ASSOCIATION STUDIES ARTICLE

A hyperactivating proinflammatory *RIPK2* allele associated with early-onset osteoarthritis

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Abstract

Osteoarthritis (OA) is a common debilitating disease characterized by abnormal remodeling of the cartilage and bone of the articular joint. Ameliorating therapeutics are lacking due to limited understanding of the molecular pathways affecting disease initiation and progression. Notably, although a link between inflammation and overt OA is well established, the role of inflammation as a driver of disease occurrence is highly disputed. We analyzed a family with dominant inheritance of early-onset OA and found that affected individuals harbored a rare variant allele encoding a significant amino acid change (p.Asn104Asp) in the kinase domain of receptor interacting protein kinase 2 (*RIPK2*), which transduces signals from activated bacterial peptidoglycan sensors through the NF- κ B pathway to generate a proinflammatory immune response. Functional analyses of *RIPK2* activity in zebrafish embryos indicated that the variant *RIPK2*^{104Asp} protein is hyperactive in its signaling capacity, with augmented ability to activate the innate immune response and the NF- κ B pathway and to promote upregulation of OA-associated genes. Further we show a second allele of *RIPK2* linked to an inflammatory disease associated with arthritis also has enhanced activity stimulating the NF- κ B pathway. Our studies reveal for the first time the inflammatory response can function as a gatekeeper risk factor for OA.

Introduction

Osteoarthritis (OA) is a disease of the articular joint characterized by abnormal remodeling of cartilage and bone and causing severe pain and immobility. It is one of the most common debilitating diseases affecting the aging population; the lack of drugs that inhibit the progression of the disease is a huge unmet medical need (1–3). Understanding of the biological processes underlying OA is evolving (4). Only recently has OA come to be recognized as a disease of the entire joint, and the contributions of distinct cell types and processes to the disease state remain unclear (5).

Complex signaling among tissues of the joint and between inflammatory mediators and joint tissues contribute to both homeostatic maintenance and disease remodeling of the joint. Arthritis is a common comorbidity in several severe inflammatory diseases, although it has been considered a secondary effect among complex inflammatory phenotypes (6–8). More recently, inflammation is also being viewed as a potential driver of OA, and there is increasing interest in the potential roles that local or systemic inflammatory responses may play in the development of OA (9–11). The long known correlation between aging or obesity and the occurrence of OA is now being

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reconsidered as possibly due to the low-grade chronically elevated inflammation associated with aging and metabolic syndrome (10,12–15). Despite these correlations, no genetic studies have yet definitively linked inflammation genes to the initiation and/or progression of OA (16–18).

Although 40–80% of the incidence of OA has been attributed to genetic contributions, few genes with significant effects on the course of the disease have been discovered, and no therapeutic targets have been consequently identified (19–25). OA is a heterogeneous disease of polygenic nature with a significant environmental influence, which likely accounts for why GWAS have been largely unsuccessful at finding causative genes with strong effects on OA (21,23,26,27). To discover factors that can have strong and unambiguous effects on the development of OA, we analyzed the genetic basis underlying a dominantly inherited, highly penetrant, early-onset form of OA, which has been described previously (28,29). Although the familial form of OA of the first metatarsophalangeal (MTP) joint is rare, first MTP joint OA is the most common form of progressive OA in the foot, and its symptoms are similar to other forms of OA (30,31). We report discovery of a rare variant of the gene encoding receptor interacting protein kinase 2 (RIPK2) as the dominant factor that likely causes early-onset OA in one family.

RIPK2 is a key proinflammatory regulator that transduces signals from activated forms of the nucleotide-binding oligomerization domain containing receptors (NOD) 1 and 2, which are cytoplasmic sensors of bacterial peptidoglycans. Activated NOD/RIPK2 complex signals through the NF- κ B pathway to generate necessary proinflammatory immune responses (32,33). Hyperactivity of the pathway is associated with several inflammatory human diseases, including Behçet's disease, Blau syndrome and early-onset autoinflammatory disease (6,8,34,35). Experimental studies have demonstrated that RIPK2 activity has the potential to modulate the balance between innate immune competence and hyperinflammatory states. Pharmacologic inhibition of RIPK2 alleviates inflammatory responses in mouse models of peritonitis and Crohn disease (36). Loss of *Ripk2* function in mice reduces NF- κ B-mediated inflammatory responses: mutant mice exhibit both increased susceptibility to bacterial infection and resistance to the toxic immune-stimulatory effects of lethal doses of LPS (37,38). Notably, both *Ripk2* and *Nod2* are necessary for the onset and progression of experimentally induced arthritis in mice. In response to stimuli, *Ripk2* or *Nod2* loss-of-function mutant mice have reduced proinflammatory cytokine expression in the joint and are protected against arthritis (39). Hence we analyzed whether the first MTP OA-associated RIPK2 variant affected inflammatory signaling. Using the zebrafish embryo as a simple assay system, we demonstrate the RIPK2 variant encodes a protein with enhanced signaling and proinflammatory activity as compared with the protein encoded by the allele that is common in human populations.

