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PILRα Negatively Regulates Mouse Inflammatory Arthritis

Yonglian Sun,* Patrick Caplazi,† Juan Zhang,* Anita Mazloom,‡ Sarah Kummerfeld,§ Gabriel Quinones,‡ Kate Senger,* Justin Leisch,* Ivan Peng,* Andrew Sebrell,* Wilman Luk,‖ Yanmei Lu,* Zhonghua Lin,* Kai Barck,* Judy Young,* Mariela Del Rio,** Sophie Lehar,†† Vida Asghari,** WeiYu Lin,* Sanjeev Mariathasan,†† Jason DeVoss,* Shahram Misaghi,‡‡ Mercedesz Balazs,* Tao Sai,* Benjamin Haley,‡ Philip E. Hass,* Min Xu,* Wenjun Ouyang,* Flavius Martin,* Wyne P. Lee,* and Ali A. Zarrin*†

Paired Ig-like type 2 receptor (PILRα) inhibitory receptor and its counterpart PILRβ activating receptor are coexpressed on myeloid cells. In this article, we report that PILRα, but not PILRβ, is elevated in human rheumatoid arthritis synovial tissue and correlates with inflammatory cell infiltration. PILRα−/− mice produce more pathogenic cytokines during inflammation and are prone to enhanced autoimmune arthritis. Correspondingly, engaging PILRα with anti-PILRα mAb ameliorates inflammation in mouse arthritis models and suppresses the production of proinflammatory cytokines. Our studies suggest that PILRα mediates an important inhibitory pathway that can dampen inflammatory responses. The Journal of Immunology, 2014, 193: 860–870.

Immune responses are modulated by a network of positive- and negative-regulatory mechanisms. Paired receptors consist of highly related activating and inhibitory receptors that are widely involved in the regulation of the immune system (1). Both inhibitory and activating receptors share high similarity in their extracellular domain, whereas their intracellular signaling domains are divergent (2, 3). Paired Ig-like type 2 receptor (PILRα) belongs to the Ig superfamily. Its intracellular domain contains two ITIMs that recruit SHP-1 and SHP-2 to trigger an inhibitory signaling cascade, resulting in reduced intracellular calcium mobilization (4, 5). PILRα and its gene-linked activating counterpart, PILRβ, share highly similar extracellular domains, suggesting that they may recognize the same ligands (5–7). PILRβ has a truncated cytoplasmic domain and a charged amino acid residue in its transmembrane domain that associates with the ITAM-bearing DAP12 adaptor molecule to deliver activating signals (8). Human and mouse PILRα share only ∼40% homology (7), yet conserved residues mediate ligand interactions (9).

PILRα and PILRβ transcripts show similar tissue expression, with high levels in spleen, liver, and lung and lower levels in the small intestine (8). PILRα and PILRβ are predominantly expressed in cells of the myelomonocytic lineage, including monocytes, macrophages, granulocytes, and monocyte-derived dendritic cells (DCs) (4, 8). Additionally, PILRβ is expressed in NK cells (8). It was shown that PILRα binds to mouse CD99 (8), PILR-associating neural protein (10), and HSV-1 glycoprotein B (11). Specific sialylated O-linked glycans on ligands are required for their binding to PILRα (6, 10, 12). We recently identified two more binding partners of PILRα, neural proliferation differentiation and control-1 and collectin-12, and found that an evolutionarily conserved PILRα domain mediates its interaction with these diverse sialylated ligands (9). This suggests that a complex network of ligands might modulate cellular functions via PILRα. It was shown that PILRα binds to HSV-1 glycoprotein B and serves as a virus entry coreceptor (11, 13). Previous studies showed that PILRβ-deficient and agonist anti-PILRα–treated mice show improved clearance of infectious reagents and improved survival (14, 15). A recent study showed that PILRα negatively regulates neutrophil recruitment during TLR-mediated inflammatory responses (16). However, the role of PILRα or PILRβ in autoimmunity is largely unknown.

Myeloid cells play a critical role in the pathophysiology of inflammation and autoimmunity. Rheumatoid arthritis (RA) is a systemic, inflammatory, autoimmune disorder manifested by chronic polyarthritis with synovial hyperplasia and joint destruction, resulting in pain, loss of joint function, and concomitant reduction of life quality (17, 18). Myeloid cells, including monocytes/macrophages and neutrophils, play an important role in various stages of arthritis development (19, 20). To investigate the role of PILRα in myeloid cell–mediated immune responses and its effects on autoimmune diseases, we studied its function in myeloid-driven models of RA, including collagen Ab–induced arthritis (CAIA) and K/BxN serum–transfer arthritis, using PILRα-deficient mice and PILRα-specific mAbs. Murine CAIA is in-
duced by i.v. injection of mAbs against type II collagen, followed by i.p. injection of LPS. This model is widely used to study the pathogenesis of autoimmune arthritis and to determine efficacy of therapeutics (21–24). Myeloid cells, FcγRs (22), and proinflammatory cytokines, especially TNF-α and IL-1β (21), are indispensable for the development and maintenance of arthritis in this model (23). The K/BxN serum–transfer arthritis model is induced by transferring K/BxN serum into normal mice; LPS is not needed in this model. The K/BxN serum–transfer arthritis model shares multiple features with human RA, including symmetrical involvement of peripheral joints, pannus formation, synovial hyperplasia, and bone and cartilage degradation (25). We show that Pilra−/− mice developed more severe arthritis than did wild-type (WT) mice in these two models, corroborating the inhibitory role of PILRα during inflammation. Accordingly, we found that administration of an anti-PILRα mAb attenuated mouse inflammatory arthritis and reduced the production of several proinflammatory cytokines, indicating that PILRα is part of a critical inflammatory axis regulating myeloid cell function.

Materials and Methods

Mice

BALB/c mice were purchased from Charles River Laboratory. Pilra-deficient mice were generated by genOway and were maintained in-house. Mice were used at 6–10 wk of age. All animal experiments were performed in accordance with institutional animal care and use committee–approved protocols.

