NFIL3 mutations alter immune homeostasis and sensitise for arthritis pathology

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INTRODUCTION
Juvenile idiopathic arthritis (JIA) is the most common of the childhood rheumatic diseases. JIA is characterised as juvenile-onset persistent arthritis with no defined cause. A high degree of clinical heterogeneity is observed within the JIA group of diseases, thought to reflect a diversity in genetic and environmental factors and mechanistic drivers. JIA shows similarities to adult autoimmune diseases, and, indeed, genome-wide association studies identify a strong overlap in the common of the childhood rheumatic diseases. and, indeed, genome-wide association studies identify a strong overlap in the common of the childhood rheumatic diseases.

Methods
Here, we characterised a kindred of two monozygotic twins with juvenile idiopathic arthritis at the genetic and immunological level, using whole exome sequencing, single cell sequencing and flow cytometry. Parallel studies were performed in a mouse model.

Results
The patients inherited a novel p.M170I in NFIL3 from each of the parents. The mutant form of NFIL3 demonstrated reduced stability in vitro. The potential contribution of this mutation to arthritis susceptibility was demonstrated through a preclinical model, where Nfil3-deficient mice upregulated IL-1β production, with more severe arthritis symptoms on disease induction. Single cell sequencing of patient blood quantified the transcriptional dysfunctions present across the peripheral immune system, converging on IL-1β as a pivotal cytokine.

Conclusions
NFIL3 mutation can sensitise for arthritis development, in mice and humans, and rewire the innate immune system for IL-1β over-production.

Key messages
- Homozygous NFIL3 mutations identified in monozygotic twins with juvenile idiopathic arthritis.
- Enhanced susceptibility to arthritis induction in Nfil3-knockout mice.
- NFIL3 loss in patients and mice is associated with elevated production of IL-1β.
- Knockdown of NFIL3 in healthy macrophages drives IL-1β production.
Peripheral blood mononuclear cells (PBMCs) were isolated from patients and healthy individuals. For intracellular staining, cells were plated with complete RPMI containing phorbol myristate acetate (PMA 50 ng/mL; Sigma-Aldrich), ionomycin (500 ng/mL; Sigma-Aldrich) and Brefeldin A (8 ng/mL; Tocris Bioscience) for 4 hours. Cells were fixed and permeabilised with the eBioscience Foxp3 staining kit (eBioscience). Anti-human antibodies included anti-NFIL3 (REA732) (Miltenyi Biotec), anti-CD14 (TuK4) (eBioscience); anti-CD3 (Miltenyi Biotec); anti-CD16 (3G8), anti-CD56 (NCAM16.2), anti-CD123 (7G3), anti-CD27 (L128), anti-CD45RA (H100), anti-CD8 (SK1), anti-CD4 (SK3), anti-CD1c (L161), anti-IFN-γ (4S.B3), anti-T-BET (4B10), anti-IL-17a (N49-653), anti-GATA3 (L50-823) (all from BD Biosciences); anti-HLA-DR (L243), anti-CD19 (HB19), anti-CD56 (NCAM16.2), anti-CD11c (3.9), anti-CCR7 (G043H7), anti-FOXP3 (206D), anti-ROTY (Q21-559), anti-TNFα (MAb11), anti-IL-4 (MP4-25D2) (all from BioLegend); purified Rabbit-anti-human NFIL3 (D5K80) (Cell Signaling Technology) followed by Donkey-anti-Rabbit-IgG (Thermo Fisher Scientific). Data were collected on BD Symphony (BD Biosciences) and analysed using FlowJo V.10.5 (Tree Star Inc.).

