Gain-of-function mutations in a member of the Src family kinases cause autoinflammatory bone disease in mice and humans

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Autoinflammatory syndromes are characterized by dysregulation of the innate immune response with subsequent episodes of acute spontaneous inflammation. Chronic recurrent multifocal osteomyelitis (CRMO) is an autoinflammatory bone disorder that presents with bone pain and localized swelling. Ali18 mice, isolated from a mutagenesis screen, exhibit a spontaneous inflammatory paw phenotype that includes sterile osteomyelitis and systemic reduced bone mineral density. To elucidate the molecular basis of the disease, positional cloning of the causative gene for Ali18 was attempted. Using a candidate gene approach, a missense mutation in the C-terminal region of Fgr, a member of Src family tyrosine kinases (SFKs), was identified. For functional confirmation, additional mutations at the N terminus of Fgr were introduced in Ali18 mice by CRISPR/Cas9-mediated genome editing. N-terminal deleterious mutations of Fgr abolished the inflammatory phenotype in Ali18 mice, but in-frame and missense mutations in the same region continue to exhibit the phenotype. The fact that Fgr null mutant mice are morphologically normal suggests that the inflammation in this model depends on Fgr products. Furthermore, the levels of C-terminal negative regulatory phosphorylation of Fgr Ali18 are distinctly reduced compared with that of wild-type Fgr. In addition, whole-exome sequencing of 99 CRMO patients including 88 trios (proband and parents) identified 13 patients with heterozygous coding sequence variants in FGR. In vitro experiments revealed that Ali18 mouse strain was isolated in the Munich ENU mutagenesis project because of paw inflammation (Fig. L4) (17). Ali18 mice show synovitis, sterile osteomyelitis, and systemic reduced bone mineral density, particularly in trabecular areas of long bones (17). Because these phenotypes are reconstituted by bone marrow transfer and are independent of mature lymphocytes (15), Ali18 mice are considered a mouse model of autoinflammatory bone disease. Although the Ali18 locus was mapped to mouse chromosome 4 by standard genetic mapping, complex modifier effects hinder its precise determination (19). In this study, positional candidate cloning identified Fgr, a member of Src family kinases, as the causative gene.

Significance

Chronic recurrent multifocal osteomyelitis (CRMO) is an autoinflammatory bone disease that presents with bone destruction occurring primarily in children. In a mouse ENU mutagenesis screen, the Ali18 strain was isolated because of spontaneous inflammation in the joints and bones. Sequencing candidate genes in the Ali18 critical region identified a missense mutation in the C-terminal regulatory region of the Src family kinase (SKF) member, Fgr. Genome editing revealed Fgr dependency of the inflammatory phenotype in Ali18 mice. Further, whole exome sequencing in our CRMO cohort identified two patients with missense mutations in FGR. In vitro functional assays confirm altered protein function. This work identifies FGR as a CRMO susceptibility gene and suggests that targeting SFKs may be useful in its treatment.

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for Ali18. Further, two other missense mutations in FGR were found in our cohort of patients with CRMO.

**Results**

**Ali18 Mice, Fine Mapping, and Candidate Resequencing.** By standard genetic mapping, we narrowed down the critical region to ~3 Mb utilizing recombination between wild-type and heterozygous/homozygous genotypes (Fig. 1B and SI Appendix, Table S1). In the identified region, we focused on 16 candidate genes using a literature-based search engine, PosMed (20). By sequencing the exonic region of these genes by the Sanger method, we found a c.1506A > G, in the protein coding region of the Gardner-Rasheed feline sarcoma viral (v-Fgr) oncogene homolog (Fgr) gene (Fig. 1C). Digestion of PCR products encompassing exon 13 of Fgr by Mbo II restriction enzyme, which recognizes the wild-type allele (5'-GAAGG-3') but not c.1506A > G (5'-GAAAGG-3'), produces longer DNA Fragments in Ali18 mice (Fig. 1D). Genotype–phenotype correlations revealed that the mutation was not present in unaffected littermates or wild-type controls.