Generation of Pilra−/− mice

The genomic region containing the murine PILRα locus was isolated by PCR from 129Sv/BAC genomic DNA (genOway, Lyon, France). The 6050-bp 5′ homology arm corresponds to nt 137843309–137837259 cluster NC_000071.6 contig GRCm38.p2 in chromosome 5 of C57BL/6J. The 1872-bp 3′ homology arm corresponds to nt 137830963–137829901 of the same region. This strategy allows for deletion of a 6.3-kb region comprising exons 1 and 2 of PILRα (containing ATG, Ig V-set domain, and extracellular domain). Neomycin-resistance clones were first screened by PCR using primers 5′-ATGCTCCAGACTGTCCGCT-3′ (neocassette) and antisense 5′-CACCATGAGGAGCCCCTTCATCG-3′ (within 3′ homology arm) and 5′-CACCATGAGGAGCCCCTTCATCG-3′ (within 3′ homology arm), which generates a 6.3-kb fragment in WT mice) or forward primer (located in exon 2, which is deleted in the knockout) and reverse primer: forward primer-1: 5′-ATGACAGTTTGTGTCTGGATCAGAAC-3′ and reverse primer: forward primer-2: 5′-ATGACAGTTTGTGTCTGGATCAGAAC-3′ (upstream of the loxp site) and the same reverse primer, which generates a 600-bp product from the knockout allele.

Generation of mAbs

Anti-PILRα mAbs were generated from Armenian hamsters and Pilra−/− mice using immunogenic fusion proteins consisting of the extracellular domain of the mouse Pilra gene (GenBank accession number: NM_153510), including amino acid positions Met 1 to Val 197, as previously described (9). Mouse anti-PILRα mAbs are IgG1 isotype. The specificity of anti-PILRα mAbs was confirmed by ELISA and flow cytometry analysis. These mAbs do not cross-react with mouse PILRβ. Hamster anti-PILRα mAb was used for staining, and mouse anti-PILRα mAb was used for in vivo and in vitro functional studies.

Gene expression analysis

For gene expression analysis, total RNA was isolated using TRizol reagent (Invitrogen). Total RNA (50 ng) was subjected to RT-PCR using Access RT-PCR System (Promega). Primers used for amplification were as follows: β-actin, sense primer: 5′-TACCTCAGAAGACCTCA-3′ and antisense primer: 5′-TGTGCGATGACCACAGGAC-3′; PILRα, sense primer: 5′-CATGAGGACACTCTGTCCTAGACG-3′ and antisense primer 5′-AAGGTCATCTGAAGTGTCACCCACCAA-3′; and Pilra, sense primer: 5′-GGAAAATCCAGAAGATACACAC-3′ and antisense primer: 5′-CAGGGTGCATGAGGATGAT-3′. For quantitative RT-PCR, total RNA (50 ng) was subjected to RT and real-time quantitative PCR assay using Access RT-PCR System (Promega), Perkin Elmer SYBR Green, and an ABI 7500 instrument. Gene-specific unlabeled primers used were as follows: β-actin, sense primer: 5′-TACCTCAGAAGACCTCA-3′ and antisense primer: 5′-TGTGCGATGACCACAGGAC-3′; PILRα, sense primer: 5′-CACCATGAGGAGCCCCTTCATCGTC-3′ and antisense primer: 5′-TACCTCAGAAGACCTCA-3′.

Cell extraction and Western blot analysis

Bone marrow (BM) cells and spleenocytes were harvested, and RBCs were lysed with ACK lysing buffer. Cells were washed twice with ice-cold PBS (pH 7.4) and then treated in Cell Extraction Buffer (Invitrogen) containing 1 mM PMSF and protease inhibitor mixture (Sigma). Lysates were incubated on ice for 30 min with vortexing at 10-min intervals, and the insoluble material was removed by centrifugation at 13,000 rpm for 10 min at 4˚C. Soluble extracts with Novex Tris-Glycine SDS sample buffer were separated by SDS-PAGE using 4–12% Bis-Tris gel and transferred to a PVDF/Filter Paper Sandwich (all from Invitrogen). Blots were blocked with 5% nonfat dried milk, 0.1% Tween-20 in TBS buffer and incubated with hamster anti-mouse PILRα mAbs, followed by HRP-conjugated anti-hamster IgG (Jackson ImmunoResearch) for 1 h. Proteins were detected by chemiluminescence with an Amersham ECL Plus Western blotting system (GE Healthcare).

Immunohistochemistry

Immunohistochemistry for PILRα was performed on formalin-fixed, paraffin-embedded tissue using in-house–generated Armenian hamster anti-PILRα mAb and an automated system (VENTANA DISCOVERY XT, Ventana Medical Systems, Tucson, AZ). Briefly, 4-μm sections were rehydrated, endogenous peroxidase was blocked with 3% H2O2, and sections were incubated with rat anti-mouse CD16/CD32 (AbD Serotec, Oxford, UK) and CD117 (eBioscience) by incubation with hamster anti-mouse PILRα mAb, followed by HRP-conjugated anti-hamster IgG (Jackson ImmunoResearch). The specificity of hamster anti-PILRα mAb was confirmed by ELISA and flow cytometry analysis. These mAbs do not cross-react with mouse PILRβ. Hamster anti-PILRα mAb was used for staining, and mouse anti-PILRα mAb was used for in vivo and in vitro functional studies.

Flow cytometry

Single-cell suspensions were prepared from BM and peritoneal lavage fluid. After RBC lysis, cells were incubated with rat anti-mouse CD16/CD32 (mouse anti-CD16/CD32) (BD Biosciences) and stained with anti–PILRα mAb followed by allophycocyanin-conjugated anti-hamster IgG (R&D Systems). BM cells were further stained with PE-conjugated Ly6G and FITC-conjugated anti-CD11b. Peritoneal cells were stained with PE-conjugated Gr-1 and FITC-conjugated anti-CD11b or FITC-conjugated CD117 and PE-conjugated FceRI (all from BD Pharmingen). Cell acquisition was performed on a FACScalibur (Becton Dickinson, Mountain View, CA), and data were analyzed with FlowJo software.