Biochemistry

Lyssates from lymphoblastoid cells were run on the NuPAGE Precast Gel System (Life Technologies). Thirty to 50 µg of lyssate were separated on 4%–12% bis-tris acrylamide gels and blotted on a PVDF membrane (GE Healthcare). Membranes were incubated with rabbit anti-NFIL3 (1:500, D5K80, Cell signaling) and mouse anti-Vinculin (1:2000, V9264, Sigma). Proteins were revealed using western Lightning Prime-ECL (GE Healthcare) and the imaging system G:Box XRQ (Syngene). Quantification was performed using the AIDA software (Raytest, V.5.0).

N-terminally FLAG-tagged human NFIL3-T2A-GFP (WT or carrying the M170I mutation) was expressed transiently from a plasmid in HEK293T. The expression was driven by chicken actin promoter with the CMV enhancer. For transfections, HEK293T cells were grown on poly-L lysine-treated (0.1%) cover slips to subconfluence. Plasmid transfection was done using Lipofectamine 3000 according to the manufacturers protocol (Thermo Fisher). Twenty-four hours after transfection, the cells were washed in PBS, fixed in 4% PFA and permeabilised in 0.1% Triton X-100 (in PBS). After blocking in PBS with 2% bovine serum albumine (BSA), 10% donkey serum and 0.1% Triton X-100 for 30 min, cells were stained with an anti-Flag polyclonal antibody (F7425; Sigma Aldrich) for 2 hours, then washed and incubated for 1 hour with Alexa Fluor 555 donkey-anti-rabbit (A31572; Molecular Probes) antibody as well as DAPI (D1306; Molecular Probes). After washing the cells, they were covered using Fluoromount (Thermo Fisher). Images were collected on an LSM 510 Meta confocal microscope (Zeiss) with a 60× immersion objective. Quantification of mean fluorescence intensity was measured using ImageJ software. Alternatively, 24 hours post-transfection, cells were stained with fixable viability dye (eBioscience), fixed and stained for human NFIL3 following the eBioscience protocol for flow cytometry analysis.

Arthritis induction in mice

CS7Bl/6 and Nfil3-/- mice were bred and housed under barrier conditions at a specific pathogen-free facility at the Walter and Eliza Hall Institute Animal Facility. Eight-to-ten week-old mice were used for all experiments. All procedures were approved by the Walter and Eliza Hall Institute Animal Ethics Committee. Serum transfer arthritis was induced by injection of arthritogenic serum from 12-week-old progeny of KRN and non-obese diabetic mice (K/BxN mice). Clinical score was assessed as a sum of the clinical score for each paw (0, no erythema and swelling; 1, mild erythema and swelling confined to the ankle, wrist or digits; 2, mild erythema and swelling extending from the ankle to the mid-foot; 3, moderate erythema and swelling extending from the ankle to the metatarsal joints; 4, severe erythema and swelling extending the entire limb and with joint ankylosis). The severity of joint inflammation was also assessed with in vivo imaging of bioluminescence using luminol, a substrate for myeloperoxidase activity (in myeloid cells), on days 4 and 7, as published previously. Arthritis of the ankle joint was evaluated histologically from two independent experiments. Front and hind limbs of mice were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 7 µm and stained with Safranin-O, according to standard practices. Histological analysis was performed on serial joint sections. Histology scores are as follows: 0=normal, 1=mild, 2=moderate, 3=severe.

Flow cytometry was performed on cells isolated from the peritoneal lavage, joints and blood. For cytokine production measurement, cells were stimulated with LPS (0.1 µg/mL) in the presence of Brefeldin A and monensin for 3 hours, stained for surface markers, followed by intracellular staining of IL-1β and TNF.