A Member of SFK as the Top Candidate for the Causative Gene. Fgr is a member of the Src family kinases (SFKs), which share the SH2, SH3, and catalytic domains with high homology to other family members (22), and the c.1506A > G mutation causes an amino acid change (p.Asp502Gly) in the terminal end of the catalytic domain of the kinase (Fig. 1C). Alignment of the amino acid sequences of Fgr with those of other SFKs indicates that the 502 aspartic acid is conserved among Src, Lyn, and Blk (Fig. 1F). The corresponding amino acid in Hck and Blk is glutamic acid and has a negatively charged side chain as does aspartic acid. The aspartic acid of Fgr is also conserved in humans (Fig. 1F, FGR). We searched sequence variants among 36 mouse inbred strains in the “mousepost.be” database (23) and no variants were detected in the Fgr locus. The PROVEAN (Protein Variation Effect Analyzer) software (24) predicts that the amino acid substitution is deleterious (score = −6.440; cutoff = −2.5). In addition, we performed whole-genome sequencing by next generation sequencing (NGS) using genomic DNA from Ali18/+ and wild-type mice on the same genetic background, and Fgr c.1506A > G (IGV, 2.3.94, mouse mm10, chr4: 133,002,924, SI Appendix, Fig. S1A) mutations were found in only Ali18/+ DNA as a heterozygous change (NGS reads, A:20 and G:24). Within the Ali18 critical region, we found three other candidate mutations (IGV, 2.3.94, mouse mm10, chr4: 133,543,428; chr4: 133,705,306; chr4: 133,919,389, SI Appendix, Fig. S1 B–D) besides the Fgr coding mutation. However, all three mutations are located in noncoding regions.

Deficiency of Fgr Abolishes the Autoinflammatory Phenotype of Ali18 Mice. To confirm whether the inflammatory phenotype of Ali18 mice is caused by the Fgr coding mutation, we used the prokaryotic antiviral system, CRISPR/Cas9, to induce additional loss-of-function mutations in the N-terminal region of Fgr besides p.Asp502Gly. Because Fgr knockout mice show no overt phenotype (25, 26), it is predicted that loss-of-function mutations in Fgr do not support the osteomyelitis phenotype in Ali18 mice. As shown in Fig. 2A and B, two pX330 related constructs containing guide RNA around exon 3 of the Fgr gene were microinjected into Ali18/Atell18 fertilized eggs. Therefore, all of the Cas9-induced mutations are in the Ali18 haplotype containing the Fgr c.1506A > G (p.Asp502Gly mutation in the protein coding region containing the transcription initiation site of Fgr. All F0 mice (nos. 405, 406, 407, 409, 410, and 411) without mutation around the Cas9-targeted PAM sites exhibit the arthritic phenotype (5/10). In contrast, half of the F0 mice harboring mutations around the PAM sites did not show the inflammatory paw phenotype (5/10).

As it is expected that somatic and germ cells of the F0 mice are chimeric with various indel mutations around the PAM, we crossed F0 mice with wild-type C3HeB/FeJ mice to obtain F1 mice without chimerism (Fig. 2B). Twelve germline-transmitted lines were established from seven F0 mice with a mutation around PAM (SI Appendix, Table S2). Among all 12 lines established, 8 lines, which have deleterious mutations in the Fgr coding region, such as frameshift (#404a, #404b, #415a, and #416a) and deletion of translational initiation site (#418) and/or splicing acceptor (#417a and #419), abolish the inflamed paw phenotype of Ali18 mice (Fig. 2C and D and SI Appendix, Figs. S2 and S3). In contrast, the other four lines with missense (#408 and #416b), in-frame (#415b), and synonymous (#417b) mutations of Fgr exhibit the inflammatory paw phenotype (Fig. 2C and D and SI Appendix, Figs. S2 and S3 and Table S2).

Interestingly, #408a and #417b F1 mice showed different severity of paw swelling compared with original Ali18 mice. Ali18/ #408a (p.Ser25Arg;Asp502Gly) (Fig. 2, D and SI Appendix, Fig. S2C) mice show more severely inflamed paws, indicating that combination of the amino acid changes enhances paw swelling. The #417b (Fig. 2C and D).
A

and SI Appendix, Fig. S2J) line, which has an in-frame 5′ UTR deletion with a newly formed ATG codon in Fgr, showed swollen paws. However, not all mice of the #417b line showed a swollen paw phenotype (4/9, SI Appendix, Table S2 and Fig. S3). It is likely that the newly formed translational initiation site or deletion in the UTR region causes low transcriptional and/or translational efficiency of the newly formed translational initiation site or deletion in the UTR re-
notype (4/9, SI Appendix, Table S2 and Fig. S3). However, not all mice of the #417b line showed a swollen paw phenotype (4/9, SI Appendix, Table S2 and Fig. S3). It is likely that the newly formed translational initiation site or deletion in the UTR region causes low transcriptional and/or translational efficiency of the Fgr protein. These results strongly support that the Fgr p.Asp502Gly mutation is responsible for autoinflammation in Ali18 mice.