Induction of CAIA

Arthritis was induced in 7–8-wk-old (age-matched for a given study) female C57BL/6N or BALB/c mice with an arthritogenic type II collagen cDNA mixture purchased from Chondrex. Pilra−/− and WT littermate mice received 4 mg a mixture of type-II collagen mAbs i.v. in 400 μl...
PBS on day 0, followed by 50 µg LPS i.p. in 100 µl PBS on day 3. To test the effect of anti-PILRα mAb on the development of CAIA, BALB/c mice received 2 mg Arthrogen-CIA Ab mixture i.v. in 200 µl PBS on day 0, followed by 50 µg LPS i.p. in 100 µl PBS on day 3. Mice were treated with 100 µg mouse anti-PILRα mAb or anti-ragweed mlgG1 isotype control i.p. daily in 100 µl sterile saline, starting on day −1. For K/BxN serum-transfer arthritis model, BALB/c mice were given 20 µl arthritisogenic K/BxN serum on days 0 and 2 by i.v. injection and were treated with 100 µg mouse anti-PILRα mAb or anti-ragweed mlgG1 isotype control i.p. daily in 100 µl sterile saline starting on day −1. Swelling of the paws was monitored for 11–12 d. Each paw was assigned a score based on the following scale: 0 = no evidence of erythema and swelling; 1 = erythema and mild swelling confined to the midfoot (tarsal) or ankle; 2 = erythema and mild swelling extending from the ankle to the metatarsal joints; and 3 = erythema and moderate swelling extending from the ankle to the metatarsal joints; and 4 = erythema and severe swelling encompass the ankle, foot, and digits. The clinical score of each mouse is the sum of the four paw scores.

Histopathological assessment

Paws were removed, fixed in formalin, decalcified, and processed to paraffin sections stained with H&E. Sagittal sections were examined by light microscopy and scored on arbitrary scales ranging from 0 to 5 for features of arthritis, including number of affected joints, extent of inflammation, extent of soft tissue proliferation including pannus, cartilage injury, and bone remodeling. Immunoreactivity for MPO or F4/80 was evaluated on arthritic paws by microscopic examination of affected synovial structures (joints, tendon sheaths). Intensity of infiltration with immunoreactive cells was estimated using an arbitrary scoring scheme ranging from 0 to 3, according to the following criteria: 0 = none detected near or in synovial structures; 1 = few, scattered positive cells near or in synovial structures; 2 = frequent disseminated or loosely clustered positive cells near or in synovial structures; and 3 = locally extensive infiltration with numerous positive cells near or in synovial structures.

Microcomputed tomography imaging

Microcomputed tomography (micro-CT) imaging was performed using a previously described micro-CT and automated analysis technique (26) to quantify bone destruction in the arthritis mouse model. Briefly, the paws were scanned ex vivo on a micro-CT 40 scanner (Scanco Medical, Brüttisellen, Switzerland). The analysis algorithm automatically locates five metatarsophalangeal joints and three metacarpophalangeal joints and segments the cortical bone and periosteal new bone. Joint cortical bone volume (JCBV) is sensitive to cortical bone erosion at the joints, and periosteal new bone volume (PNBV) estimates the amount of periosteal new bone formation that results from a repair response to the bone erosion. The analysis technique was modified in such way that JCBV, as well as the PNBV estimate, was limited to the joints.

Measurement of cytokine concentration in arthritic hind paws

Hind footpads were obtained from mice by cutting at the borderline of fur growth. The footpads were hammer smashed and shaken at 20 Hz for 30–60 min with 5-mm steel beads at 4˚C in ice-cold cell lysis buffer (Cell Signaling Technology) containing Protease Inhibitor Cocktail Set I (Calbiochem). Homogenized tissue was kept on ice for 30 min, vortexed occasionally, and then spun down at high speed for 15 min at 4˚C. Supernatants were collected, and all samples were frozen at −80˚C and thawed to room temperature before analysis. Cytokine levels were analyzed by ELISA (R&D System). The concentration of total protein in the supernatants was measured using a Pierce BCA kit and was normalized against the concentration of BSA. The concentration of cytokines was expressed as pg/mg of protein.

In vitro stimulation of BM-derived DCs

BM-derived DCs (BMDCs) were generated as described elsewhere (27). In brief, femurs of mice were removed, and BM was flushed out with PBS containing 10% FCS. RBCs were lysed with ACK lysis buffer. Cells were cultivated in bacterial culture dishes for 9–10 d at 37˚C in a humidified
atmosphere containing 5% CO₂, using RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml 2-ME (Sigma; Taufkirchen, Germany), and 20 ng/ml recombinant mouse (m)GM-CSF (R&D Systems). At days 9–10, the nonadherent cells were harvested and stimulated with mLgG (60 μg/ml; Jackson ImmunoResearch Laboratories) plus various concentrations of mouse anti-mouse PILRα mAb coated on Protein G Agarose beads (Roche) for 18 h. Cytokine production in supernatants was detected by ELISA (R&D) or Luminex (Bio-Rad).

**PILRα and PILRβ expression in synovial tissues of RA and osteoarthritis patients**

Patients and synovial tissues. Synovial tissues were obtained from RA subjects undergoing arthroplasty and/or synovectomy of affected joints (University of Michigan, two sequential cohorts). RA was diagnosed based upon the 1987 American College of Rheumatology criteria (28). Patients were treated using the standard of care for RA (nonsteroidal anti-inflammatory drugs and disease-modifying antirheumatic drugs), and some patients also were treated with biologics (adalimumab, etanercept, infliximab, anakinra, and rituximab). Patients were diagnosed with RA ≥3 y before surgery, and 70% of patients for whom data were available were positive for rheumatoid factor. All procedures to collect human specimens were performed under a protocol approved by the University of Michigan Institutional Review Board. Excised tissues were immediately snap-frozen in liquid nitrogen and stored at −80°C. Each tissue was used for both histology and RNA extraction. For cryosectioning, samples were brought briefly to 20°C, sectioned, and immediately returned to −80°C to maintain RNA integrity. All tissues used for downstream studies were prospectively randomized during processing and sectioning, prior to expression analysis, to minimize technical batch effects in the data.