Single cell sequencing

Peripheral blood was collected by venipuncture, and the PBMC fraction was isolated using LSM-Lymphocyte Separation Medium (MP Biomedicals). PBMC were then viable frozen and stored in liquid nitrogen prior to single cell sequencing. On thawing, the PBMC were counted using a Countess II Automated Cell Counter (Thermo Fisher), and 8700 cells for each sample were loaded individually onto the Chromium Controller (10x Genomics).
Analysis of single-cell RNA-seq data from patient and control PBMCs

Sequence data were preprocessed with Cell Ranger V2.0 (10x Genomics). The resulting count matrices were analysed with R V3.4 and the package Seurat V2.2 (6), following the standard pipeline with default parameters, unless stated otherwise. Genes detected in less than five cells as well as cells with less than 500 genes detected were filtered out, leaving 15 216 genes across 4743 cells in the control and 14 367 genes across 2165 cells in the patient. Gene expression was normalised across genes by dividing the total expression per cell, log-transformed and standardised across cells. The 1000 most variable genes were used to align the expression levels of both samples, through the components of a canonical correlation analysis (CCA). The tSNE plots were calculated on the first 20 components of the CCA, and clusters were identified by the community-detection algorithm implemented by Seurat.

Gene set enrichment analysis was carried out for each cluster (cell type) with GSEA v 3.0 (Broad Institute, Cambridge, Massachusetts, USA) (7). Gene sets with size larger than 1000 or smaller than 10 were excluded. Detection of variation in gene sets was controlled to have a false discovery rate lower than 0.25. Gene sets were prioritised according to the normalised enrichment score provided by GSEA.

KEGG pathways were analysed with Pathview, through the web server API. First, each cluster (cell type) was analysed separately, using all genes with detected fold-change, for the pathways corresponding to signal transduction, immune system and immune diseases. Then, the final pathway representation was obtained by merging the expression levels of the genes directly related to each cell type.

RESULTS

NFIL3 mutations in monozygotic twins with juvenile idiopathic arthritis

Monozygotic twins were identified with JIA (figure 1A). Both sisters were diagnosed with oligoarticular JIA at the age of 4 years (P1) and 6 years (P2), respectively. Systemic inflammation at onset (sedimentation 49; <20 mm/hour, CRP 15.2; <5 mg/L, IgG 14.8; 4.78–11.29 g/L (P1); sedimentation 32; <20 mm/hour, CRP 2.9; IgG 17.50; 5.58–12.54 g/L (P2)) and antinuclear antibodies (table 1) were present in both. There was no occurrence of uveitis. Autoimmune thyroiditis developed at age 9 years (P1) and 11 years (P2). P1 was initially treated with intra-articular steroids and methotrexate, with adalimumab added after a relapse. At 11 years of age, laboratory tests revealed mildly increased liver enzymes with normal bilirubin levels, normal NSE, αFP and coagulation tests. Liver ultrasonography with duplex Doppler showed a large well-marginated lesion in the left liver lobe displacing the left subhepatic vein, with a characteristic spoke wheel vascularisation pattern compatible with focal nodular hyperplasia. MRI confirmed a T2 isointense multilobulate tumour with a central T2 hyperintense scar, occupying

Real time PCR

RNA has been isolated from sorted CD14+ monocytes, differentiated macrophages or NIH3T3 cells using the ReliaPrep RNA Cell Miniprep System (Promega). cDNA synthesis was performed using the Superscript III RT System (Thermos Fisher). Expression of STX11, TGFB1, CSF2RB, CEBSG, CD224, NFIL3, TNF, IL1B, HPRl, RPL0, ACTB and plasmid-encoded ncRNA was measured by PrimeTime qPCR Probe Assays (IDT) and IL1B by SYBR green qPCR (Thermo Fisher). The expression of HPRl, RPL0 and ACTB was used to normalise mRNA expression.

