase pathway inhibitor Fhl2.⁴ The increased manifestation of MMPs, along with CCL2 and IL-10, in *ihTNFtg/Fhl2^{KO}* mice (ie, proteins intimately involved in activation and attraction of immune cells at inflammation) fits well with increased infiltration of skin and joints with macrophages and Tregs and with the more severe disease in *Fhl2* knockout mice. Interestingly, in *ihTNFtg/RAG1^{KO}* mice, the increased infiltration of psoriatic skin by macrophages because of the absence of inflammation inhibitory Tregs also coincided with increased expression of MMP9 and CCL2.¹⁴ Whether Fhl2 inhibits, similar to MMP transcription, the TNF-mediated expression of CCL2 and IL-10 is not clear yet, although a clear reduction of Fhl2 expression in macrophages on TNF burden was demonstrated in this work. It is also unclear whether the increased infiltration of immune cells into diseased tissues of *ihTNFtg/Fhl2^{KO}* mice might be caused, in addition to increased levels of MMPs and cytokines, by altered integrin or other protein functions, the association of which with Fhl2 is disturbed in *Fhl2^{KO}* mice. Although we

cannot exclude that altered cytoskeletal rearrangement or cell-cell and cell-matrix interactions might play a role in increased tissue infiltration in *ihTNFtg/Fhl2^{KO}* mice, the question probably has to be analyzed separately for distinct cell types, because Fhl2, as a multifaceted adaptor protein, might both support and inhibit migration of cells.^{9,13}

An additional interesting observation of this work is that the type and kind of inflammation in different types of tissue, as skin and joints, were actually similar during the TNF-mediated disorder. Both skin and DIP joints showed a strong up-regulation of the same range of cytokines, such as IL-1 β , IL-6, or IL-23. Also, the pattern of immune cells that infiltrated the affected skin and DIP joints was similar. Only the relative amount of cytokine induction and immune cells in the skin was much higher than in DIP joints. The cause for that, however, might rather have technical than physiological reasons. It was easier to localize the inflammation and to gain large numbers of single cells for flow cytometry analyses from skin samples than from digits. In the last case, always complete forepaws were digested, although the major inflammation was localized in DIP joints, as histological analysis indicated (Supplemental Figure S2).

In summary, we showed herein that Fhl2 inhibits the cleavage of the TNF ectodomain by Adam17. The decreased TNF release, in turn, reduces the systemic induction of proinflammatory cytokines and MMPs, thus dampening the development of inflammation and tissue degradation. Besides this, Fhl2 blocks the transcription of MMPs, additionally contributing toward minimizing the inflammation. However, on sustained TNF expression, Fhl2 itself is down-regulated, which promotes the expansion of inflammation or turning it into chronification. Hence, the results of this work not only confirmed the anti-inflammatory properties of Fhl2 but also disclosed a novel site of the versatile adaptor protein Fhl2, anticipating the emerging inflammation by impeding the Adam17-mediated release of TNF- α . Altogether, our results indicate that FHL2 has a strong regulatory effect on tissue damage *in vivo* and provides a promising new target in the treatment of psoriasis and rheumatoid arthritis.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.07.015>.

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