**RNA isolation.** Frozen samples were weighed and homogenized in RLT (QiAGEN, Valencia, CA) + 2-ME (10 μl/ml) at a concentration of 100 mg/ml. RNA was isolated using an RNeasy Mini Kit (QIAGEN) with on-column DNase digestion.

**Microarray hybridization.** The protocols for preparation of cRNA and for array hybridization were followed as recommended by Affymetrix (Santa Clara, CA). Samples were hybridized to GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix). Arrays were washed and stained in the Affymetrix Fluidics station and scanned on a GeneChip scanner 3000. Expression signals were obtained using the Affymetrix GeneChip operating system and analysis software. Data have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus under accession no. GSE48780 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48780).

**Computational analysis.** Computational analysis was carried out using the R programming language (29) and libraries from the open-source Bioconductor project (30). Quality assessment of microarray data was conducted using the Bioconductor affy package (31), and the expression data were normalized using the robust multia array average method (32, 33). PILRα and PILRβ are each represented by two probes on the Affymetrix U133Plus arrays.

To compare the expression levels of PILRα and PILRβ in RA and osteoarthritis (OA) samples, we used a linear model with terms for diagnosis (OA or RA) and inflammation status (inflamed or noninflamed) based on histology. Conceptually, this can be thought of as a t test comparing OA and RA expression with correction for inflammation status. The limma Bioconductor package was used for the computation (34).

**PILRα ligand screen**

Recombinant human PILRα-Fc was screened for interacting partners using the Genentech human full-length cDNA expression library. COS-7 cells were transfected with 6 ng/ml individual cDNA expression vectors and FaGene 6 (Roche) in a 384-well format. Forty-eight hours after transfection, COS-7 cells were exposed to 10 ng/ml PILRα-Fc, followed by fixation in 4% formaldehyde. Protein–protein interactions were detected upon binding of an Alexa Fluor 488–conjugated anti-mIgG2a secondary Ab (Invitrogen) to the cell surface using an MD Isocyte plate scanner or, in follow-up experiments, using a GE IN Cell 2000 Analyzer.

To confirm the binding of screening hit to PILRα, HEK 293T cells were transfected with C-type lectin domain family 4 (CLEC4G) cDNA, and transfec tants were stained with human (h)PILRα-Fc/mPILRα-Fc, hPILRαR126A-Fc/mPILRαR133A-Fc, followed by FITC-conjugated anti-mIgG2a. Cell acquisition was performed on a FACSCalibur (Becton

**FIGURE 2.** Expression of PILRα in various tissues and cells. (A) Immunohistochemistry of normal murine tissues. Tissue sections from WT mouse spleen, liver, lung, and BM were stained with anti-PILRα Ab and compared with control tissues from Pilra−/− mice. Spleen red pulp and tissue-resident macrophages, such as Kupffer cells in liver (inset) or exfoliated alveolar macrophages (inset) in lung, express PILRα. In BM, most PILRα− cells are identified as granulocytes or their precursors. Scale bars, spleen = 500 μm; liver and lung = 50 μm; BM = 20 μm. (B) PILRα is expressed on murine granulocytes and monocytes from BM and peritoneal macrophages, neutrophils, and mast cells, but it is not expressed on spleen lymphocytes. Data are representative of three experiments.
Dickinson, Mountain View, CA), and data were analyzed with FlowJo software.

Statistical analysis

Statistical significance was determined by the parametric Dunnett test for the comparison of joint cytokine concentrations, by the nonparametric Dunnett test for the comparison of arthritis scores and histopathological as well as micro-CT results, and by Student t test for the comparison of in vitro cytokine data. The p values < 0.05 were considered significant.

Results

Generation and characterization of Pilra−/− mice

To generate the PILRa-knockout mouse, exon 1 and exon 2 of the PILRa gene were deleted by homologous recombination in C57BL/6J ES cells (Fig. 1A), and the targeted locus was confirmed by Southern blot analysis using internal (Fig. 1B, left panel) or external (Fig. 1B, right panel) probes, as described in Materials and Methods. Chimeric mice were bred to C57BL/6N mice to generate PILRa-deficient mice on a pure genetic background. Given the close proximity of PILRβ, the neo-pgk cassette was removed by breeding PILRa-knockout mice to Rosa-26-cre knockin mice to avoid nonspecific effects on PILRβ or other surrounding genes (35). RT-PCR analysis revealed that PILRa mRNA production was abolished in the spleen and BM of Pilra−/− mice, whereas PILRβ expression was not affected (Fig. 1C). Real time RT-PCR was done to better quantitate PILRβ levels in WT and Pilra−/− mice. These expression studies showed that PILRβ expression in spleen and BM was not affected (Fig. 1D). PILRa protein was abundant in WT BM and was detected in spleen at lower levels. However, in Pilra−/− mice, no PILRa protein was detected in either BM or spleen, and Pilra+/− heterozygous mice displayed intermediate levels of protein (Fig. 1E). Pilra−/− mice are viable, fertile, and have a normal lifespan. They did not show...
any appreciable developmental defects in myeloid or lymphoid cell populations (Supplemental Table 1).