Figure 1 NFIL3 mutations in a pedigree with juvenile idiopathic arthritis. (A) Family pedigree of the affected patients (grey). (B) Sanger sequencing of NFIL3 indicating the site of mutation. (C) Schematic of NFIL3 domains and the site of mutation. (D) Cross-species conservation of NFIL3 in the region flanking M170 (ClustalW). Amino acids with >50% conservation are indicated in blue. (E) Western blot indicating protein expression of NFIL3 in LCLs from control individuals, the patient (homozygous) and mother (heterozygous), with quantification normalised against vinculin.
Nfil3 knockout mice have been found to have enhanced susceptibility to arthritis. Cell lines demonstrated a ~50% reduction in NFIL3 expression at transcriptional level (figure 1B). The mutation is in the Ser-rich region (figure 1C), leading to a methionine to isoleucine mutation at residue 170 (M170I). This mutation was confirmed by Sanger sequencing as homozygosity, with a G510A mutation in the M170I allele (online supplementary figure S2). Together, these results indicate that M170I NFIL3 is unstable, without excluding additional functional loss from the amino acid change.

**NFIL3 mutations drive elevated IL-1β production in myeloid cells**

In order to determine the immunological impact of NFIL3 loss of function on the peripheral immune system, we ran a single cell sequencing experiment on P1 and a healthy age-matched control. After data curation, data from 4743 cells from the healthy individual and 2165 cells from the patient were clustered using a tSNE approach (figure 3A). Clusters were manually annotated into leucocyte populations based on the expression of key lineage markers (online supplementary figure S4). Quantification of the clustered leucocyte populations revealed multiple immunological abnormalities in the patient (figure 3B,C). The adaptive immune system gave indications of defective activation, with increased naïve B cells and T cells, while memory B cells and activated T cells were normal and activated CD8 T cells were reduced. Changes were also observed in the innate immune system, with a shift from the CD14+ monocyte cluster to the CD16+ monocyte cluster, a relative defect in the CD56 bright NK cell population (figure 4). As was observed using single cell sequencing, the innate immune system was disturbed in the NFIL3 patients, with an increased frequency of CD16+ monocytes (figure 4B) and a selective reduction in the CD56bright NK population (figure 4C). Analysis of T cell populations with flow cytometry picked up an increase in T cell activation not apparent at the transcript level. Th1, Th17, IFNγ-producing CD8 and TNF-producing CD8 T cells were all increased (figure 4G,I–K). These results validated and extended the single cell analysis, identifying an inflammatory milieu in NFIL3 patients.

Beyond changes in leucocyte population frequency, we used a flow cytometric analysis on both P1 and P2 and four healthy controls (figure 4). As was observed using single cell sequencing, the innate immune system was disturbed in the NFIL3 patients, with an increased frequency of CD16+ monocytes (figure 4B) and a selective reduction in the CD56bright NK population (figure 4C). Analysis of T cell populations with flow cytometry picked up an increase in T cell activation not apparent at the transcript level. Th1, Th17, IFNγ-producing CD8 and TNF-producing CD8 T cells were all increased (figure 4G,I–K). These results validated and extended the single cell analysis, identifying an inflammatory milieu in NFIL3 patients.

Previous studies have suggested that NFIL3 deficiency can lead to a loss of NK cell-mediated cytotoxicity. Here, we confirmed this by showing a ~50% reduction in the mRNA/protein ratio of NFIL3 in Nfil3 knockout mice (figure 2F,G). These changes in the NK cell population suggest a role for NFIL3 in the regulation of innate immune responses.