Abnormal C-Terminal Phosphorylation of Fgr<sup>Ali18</sup> Reveals Gain of Function. To elucidate how the p.Asp502Gly mutation affects Fgr function, we analyzed the tyrosine kinase activities of wild-type and p.Asp502Gly Fgr proteins by standard kinase assay using radioisotope-labeled ATP. Fig. 3C shows tyrosine kinase activity of in vitro trans-
lated Fgr proteins against affinity purified enolase as a substrate of SFKs. Unlike oncogenic mutations, the kinase activity of p.Asp502Gly Fgr was not dramatically changed. These results were also supported by Western blot analysis using antiphosphotyrosine in Ali18/Ali18 organs and Fgr p.Asp502Gly expressing cultured cells (Fig. 3A and B). We then focused on efficiency of the C-terminal phosphorylation, because p.Asp502Gly is located close to this phosphorylation site. SFKs are normally inactive with the C-terminal phosphorylation by Src (27). To detect the C-terminal phosphorylation, a kinase-ablation mutation (KD, kinase dead) is necessary to avoid autophosphorylation of Src (27). Therefore, we used affinity purified Fgr protein with a p.Lys279Met (K279M) kinase dead mutation (Fgr<sup>KD</sup>, WT) and Fgr<sup>KD</sup> with the p.Asp502Gly mutation (Fgr<sup> KD</sup>, p.Asp502Gly) as substrates for kinase assays by Csk. As shown in Fig. 3D, specifically phosphorylated products were detected in the samples containing Csk without autophosphorylation. Further, the efficiency of phosphorylation of Fgr<sup>KD</sup> (WT) by Csk is approximately two times more than that of Fgr<sup>KD</sup>, p.Asp502Gly (mut) (relative activity ratio: 0.472 ± 0.072). The decrease of C-terminal phosphorylation indicates that Fgr<sup>KD</sup>, p.Asp502Gly is more active than wild-type protein. These results strongly suggest that p.Asp502Gly is a gain-of-function mutation of the Fgr tyrosine kinase.

FGR Coding Variants Were Detected in Autoinflammatory Bone Disease Patients. As part of a human CRMO genetic study, whole-exome sequencing by NGS was performed on 99 affected individuals and their family members. The families included 88 family trios (affected and both unaffected parents) and 11 dyads (affected and one unaffected parent). FGR variants from affected subjects were queried, and 11 rare exonic variants in total were identified in 13 probands (SI Appendix, Table S3). These include two missense variants, p.Arg118Trp and p.Pro525Ser (Fig. 4H). For the patient with the p.Arg118Trp mutation, radiographs show osteolytic lesion with sclerosis and peri-
osteal reaction in distal femur (Fig. 4A and B) which demonstrated abnormal signal intensity and enhancement on postcontrast MRI compatible with CRMO; an additional MRI shows microinjected into Ali18/Ali18 oocytes in C3H (C3HeF6/Hej) genetic background from in vitro fertilization. The founder mice (F0) derived from microinjection were bred with wild-type C3H mice to obtain F1 mice. (C and D) Cor-
relation of Fgr genotypes and lower limb morphology of F1 mice. Loss-of-function mutations show no mor-
phological abnormality (red font). In contrast, missense, in-frame deletion, and synonymous mutations exhibit autoinflammatory paws. SA, splice acceptor; ATG, the translational initiation site. See also SI Appendix, Table S2 for detail.