Previous work showed that Pilra transcripts are relatively high in spleen, liver, and lung (8). We tested Pilra protein expression in these tissues by immunohistochemistry (Fig. 2A) using Pilra−/− mice as a negative control and in-house–generated Armenian hamster anti-mPilra mAb, which did not show cross-reactivity to mPILRβ. In the spleen, Pilra+ cells are mainly localized in the red pulp, consistent with expression predominantly by myeloid cells. In liver and lung, Pilra is expressed in tissue-resident macrophages, such as Kupffer cells or exfoliated alveolar macrophages, respectively. In BM, most Pilra+ cells are identified as granulocytes or their precursors. These data corroborate the notion that Pilra is expressed primarily in cells of the myeloid lineage. Correspondingly, Pilra protein is detected on granulocytes (CD11b+Ly6G+) and monocytes (CD11b+Ly6G−) from BM, as well as peritoneal cavity macrophages (CD11b+Gr-1−), neutrophils (CD11b+Gr-1+), and mast cells (CD117+FcεR1+), but not on spleen lymphocytes, including T, B, NKT, and NK cells (Fig. 2B).

Pilra−/− mice develop enhanced autoimmune arthritis

To investigate the biological function of Pilra in myeloid cell–mediated immune responses and its role in autoimmune diseases, we made use of a CAIA model, which is a myeloid-dependent preclinical disease model. We observed an infiltration of Pilra-producing cells in mouse arthritic tissues, specifically periarticular parameter, including number of affected joints/paw, periarticular infiltration with mixed inflammatory cells, soft tissue proliferation (including pannus formation), cartilage injury, and bone remodeling (Fig. 3C, 3D). Consistent with the histological evidence for more extensive bone remodeling, micro-CT revealed more severe osteolysis (i.e., smaller JCBV), as well as increased new bone formation (i.e., greater PNBV), in Pilra−/− mice compared with WT mice (Fig. 3E). These results support the regulatory role of Pilra in downregulating myeloid-dependent inflammation.

PILRa deficiency results in an increase in intra-articular proinflammatory cytokine levels at the onset of arthritis

To better understand how Pilra deficiency impacts CAIA, we tested inflammatory cytokine levels in joint tissue at the onset of disease. Onset of disease was similar in WT and Pilra−/− mice, but they exhibited more severe disease, with maximum average clinical scores and end point clinical scores compared with WT mice (Fig. 3B, middle and right panels).

On termination of the experiment at day 12, histopathology confirmed the clinical observations. Compared with WT mice, Pilra−/− mice had more severe disease, as shown by all evaluated parameters, including number of affected joints/paw, periarticular infiltration with mixed inflammatory cells, soft tissue proliferation (including pannus formation), cartilage injury, and bone remodeling (Fig. 3C, 3D). Consistent with the histological evidence for more extensive bone remodeling, micro-CT revealed more severe osteolysis (i.e., smaller JCBV), as well as increased new bone formation (i.e., greater PNBV), in Pilra−/− mice compared with WT mice (Fig. 3E). These results support the regulatory role of Pilra in downregulating myeloid-dependent inflammation.

**FIGURE 4.** Elevated levels of inflammatory cytokines were detected in joints of Pilra−/− mice at the onset of disease. (A) C57BL/6N WT (n = 9) and Pilra−/− mice (n = 9) were given arthritogenic anti–type II collagen mAb mixture to induce arthritis (CAIA). On day 5, hind footpads were obtained from mice for homogenization in cell lysis buffer containing protease inhibitors. Cytokine levels in lysate supernatants were analyzed by ELISA. The cytokine concentrations are expressed as pg/mg of protein. (B) Kinetics of neutrophils and macrophages in the early phase of CAIA show a similar degree of infiltration in arthritic joints of Pilra−/− and WT mice. WT (dark gray bars, n = 4) and Pilra−/− mice (light gray bars, n = 4) were given arthritogenic anti–type II collagen mAb mixture to induce arthritis. On days 3, 4, and 5, paws were collected for immunohistochemical staining of MPO and F4/80. Intensity of infiltration was estimated using an arbitrary scoring scheme ranging from 0 to 3. Bars represent mean of four arthritic paws. Error bars are SEM. Results are representative of two or three separate experiments. The p values are based on the parametric Dunnett test.
jection of type II collagen–specific Abs. At this time point, hind paw lysates of Pilra−/− mice exhibited significantly increased production of proinflammatory cytokines, including IL-1β and IL-6, as well as neutrophil and monocyte chemokines KC and MCP-1 (Fig. 4A). The increased inflammatory cytokine and chemokine production could be due to either increased inflammatory cell number or activity. It was shown that PILRα plays an important role during acute inflammatory responses in regulating neutrophilic migration (16). We then asked how cellular infiltrates are impacted in the ankle joints at early time points each day from day 3 to day 5 using immunohistochemistry MPO to stain neutrophils or F4/80 to localize macrophages (Fig. 4B). Neutrophils were slightly reduced in PILRα-knockout joints on day 3; however, their numbers were transiently increased on day 4 compared with WT mice. Macrophages in PILRα-knockout joints also showed a similar trend on day 3; however, their numbers were slightly increased on day 5. Overall, the changes in macrophage and neutrophil cellularities were not statistically significant between WT and Pilra−/− mice (Fig. 4B). The slight reduction in neutrophil/macrophages in Pilrα-knockout mice on day 3 might be secondary to the systemic effect of LPS on the increased migration of these cells to other tissues, such as liver, in these mice (16). In addition, we did not observe any difference between WT and Pilra−/− neutrophil and macrophage cellular infiltrates in an acute localized inflammatory model of Staphylococcus aureus (Supplemental Fig. 1). These observations support the notion that the lack of PILRα has a global effect on the severity of arthritis, mainly through enhanced cytokine production. In summary, our data suggest that the joint-infiltrating Pilra−/− myeloid cells are functionally hyperresponsive, further highlighting the regulatory role of PILRα in myeloid cells.