**NFIL3 knockout mice have enhanced susceptibility to arthritis induction**

In the absence of a second family with NFIL3 mutations, we turned to a mouse model. Nfil3 knockout mice have been previously characterised as possessing a diverse set of immunological alterations. Here, we challenged 8–10-week-old C57BL/6 and Nfil3 gene deleted mice with arthritogenic serum antibodies derived from the K/BxN mouse strain. This model bypasses early priming stages and compares sensitivity to downstream arthritis pathology processes. Compared with wildtype mice, Nfil3 knockout mice developed inflammatory arthritis earlier and had more severe joint inflammation, as assessed by paws and hindlimbs (figure 2A-B). In Nfil3 knockout mice, we found an elevated myeloid infiltrate, dominated by neutrophils (figure 2D,E). Infiltrating neutrophils and monocytes/macrophages demonstrated enhanced production of IL-1β and TNF in the Nfil3 knockout joint (figure 2F,G). These changes in the joint were reflected in the serum, with elevated IL-1β and TNF in the arthritic Nfil3 knockout mice (figure 2H). Together, these results support NFIL3 as a genetic contributor to inflammatory arthritis in the patient pedigree and identify innate inflammatory cytokines as a potential mechanism.
Figure 2  Nfil3<sup>−/−</sup> mice have increased susceptibility to arthritis induction. Wildtype and Nfil3<sup>−/−</sup> mice were injected with serum from K/BxN mice. (A) Mice were scored for clinical arthritis daily for 7 days. Each paw was scored on a scale of 0–4 based on signs of swelling and inflammation (n=9/group). (B) Mice were imaged for MPO activity in paws using luminol sodium salt solution and were imaged for bioluminescence using the IVIS spectrum imaging. Representative picture and (C) average RADIANCE at days 4 and 7. (D) Wild-type and Nfil3<sup>−/−</sup> mice were assessed by flow cytometry 5 days after injection of K/BxN serum. Data are representative of two independent experiments with 6 wild-type and 2–3 Nfil3<sup>−/−</sup> mice per experiment. Representative gating of neutrophils, macrophages and monocytes, and (E) quantification of joint-infiltrating cells. (F) Representative flow cytometry analysis showing the intracellular expression of IL-1β and TNF in monocytes and macrophages (CD88<sup>+</sup>Ly6G<sup>−</sup>CD64<sup>+</sup>) and neutrophils (CD88<sup>+</sup>Ly6G<sup>+</sup>CD64<sup>−</sup>) from joints of wild-type and Nfil3<sup>−/−</sup> mice. (G) Total numbers of IL-1β-producing and TNF-producing leucocytes are shown from wild-type and Nfil3<sup>−/−</sup> mice in peritoneal lavage, joints and blood. (H) Concentrations of IL-1β and TNF were determined from joint lavage of mice 5 days after injection of K/BxN serum by ELISA. Mean±SD, *p<0.05. MPO, myeloperoxidase.
Figure 3  Peripheral immune alterations with NFIL3 mutation. Integrated analysis of single cell sequencing transcriptomics data from patient and control PBMCs. (A) tSNE projection of 6908 PBMCs. After alignment, each cell is grouped into clusters (distinguished by colour). Single joint clustering revealed 14 immune populations annotated according to the expression of key lineage markers. (B) tSNE projection of 6908 PBMCs, split between patient and control after alignment. (C) Proportion of the total number of cells from each sample belonging to each leucocyte population. (D) Proportion of known NFIL3 target genes with a 2-fold (light blue/light red) or 4-fold (dark blue/dark red) expression change, within each leucocyte cluster. Only NFIL3 targets expressed within the cluster were considered. PBMCs, peripheral blood mononuclear cells.

Many biological pathways were altered in the myeloid compartment, with the upregulation of components of the MAPK pathway the key feature (online supplementary figure S6), again indicative of excessive activation. When transcriptional changes were mapped onto the Rheumatoid Arthritis KEGG pathway, excessive production of IL-1β and TNF by innate leucocytes was identified as a key change (figure 5A), corresponding with the changes observed in mice (figure 2). Due to the known arthritogenic role of IL-1β, we tested whether a direct link could be established between NFIL3 expression in macrophages and IL-1β production. Using an siRNA approach, we knocked down NFIL3 expression in primary macrophages cultured from a healthy individual and found that ~50% reduction in NFIL3 primed macrophages for excessive IL-1β and TNF expression (figure 5B). This mechanistic analysis suggests that the effects of NFIL3-deficiency may be pleiotropic, with differential rewiring of multiple leucocyte populations culminating in dysregulated IL-1β and TNF production in an arthritogenic reaction.