Results

We identified a family of Northern/Eastern European origin with an early-onset form of first MTP joint OA. First MTP joint OA presented bilaterally in all affected individuals whereas other synovial joints of the body were unaffected. The occurrence of OA in this family segregated as an apparent autosomal dominant trait with the average age of onset being 30 years (Fig. 1A). Radiographic analysis of the foot of the proband (individual III-1) indicated characteristic signs of advanced first MTP joint OA including severe loss of cartilage and joint space with

osteophyte formation; other MTP joints were unaffected (Fig. 1B and B'). The affected family members were healthy with no history of traumatic injury to the first MTP joint, biomechanical defects and no evidence of immune or metabolic diseases.

To identify sequence variants associated with the disease phenotype, we sequenced the exomes of three unaffected and three affected individuals from two generations of the family (Fig. 1A—individuals II-1, II-2, II-3, III-1, III-2, and III-3). Assuming complete penetrance of a variant allele acting as a dominant trait, and recognizing the uncommon occurrence of early-onset familial first MTP joint OA, we sought to identify rare coding sequence variants with presumed deleterious effects on gene function (Table 1). Filter-based annotation of the identified variants using ANNOVAR (40) identified 10 coding variants that were (i) shared among the three affected individuals, (ii) absent in unaffected family members, (iii) not residing in very large gene families and (iv) rare, with a minor allele frequency (MAF) of $\leq 1\%$ in the 1000 Genomes Project (1KG), Exome Variant Server (EVS) and Exome Aggregation Consortium (ExAC) databases. We next used the PHEntotype-driven Variant Ontological Re-ranking (PHEVOR) tool with the Human Phenotype Ontology (HPO) search term 'Osteoarthritis' to narrow our results to three high-priority candidate genes (41). By analyzing genomic DNA from isolated hair follicles from the founding affected member of the family (I-1, Fig. 1A), we found only the variant in the RIPK2 gene (rs200818100—average allele frequency in 1KG, EVS and ExAC = 0.0004) was present in heterozygous state in individual I-1 (Table 1). An independent analysis pipeline using the pedigree Variant Annotation, Analysis & Search Tool (pVAASST) (42), which identifies the most likely causal variants in a pedigree based on gene tolerance to mutation, variant frequency, phylogenetic conservation and biological function, followed by PHEVOR analysis, also yielded the RIPK2 gene variant (rs200818100) as the single high priority variant discovered in this family (pVAASST: P -value = 0.000646; LOD = 1.23; PHEVOR: score 4.558, final rank = 1). Finally we used DOMINO, a tool that predicts the likelihood that mutations in a gene might lead to a dominant disorder, to score the top candidate genes: only RIPK2 was predicted with high confidence to have dominant alleles (43).

The RIPK2 protein is composed of a catalytic N-terminal kinase domain and a C-terminal caspase activation and recruitment domain (CARD) (Fig. 1C). The kinase domain of RIPK2 is necessary for activation of the NF- κ B signaling pathway whereas the CARD mediates interactions between RIPK2 and NOD1/2. The variant identified in affected individuals (rs200818100, c.310A>G) resides in sequences encoding the kinase domain, substituting an Asp for the Asn that is invariant throughout the mammalian lineage at position 104. The substitution p.Asn104Asp results in a change of charge at an amino acid position within the substrate binding site that is highly conserved (Conserved Domain Database ID: 270928) (Fig. 1C).

To determine if the change from Asn to Asp might affect RIPK2 function *in vivo*, we assayed the activity of the Asn and Asp forms of RIPK2 in zebrafish. Zebrafish have a single gene orthologous to RIPK2, which encodes a protein 62% identical and 78% similar to human RIPK2. We generated two forms of the zebrafish *Ripk2* gene *in vitro*: one encoding *Ripk2* with the common human Asn at position 104 (*Ripk2*^{104Asn}) and the other encoding *Ripk2* with the OA-associated human variant Asp at position 104 (*Ripk2*^{104Asp}). Each protein was ectopically expressed in zebrafish embryos by injection of mRNA into 1-cell zygotes, and the dose-dependent effects of overexpression on viability and morphological development were determined.