Engagement of PILRα suppresses the production of inflammatory cytokines and chemokines

To confirm the regulatory role of PILRα in modulating cellular function, we tested how the production of cytokines and chemokines is affected in Pilra−/− cells or upon its engagement in vitro. We used various PILRα-expressing cell types, such as BMDCs, BM-derived monocytes (BMDMs), and neutrophils, which are implicated in the pathology of anti-CAIA. First, we tested how these cells respond to LPS, which is used to activate myeloid cells in the same CAIA model. The results showed that Pilra−/− BMDCs produced increased TNF-α and MCP-1 in response to LPS but not IL-1β, IL-6, or KC (Supplemental Fig. 2). A subtle trend of cytokine elevation was seen in neutrophils and BMDMs (Supplemental Fig. 2). The state of cellular hyperresponsiveness, as measured by cytokines/chemokines, appears to vary in different PILRα-expressing cells. BMDCs were more sensitive to this pathway, which might be explained by the high expression of PILRα (Fig. 5A) in these cells.

We then asked how engagement of PILRα receptor in BMDCs modulated cytokine production. To couple stimulatory and putative inhibitory pathways, we used mlgG-coated beads as the stimulus. WT and Pilra−/− BMDCs produced similar levels of the proinflammatory cytokine TNF-α and chemokine MCP-1 in response to mlgG-coated beads (Supplemental Fig. 2). However, stimulation of BMDCs with beads coated with anti-mPILRα mAb, but not isotype control, showed a dose-dependent inhibition of TNF and MCP-1 production in WT cells. The inhibitory effect of anti-PILRα Ab was not seen in the BMDCs derived from Pilra−/− cells, again confirming that the inhibitory effect is PILRα dependent (Fig. 5B). This result indicates that engagement of PILRα downregulates proinflammatory cytokine and chemokine production when it is proximal to an activating receptor, such as FcyR.

A recent study showed that PILRα associates with its ligands in cis on neutrophils (16). Because BMDCs express both PILRα and its ligands (Supplemental Fig. 3A), we predicted that PILRα would interact with its ligands in cis, and this may play a role in negatively regulating BMDC function. If this were the case, dissociation of the interaction between PILRα and its ligands would increase BMDC cytokine production. We showed previously that sialic acid is an essential component of PILRα ligands in primary cells, and treatment of primary cells with sialidase A, which cleaves sialic acid from surface proteins, abolished their binding to PILRα (9). Therefore, we used sialidase A treatment to dissociate PILRα–ligand interactions on BMDCs. We found that sialidase A treatment increased cytokine production by BMDCs in response to mlgG coated on protein G beads, suggesting that the interaction of sialylated ligands with their receptors inhibits cellular function (Supplemental Fig. 3B). Given the fact that PILRα is able to interact with a network of ligands expressed by different tissues, including hematopoietic cells other than myeloid cells (9), cis and trans interactions may both be involved in the regulation of PILRα-expressing myeloid cell function.

Anti-PILRα mAb treatment attenuates arthritis

We then tested whether treatment with anti-PILRα mAb affects inflammation in vivo using the CAIA model. Because C57BL/6N WT mice develop only mild disease, we used BALB/c mice instead for anti-PILRα mAb treatment. We found that anti-PILRα mAb treatment significantly reduced inflammation compared with isotype control treatment (Fig. 6A). Isotype control–treated mice started to develop clinical signs of arthritis 4 d after injection of type II collagen–specific Ab mixture or 2 d after LPS administration and reached maximum clinical scores of 14.6 ± 2.2 (mean ± SD) between days 5 and 7. By contrast, anti-PILRα mAb-
treated mice exhibited delayed disease onset, which gradually increased to a much reduced maximum clinical score of 4.4 ± 4.3 (mean ± SD) by the end of the study. Anti-PILRα mAb–treated mice also showed significantly reduced average clinical scores and end point clinical scores compared with isotype control–treated mice (Fig. 6A, middle and right panels). In accordance with in vivo findings, histopathological assessment of hind paws at the termination of the study at day 11 showed significantly decreased scores for all scored features in anti-PILRα mAb–treated mice compared with isotype control–treated mice (Fig. 6B).

Finally, micro-CT imaging of paws at day 11 showed that anti-PILRα mAb–treated mice exhibited less severe bone remodeling compared with isotype control Ab–treated mice (Fig. 6C), as measured by JCBV and PNBV (p < 0.01 for both parameters). The observation that anti-PILRα treatment attenuates arthritis development, whereas Pilra−/− mice show enhanced disease, suggests that the anti-PILRα mAb does not block the receptor but rather engages it. To measure the impact of PILRα modulation on other arthritis models, we tested the effect of anti-PILRα treatment on mouse K/BxN serum–transfer arthritis. The K/BxN serum–transfer model is a widely accepted model of inflammatory arthritis that shares features of human RA (25). In K/BxN mice, the induction of disease does not require LPS, and this disease depends highly on the innate cells in a TNF-independent fashion (36–39). Mice started to develop clinical signs of arthritis 1 d after K/BxN serum transfer. Isotype control–treated mice reached maximum clinical scores of 14.7 ± 1.0 (mean ± SD), whereas anti-PILRα mAb–treated mice exhibited a reduced maximum clinical score of 8.5 ± 4.0 (mean ± SD) between days 5 and 7 (Fig. 6D, left panel). Anti-PILRα mAb–treated mice also showed significantly reduced average clinical scores and terminal clinical scores compared with isotype control–treated mice (Fig. 6D, middle and right panels). Additional studies are required to understand how PILRα is relevant in the context of additional inflammatory diseases.