Genomic DNA from the patient and parents was validated by Sanger sequencing, and the chromatograms indicate a de novo mutation in the child with the p.Arg118Trp FGR mutation (Fig. 4D). A p.Pro525Ser variation is detected in another child for which DNA from only one parent was available, so it is not known if the variant is inherited or de novo. MRI abnormalities in the fibula of the child are shown in Fig. 4 E–G. Both variants are rare (SI Appendix, Table S3); the p.Arg118Trp (rs774209795) and p.Pro525Ser (rs143850913) variants have minor allele frequencies of 1.2 × 10<sup>−3</sup> and 3.5 × 10<sup>−3</sup>, respectively, according to gnomAD (28). The PROVEAN software predicts that both amino acid substitutions are deleterious; the score of p.Arg118Trp is −5.015 (cutoff = −2.5); the score of p.Pro525Ser is −2.716 (cutoff = −2.5) and the other predictors including SIFT, Polyphen2, and CADD also predict pathogenicity (SI Appendix, Table S5). There were no mutations in the known CRMO susceptibility genes including FBLIM1, IL1RN, LIPIN2, or PSTPIP2 in these two patients (SI Appendix, Table S3). However, the same missense variant in FBLIM1 was found in two patients with FGR splice or synonymous mutations, and a missense mutation in LIPIN2 was detected in a patient with an FGR 5′ UTR mutation (SI Appendix, Tables S3 and S4). All three variants are in trans with the coding FGR variant based on parental genotypes. The FBLIM1 variant identified in two probands induces a p.Gly311Arg amino acid change and the LIPIN2 variant induces a p.Cys874Phe change.

To elucidate functional abnormality of the variants found in CRMO patients, kinase assay experiments were performed using affinity purified mutant FGR proteins and enolase (Fig. 4 I and J). The p.Arg118Trp FGR protein (RW, Fig. 4H) showed decreased phosphorylation compared with that of wild-type protein (WT) (relative activity ratio: 0.39 ± 0.12, P < 0.0001, t test, Fig. 4 I and J). As shown in SI Appendix, Figs. S4 and S5, the structural model of p.Arg118Trp may lead to disruption of SH3-kinase linker interaction which destabilizes the protein functions (see also SI Appendix, Results and Discussion). In contrast, p.Pro525Ser (PS, Fig. 4H) may inhibit the closed-inactive formation of FGR as predicted by the structural model (SI Appendix, Fig. S6). Actually, the kinase assay
experiments of PS show increased levels of phosphorylation compared with WT (relative activity ratio: 1.20 ± 0.05, P = 0.015, t test, Fig. 4 J). Unlike the All8 mutation, the decreased levels of C-terminal phosphorylation of kinase dead RW and PS by Csk were not observed (Fig. 4 K). However, both murine p.Arg106Trp (corresponding to PS) and p.Arg106Trp;p.Asp502Gly Fgr constructs showed an ~10% decrease in C-terminal phosphorylation by Csk compared with the wild-type and p.Asp502Gly Fgr constructs, respectively (SI Appendix, Fig. S7).

Discussion

In this study, we identified Fgr as the causative gene for inflammatory disease in the All8 mutant mice by positional candidate cloning despite complex modifier effects from the genetic background. Further genetic studies by genome editing confirmed that multiple null alleles of Fgr disrupt the inflammatory paw phenotype in All8 mice. Previously, it was shown that the All8 phenotype occurs independently of the adaptive immune system (disease occurs in All8/Reg1−/− mice that lack B and T cells); further, granulocytes are increased in peripheral blood of All8 mice (18). Fgr is predominantly expressed in myeloid cells, and a database search for All8 mutations contained a frameshift mutation in Fgr, a common cause of All8 (16). Therefore, we decided to further characterize this mouse strain and identify Fgr as the causative gene for inflammatory disease in All8 mice. The frameshift deletion of Fgr found in All8 mice disrupts the tyrosine kinase domain, resulting in a truncated kinase that is predominantly expressed in myeloid cells, as shown by Western blot analysis (Fig. 1 A and B). Fgr has been shown to play a critical role in innate immune responses, and its loss results in the disruption of the inflammatory paws in All8 mice. We also identified a second frameshift mutation in Fgr in All8 mice, which results in the loss of the kinase domain and further supports the role of Fgr in inflammatory disease. In addition, we observed that Fgr expression is increased in peripheral blood mononuclear cells from All8 mice compared to control mice, which further supports the role of Fgr in the inflammatory phenotype. This study provides insights into the role of Fgr in innate immune responses and its potential as a therapeutic target for inflammatory diseases.
lineage cells such as granulocytes, monocytes, and dendritic cells (29). This expression pattern fits the cell populations implicated in the auto-inflammatory phenotype of Ali18 mice. Because Fgr plays an important role in mast cell activation (30, 31) and neutrophil adherence (29), these cell types are likely contributing independently, or in combination, to autoinflammation in Ali18 mice and is the subject of ongoing research.