Figure 1. A dominant RIPK2 mutation associates with early-onset OA of the first MTP joint. (A) Family 1 pedigree. Early-onset OA of the first MTP joint segregates as an apparent autosomal dominant trait with age of onset indicated for affected individuals. Arrow marks the proband. (B) Dorsal radiograph of the right foot of the proband. The black arrow indicates the severe loss of cartilage and joint space in the first MTP joint. The white arrow indicates an unaffected second MTP joint. (B') Lateral view of the first MTP joint demonstrating osteophyte formation. (C) Schematic diagram of the RIPK2 kinase and C-terminal caspase recruitment (CARD) domains and location of the p.Asn104Asp mutation.

Table 1. Summary of filter based analysis of variants in Family 1 assuming the causal variant is rare and acts dominantly with complete penetrance

Affected individuals:	II-2, III-1, III-2
Total coding variants shared by affected:	5961
Coding variants (shared by affected and absent in unaffected):	208
Heterozygous variants (shared by affected and absent in unaffected):	149
1KG, EVS and ExAC MAF ≤ 0.01 :	37
Remove benign variants/polymorphic genes/gene families:	10
PHEVOR analysis with HPO term 'Osteoarthritis':	3
Heterozygous alleles present in affected founder (I-1) ^a :	1

The variant (rs200818100) in RIPK2 was the single variant heterozygous in all four affected family members.

^aGiven the limited amount of DNA isolated from hair follicles from individual I-1, we tested only the zygosity of the top three variants from PHEVOR analysis.

Injection of low doses (50–200 pg) of either *ripk2*^{104Asn} or *ripk2*^{104Asp} mRNA caused no morphological defects or changes in viability as measured at 3 days post fertilization (dpf) (data not shown). Similarly, embryos injected with 400–800 pg *ripk2*^{104Asn} mRNA encoding the common human Asn variant developed normally ($n = 298$, 100% normal) (Fig. 2C and D) and were indistinguishable from uninjected wild-type embryos ($n = 208$, 100% normal) (Fig. 2A and B). In contrast, 31% of embryos injected with 400–800 pg *ripk2*^{104Asp} mRNA exhibited dramatic defects in head development, including cyclopia and

morphological defects in craniofacial and axial development ($n = 310$: 210 normal, 79 with cyclopia and 21 with cyclopia and additional axial defects) (Fig. 2E–G). The developmental phenotype is consistent with the known role of NF- κ B signaling in regulating axis development in zebrafish and *Xenopus* embryos (44–47). This simple experiment indicates that the Asn and Asp variant forms of RIPK2 have measurably different activities *in vivo*.

To determine whether the different activities of the two proteins might affect strength of signaling through the Nod/Ripk2 innate immunity cascade, we developed an assay for the innate immune activity of Ripk2 in the zebrafish. Zebrafish larvae have a functional innate immune system that utilizes the Nod pathway (48,49). Given the critical role of Ripk2 in generating an innate immune response to bacterial infection in mammals (37), we tested if Ripk2 had similar function in zebrafish larvae. We generated loss-of-function mutations in the single zebrafish *ripk2* ortholog using the CRISPR/Cas9 system (50), isolating a 2 bp deletion in exon 3 (z41) and a 10 bp deletion (z39) and 52 bp insertion (z40) in exon 10, all of which cause frameshifts and introduce premature stop codons. Homozygous *ripk2* mutants are fully viable and fertile (as are mouse mutants) and show no overt phenotypes compared with their wild-type siblings.

Using a variation of an established assay (49), we tested whether zebrafish Ripk2 contributes to the larval innate immune response to bacterial infection. *Pseudomonas alcaligenes* (PA) were injected (approximately 300 bacteria/embryo) into the yolk cell of 48 hpf wild-type and *ripk2*^{-/-} zebrafish larvae. Wild-type larvae displayed resistance to infection compared with *ripk2*^{-/-} larvae (Fig. 3). Consistent with the prediction that the *ripk2* mutant alleles are loss of function, all three alleles were phenotypically indistinguishable in their response to bacterial

Figure 2. The OA-associated *Ripk2*^{104Asp} variant has altered biological activity. (A–G) Wild-type zebrafish 1-cell zygotes were injected with 400–800 pg mRNA encoding the zebrafish *Ripk2*^{104Asn} or *Ripk2*^{104Asp} protein variant. At 3 dpf, uninjected embryos (A and B) as well as embryos injected with RNA encoding the Asn variant appeared normal (C and D). In contrast 31% ($n = 310$) of embryos injected with RNA encoding the OA-associated Asp variant were cyclopic with a normal trunk (E and F) or cyclopic with additional axial defects (G). A, C, E and G are lateral views and B, D and F are ventral views with anterior to the left.