PILRα is expressed in human inflammatory arthritic tissues and correlates with monocyte and DC levels

To test whether PILRα is involved in human inflammatory arthritis, we assessed the expression patterns of PILRα and its counterpart PILRβ using mRNA microarrays on human synovial tissue. Samples were obtained during joint resection surgery from OA and RA patients. Sections were taken for both histological observation and mRNA extraction. The Affymetrix expression microarrays included two probes each for PILRα and PILRβ. PILRα consistently showed significantly higher expression in the RA patients compared with the OA patients, including a correction for inflammation status (p < 1e−7) (Fig. 7A, upper panels). In contrast, PILRβ showed a modestly higher level of expression in OA patients (p < 0.02) (Fig. 7A, lower panels). The differential expression of PILRα and PILRβ in inflammatory RA suggests that PILRα might function as a negative-feedback regulatory loop to dampen inflammation. Cell-specific gene signatures can be used as an estimate of cellularity (40). For each cell type, we calculated a summary gene set score

**FIGURE 6.** Anti-PILRα mAb treatment attenuates arthritis development. (A–C) BALB/c mice were given arthritogenic anti–type II collagen mAb mixture to induce arthritis (CAIA) and were treated with anti-PILRα mAb (n = 5). (A) Anti-PILRα mAb–treated mice exhibited reduced disease development compared with isotype control. Anti-PILRα–treated mice showed decreased average clinical score (left panel), average daily clinical score (days 4–11, middle panel), and end point clinical score (day 11, right panel) compared with isotype control–treated mice. (B) Pathological scores of isotype control and anti-PILRα mAb–treated mice on day 11. Data points represent average scores for two paws/mouse. Two limbs per animal, four to six sections/animal, and a total of 10 mice were examined. (C) Hind paws from mice harvested on day 11 were subjected to micro-CT analysis. Images show high-resolution three-dimensional rendering of micro-CT scans. Quantification of bone integrity, including JCBV and PNBV. (D) Anti-PILRα mAb treatment inhibited disease development in a K/BxN serum–transfer arthritis model. BALB/c mice were given 20 μl of arthritogenic K/BxN serum on days 0 and 2 by i.v. injection and were treated with anti-PILRα mAb (n = 6). Mean clinical score (left panel), average daily clinical score (middle panel), and day-7 clinical score (right panel) are shown. In vivo arthritis scores and micro-CT parameter estimates for the treatment groups were compared with the control group using the Dunnett test. Results are representative of two experiments.
using a quartile-trimmed mean of the normalized probe set values present in the gene set. This summary score provides an estimate for the relative abundance of the given cell type across samples. Correspondingly, we found that PILRa expression was highly correlated with the tissue-specific gene sets obtained from different inflammatory cell types, such as DCs (Spearman $r = +0.7$, $p < 2e^{-16}$), neutrophils ($r = +0.5$, $p < 2e^{-5}$), and monocytes ($r = +0.8$, $p < 2e^{-16}$) in RA samples (Fig. 7B, upper panels). In contrast, PILRb expression was minimally inversely correlated with DC-specific and monocyte-specific genes ($r = -0.2$ for both, $p < 0.01$), and it was not significantly correlated with neutrophil-specific genes ($r = -0.1$, $p < 0.3$) (Fig. 7B, lower panels). These trends held true when we considered all samples together or RA and OA samples in isolation. Our human expression study shows that PILRa expression may dissociate from PILRb, and this inhibitory pathway is overrepresented in myeloid cells in human RA.

**Diverse ligands are involved in PILRa pathway**

We (9) and other investigators (8, 10, 41) reported that PILRa engaged multiple ligands with common molecular determinants.

**FIGURE 7.** PILRa-inhibitory versus PILRb-activating pathways in human RA joints and their correlation with pathogenic cell types. Synovial samples were taken from RA ($n = 81$) and OA ($n = 121$) patients during joint resection surgery. (A) The Affymetrix expression microarrays included two probes each for PILRa and PILRb. PILRa expression is significantly higher ($p < 1e^{-07}$) in RA patients compared with OA patients; this also holds when corrected for inflammation status. PILRb expression is modestly higher in OA patients compared with RA patients ($p < 0.02$), correcting for inflammation status. (B) The Affymetrix expression microarrays of PILRa and PILRb. In RA patients, PILRa expression is significantly correlated with DC-specific genes ($r = +0.7$, $p < 2e^{-16}$), neutrophil-specific genes ($r = +0.5$, $p < 2e^{-5}$), and monocytes ($r = +0.8$, $p < 2e^{-16}$). This trend also holds true for all of the inflammatory RA subset of patients. In contrast, PILRb expression was not positively correlated with DC, neutrophil, or monocyte gene expression ($r = -0.2$, $p = 0.01$; $r = -0.1$, $p = 0.2$; and $r = -0.2$, $p < 0.01$). Scores were calculated using a quartile-trimmed mean of the normalized probe set values present in the gene set.
Discussion

In this study, we reported that PILRα represents a potent inhibitory receptor that negatively regulates the function of myeloid cells to control inflammation in mouse arthritis models. PILRα is predominantly expressed by macrophages, DCs, and neutrophils, all of which are important in the pathogenesis of arthritis. Using PILRα-knockout mice in a CAIA model, we demonstrate that the absence of PILRα leads to dysregulation of inflammatory processes in inflamed tissues, resulting in increased inflammatory cytokine production and severity of disease. Conversely, engaging PILRα with an anti-PILRα mAb reduces disease severity in both CAIA and K/BxN serum-transfer models.

In preclinical arthritis models, as well as in human inflammatory arthritis, PILRα gene expression profiling supports the notion that this receptor acts in a counter-regulatory fashion to dampen inflammation. It is interesting that PILRα (inhibitory receptor), but not PILRβ (activating receptor), expression is positively correlated with monocytes or neutrophil gene signatures in human RA, suggesting that this pathway is highly regulated via its ligands to possibly keep these inflammatory cells in check. The usefulness of the PILRα pathway in human RA or additional inflammatory models requires addition studies.

A complex network of ligands expressed in a variety of tissues has been implicated in PILRα interactions (8–10). Although we do not fully understand the consequence of the interaction of PILRα with individual ligands, it is interesting that a conserved domain in PILRα is critical for its interaction with all known ligands, a property shared with the Siglec family of inhibitory receptors (9, 43). A recent study showed that PILRα interacting domain in cis engaged its ligands in the same cell (16). The new ligand CLEC4G is expressed in macrophages, it might be able to interact in cis with PILRα. CLEC4G is implicated in Ebola virus entry receptor, as well as clearance and presentation of various Ags (44). Given the complexity of the PILRα receptor/ligand network, PILRα appears to have a diverse function in that it fine tunes the immune response depending on inflammatory cues, similar to other paired receptors (45). Additional studies are required to determine how cis/trans interactions of PILRα ligands are modulated during inflammation where this pathway is up-regulated.