In SFKs, the C-terminal region is important for inactivation of tyrosine kinase activity; therefore defects in the C-terminal region cause constitutive activation, which leads to cancerization (32, 33). Csk is another member of SFKs and specifically phosphorylates a C-terminal constitutive activation, which leads to cancerization (32, 33). Csk is activation of Fgr leads to the inflammatory phenotypes of Ali18 mice (34). Without C-terminal phosphorylation by Csk, SFKs cannot form a folded inactive conformation. For this phosphorylation, it is known that docking between the C-terminal region of SFKs and Csk is necessary (27). In our results, Fgr-Cbl (p.Arg106Trp) was less phosphorylated than wild-type Fgr by Csk. The corresponding amino acid substitution in Src (p.Asp518Ala) also shows decreased phosphorylation of the C-terminal region (27). Thus, it strongly suggests that constitutive activation of Fgr leads to the inflammatory phenotypes of Ali18 mice.

In the cmo (chronic multifocal osteomyelitis) mouse model, the proinflammatory cytokine IL-1β mediates autoinflammation but does not require the Nlrp3 inflammasome (35). In Fgr<sup>−/−</sup> mice, IL1β is not up-regulated in peripheral blood (18). Although Fgr is not known to activate the inflammasome directly, NEK7 serine-threonine kinase and two tyrosine kinases, Btk and Syk, control inflammasome activation (36). Thus, the C-terminal phosphorylation network is important for regulation of inflammasome activation in myeloid cells. Recently, it was reported that phosphorylation of CBL ubiquitin ligase at Y371 by SFKs negatively controls NLRP3 inflammasome activation (37). Further, Cbl ubiquitinates Fgr directly for its activation (38). Thus, gain of function of Fgr may lead to the activation loop of Fgr-Cbl which suppresses the NLRP3 inflammasome. These suggest that autoinflammation caused by Fgr activation occurs independent of Nlrp3 inflammasome activation.

In the cohort of patients with CRMO, we found two FGR coding variants, p.Arg118Trp and p.Pro525Ser, which are located in the SH3 domain and C-terminal tail, respectively. Since decreased C-terminal phosphorylation by Csk was not observed using human FGR constructs, it is likely that mouse and human mutations have different functional properties. Increased kinase activation was observed in p.Pro525Ser FGR. Pro525 is the only hydrophobic amino acid in the C terminus of FGR. In the inactive form of Src, the C-terminal region is bound to two hydrophobic pockets of the protein surface in the SH2 domain (39, 40). The substitution of the sub- stitution of Pro525 for hydrophilic Ser could be repellent to the surface in the SH3 domain (39, 40). The substitution of the sub-

Positional Candidate Cloning. Standard genetic mapping was used to narrow down the critical region (17, 19). In the region, we used a literature-based search engine, PosMed (20), to select candidate genes. Because bone marrow cells from Ali18 mice could reconstitute the inflammatory phenotype of Ali18 mice in irradiated wild-type mice (18), we used “bone marrow” as a keyword for search by PosMed. Sixteen genes were selected, and PCR primer pairs spanning the exonic region were designed by Exon Primer (https://ihg.helmholtz-muenchen.de). The PCR products from wild-type and Ali18/ Ali18 templates were then sequenced by standard Sanger sequencing.

Genome Editing of Ali18 Mice. A standard DNA microinjection method into Ali18/Ali18 fertilized eggs was performed (48) using pX330-based constructs described in SI Appendix, Methods. To obtain Ali18/Ali18 fertilized eggs, in vitro fertilization with Ali18/Ali18 oocyte and sperm was done. The CRISPR/Cas9-mediated mutations of F0 and F1 mice were confirmed by Sanger sequencing. We only used F0 mouse (Ali18/Ali18) with newly introduced mutations in exon 3 of FGR for further mating with CB6F1 mice to obtain F1 mice (Ali18<sup>+/−</sup>).

Western Blotting. Western blotting was done by a standard procedure as previously described (49). Antibodies used in this study were as follows: anti-Fgr (M60) (sc-50338, Santa Cruz Biotechnology, Inc.), anti-phosphotyrosine (427, Upstate), and anti-actin (A5471, Sigma). For protein preparation Western Blotting.

Protein Purification for Kinase Assays. The in vitro transcription/translation system was used to synthesize mouse Fgr protein (TNT Quick Systems, Promega). GST-Csk (mouse), GST-FGR<sup>−</sup> (mouse), and GST-FGR and GST-FGR<sup>−</sup> (human) were expressed in Escherichia coli BL21(DE3), purified by glutathione.
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