DISCUSSION
In this study, the in vivo immunological role of NFIL3 has been characterised, with deficiency in NFIL3 sensitising to arthritis development in mice and in patients. Mechanistic analysis in both species converged on IL-1β overproduction by innate leucocytes as a potential disease mechanism. It is likely, however,
Distinct immunological profiles of patient peripheral blood. Peripheral blood from healthy controls (black squares) and the two patients (open circles) were assessed for immune phenotype by flow cytometry. (A) CD14+ monocytes (CD14+CD16HLADR+), (B) CD16 monocytes (CD16+CD14HLADR+), (C) CD56bright NK cells (CD3 CD19 CD14 CD16 CD56bright), (D) plasmacytoid DCs (CD3 CD19 CD14 CD56 HLADR+CD11cCD123+), (E) CD1c+ myeloid DCs (CD3 CD19 CD14 CD56 HLADR+CD11c+CD1c+CD123+), (F) naive B cells (CD19+CD14CD27), (G) Th1 (CD3+CD4+IFNγ+TBET+), (H) Th2 (CD3+CD4+IL4+GATA3+), (I) Th17 (CD3+CD4+RORγ+IL17+), (J) CD3+CD8+IFNγ+TBET+, (K) CD3+CD8+TNFα+. Median and individual data points are shown.

Mapping of transcriptional changes in NFIL3 patient onto arthritogenic pathways. (A) Single cell sequencing transcriptomics data from patient and control PBMCs was mapped onto KEGG pathways. Transcriptional changes in the KEGG rheumatoid arthritis pathway were visualised using an adapted Pathview. In blue are shown labels for mapped cell types, corresponding to annotated single cell clusters. Differential gene expression within each annotated cell type is visualised with colour, with green indicating overexpression in healthy control and red indicating overexpression in patient. Synovial stromal cells, not present in the single cell RNAseq dataset, are represented but with annotated genes indicated as transcript not detected (white). (B) Healthy control PBMCs were differentiated into macrophages and treated with either scrambled siRNA or NFIL3 siRNA, and NFIL3 mRNA knockdown was confirmed by qPCR. Treated macrophages were stimulated with LPS for 24 hours, following which IL1β and TNFα mRNA expression was assessed by qPCR. PBMCs, peripheral blood mononuclear cells.
that the effect of NFIL3 is more pleiotropic, with multiple complex interactions. For example, the adaptive immune system in these patients also demonstrated a Th1/Th17 skew, which may also contribute to disease. A proinflammatory phenotype of NFIL3 deficiency is consistent with both the murine model, which develops colitis, and correlative data in humans, where NFIL3 expression is reduced in patients with colitis. While the patients described here have not presented with colitis, it is increasingly recognised that the clinical presentation of auto-inflammatory diseases is diverse, with the underlying biological defect manifesting as different clinical symptoms in different individuals. The identification of NFIL3 as an autoinflammatory gene opens up further investigation of monogenic patients, who may present with inflammatory phenotypes across the spectrum.

Independent of the role of NFIL3 mutations in disease, the identification of an NFIL3-deficient family allows the first analysis of the in vivo functions of NFIL3 in humans. In vitro gene silencing on NFIL3 in human T cells and B cells has been performed,; however, in vivo experiments on NFIL3-deficiency have been restricted to mice. Comparison of the NFIL3-deficient patients assessed here with the Nfil3-deficient mice reveals both cross-species similarities and species-specific functions. The patients, as with the mice, have defects in NK cells, with a reduction in maturation to the CD56bright population. Likewise, in patients, NFIL3 deficiency results in a major loss of the cDC population, phenocopying mice. Here, we demonstrated a mechanistic link between NFIL3 expression and proinflammatory cytokine production, and an association between NFIL3 deficiency with arthritis in mice and patients.

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Contributors SS, EP, OB, TP, CS, CL, JG, SJ and KL performed experiments. SS, VL, CPR, IPW, GB and AL analysed data. EVN, SH-B and CW provided clinical information. SH-B, CW and AL designed and led the study.

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