In the context of inflammatory arthritis, the main effect of PILRα knockout is identified as an enhanced production of pro-inflammatory cytokines, resulting in more severe disease. At the onset of arthritis, PILRα-knockout mice produced higher levels of proinflammatory cytokines, such as IL-6 and IL-1β, as well as chemokines, such as KC and MCP-1, in joint tissues, even though both WT and Pilra−/− cellular infiltrates in arthritic joints were similar in WT and Pilra−/− mice by histopathology. This suggests that, in the absence of PILRα, myeloid cells infiltrating the joints are hyperresponsive, supporting a regulatory role for PILRα in these cells. Although the myeloid cells in joint tissues at the onset of disease are hyperresponsive in terms of proinflammatory cytokine production, we did not detect robust hypersensitivity of PILRα-expressing cells in the absence of PILRα in vitro. Pilra−/− BMDCs produced mildly, but consistently, increased levels of TNF and MCP-1 in response to LPS, whereas WT and Pilra−/− BMDCs produced similar levels of these cytokines in response to mlgG binding to Protein G beads (Supplemental Fig. 2). For BMDCs, Pilra−/− mice showed a trend toward increased proinflammatory cytokine and chemokine production in response to LPS (Supplemental Fig. 2). Therefore, the cumulative effect of this augmented response may explain the robust phenotype in the context of experimental arthritis. The inhibitory effect of PILRα receptor mediated by Ab cross-linking in vitro suggests that, when proximal, stimulatory pathways, such as FcγR, can be diminished. PILRβ was shown to promote cytokine production in myeloid cells, and PILRβ−/− mice and BMDMs produced fewer proinflammatory cytokines (14). In addition, PILRα is able to regulate neutrophil infiltration during acute inflammation via modulation of integrin activation in an acute inflammatory peritonitis model (16), in which PILRα-knockout mice show increased infiltration of neutrophils upon thioglycollate challenge (16). In contrast, in CAIA we did not see a difference between WT and PILRα-knockout mice with regard to the number of neutrophils and macrophages in arthritic joints. In support of this finding, we did not observe any difference between WT and Pilra−/− neutrophil and macrophage cellular infiltrates in an acute localized inflammatory model of S. aureus (Supplemental Fig. 1). Despite these differences, both studies suggest that PILRα plays a negative regulatory role in the inflammatory process, likely by complex mechanisms under different inflammatory cues. Our findings suggest that, in the context of sterile inflammation, PILRα is a regulatory receptor that modulates inflammatory cytokine responses; however, we cannot rule out that PILRα might have additional functions outside of cytokine production to affect inflammatory responses.

Anti-PILRα treatment reduced inflammatory arthritis. Consistent with this observation, engaging PILRα on BMDCs, which highly express PILRα on their surface, inhibits their function when a stimulating receptor, such as FcγR, is coengaged. Thus,
PILRα-knockout and anti-PILRα-agonizing studies complement each other in demonstrating the regulatory effect of this factor in two rodent models of RA. Similarly, engagement of PILRα reduces serum or bronchoalveolar lavage fluid levels of proinflammatory cytokines, including IL-1β, TNF, and IL-6, in a model of S. aureus–induced pneumonia (14). Further studies are required to understand what counter-regulatory receptors might specifically interact with PILRα to modulate its function in myeloid cells and how this pathway contributes to other inflammatory diseases. The contribution of the PILRα pathway may vary, depending on the type of myeloid cell, inflammation type and stage, stimulation strength, and the ligand involved. Thus, PILRα is emerging as an important regulatory pathway in myeloid cells and, therefore, is an attractive target in arthritis or potentially other inflammatory diseases.

Disclosures

The authors have no financial conflicts of interest.

References


SUPPLEMENTAL MATERIAL

**PILRα negatively regulates mouse inflammatory arthritis**


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Running title: PILRα regulates inflammation

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The percentage and absolute numbers of T cell subsets in the thymus and the percentage of various cell types and total cell numbers in other tissues are shown. The values are the mean of 3 animals for each genotype with STDs.
Supplementary Figure 1. *Pilrα*−/− and WT mice show similar neutrophil and macrophage infiltration upon *Staphylococcus aureus* infection. *Pilrα*−/− and WT littermates (*n*=5) were intraperitoneally infected with 1X10^8 CFU USA300 NRS384 *Staphylococcus aureus*. The recruitment of neutrophils (Gr-1⁺Mac-1⁺) and macrophages (Gr-1⁺Mac-1⁺) was analyzed in the peritoneal lavage at 2h and 6h post infection. The percentage (A, Mean±SEM) and cell count (B) of macrophages and neutrophils were shown.
**Supplementary Figure 2.** Cytokine production by various cell types in Pilra−/− and WT. BMDC (generated in the presence of 20 ng/ml of rmGM-CSF for 9 days), Neut (isolated from bone marrow with Miltenyi mouse Neutrophil isolation kit) or BMDM (generated in the presence of 10 ng/ml of rmM-CSF for 8 days) from WT (open dots) or Pilra−/− (closed dots) were stimulated with various doses of LPS or mouse IgG coated on protein G beads for 18 hours. The various cytokines and chemokines were measured by luminex. The results are representative of three experiments, using three mice per group.
Supplementary Figure 3. Removal of Sialic acid increases BMDC cytokine production. (A) BMDC express both PILRα and PILRα ligand. (B) WT BMDCs were treated with/without Sialidase A and then stimulated with various doses of mlgG coated on protein G agarose beads for 18 hrs, and cytokine production in the supernatants was detected by Luminex.