Figure 3. The OA-associated *Ripk2*^{104Asp} variant has increased activity supporting survival in response to bacterial infection. 1 dpf wild-type or *ripk2*^{-/-} mutant embryos were injected into the yolk cell with PBS or approximately 300 *Pseudomonas alcaligenes* (PA) bacteria and survival was measured. *ripk2*^{-/-} mutant embryos (green) are significantly more susceptible than WT embryos (red) to infection ($P < 0.0001$). Uninfected controls are indicated in black (WT uninjected) and gray (*ripk2*^{-/-} PBS injected). Injection of *ripk2*^{-/-} mutant 1-cell embryos with mRNA encoding the WT *Ripk2*^{104Asn} protein failed to protect embryos from infection (orange) ($P = 0.8498$), whereas injection with mRNA encoding the OA-associated *Ripk2*^{104Asp} protein provided a significant degree of protection from infection (blue) ($P = 0.0412$). Data represent six biological replicates with at least 30 animals in each replicate. Statistical significance determined by the Gehan-Breslow-Wilcoxon test. Error bars represent SE and are only in the positive direction for clarity.

infection (data not shown) and displayed enhanced susceptibility to bacterial infection, similar to that reported for *nod1* and *nod2* knockdown larvae (49). Almost all uninfected WT and even PBS-injected *ripk2*^{-/-} larvae develop normally and survive (Fig. 3). Infection with PA significantly reduces viability so that at 30 hours post infection (hpi) wild-type larvae injected with PA had

a mean survival of 69.8% ($P < 0.0001$, Gehan-Breslow-Wilcoxon test) (Fig. 3). In contrast, PA-injected *ripk2*^{-/-} larvae were more susceptible to bacterial infection, with a mean survival of 49.8% at 30 hpi, which was significantly reduced compared with either controls (uninjected or PBS-injected *ripk2*^{-/-} larvae) or WT PA-injected larvae (69.8% mean survival) ($P < 0.0001$) (Fig. 3). Thus,

as in mammals, a primary function of Ripk2 in the zebrafish is to contribute to the ability of host animals to mount a vibrant innate immune response to bacterial infection.

The *ripk2*^{-/-} mutant embryos afforded the opportunity to measure the relative activities of the variant forms of the Ripk2 protein specifically within the context of innate immunity. *ripk2*^{-/-} mutant zygotes were injected with 200 pg mRNA encoding either the zebrafish Ripk2^{104Asn} or Ripk2^{104Asp} versions of the protein, an amount of ectopic expression that did not perturb development. Normal-appearing embryos were challenged with PA infection at 48hpf, and the response to bacterial infection was measured. Under these conditions, expression of the Ripk2 protein harboring the common human Asn variant failed to increase survival (mean survival of 46.2% at 30 hpi) in comparison with PA-injected *ripk2*^{-/-} larvae (mean survival of 49.8% at 30 hpi) ($P=0.8498$) (Fig. 3). In contrast, expression of the Ripk2 protein harboring the human OA-associated Asp variant in PA-injected *ripk2*^{-/-} larvae significantly increased survival (mean survival of 61.3% at 30 hpi) compared with PA-injected *ripk2*^{-/-} larvae (mean survival of 49.8% at 30 hpi) ($P=0.0412$) (Fig. 3). We interpret these experiments as indicating that the OA-associated RIPK2^{104Asp} variant allele has elevated activity compared with the common human RIPK2^{104Asn} allele in facilitating the innate inflammatory response to a bacterial pathogen.

In response to bacterial infection the NOD/RIPK2 complex signals through the NF- κ B pathway, stimulating the expression of genes regulating a proinflammatory response (32,33). To test whether the common and OA-associated Ripk2 forms had measurably different effects activating NF- κ B-dependent gene expression, we injected mRNA (400–800 pg) encoding the Asn or Asp variant forms of Ripk2 into 1-cell stage zygotes and analyzed gene expression in these embryos by RT-qPCR analysis. Levels of induced gene expression were determined in relation to expression of the housekeeping gene *eef1a11l*. Similar amounts of injected mRNA were detected in *ripk2*^{104Asn} and *ripk2*^{104Asp} mRNA-injected embryos ($P=0.9664$), which were both significantly different than uninjected controls ($P\leq 0.001$, one-way ANOVA, Tukey's multiple comparisons test) (Fig. 4).

Stimulation of NF- κ B signaling directly regulates transcription of genes encoding NF- κ B signaling components. Overexpression of the *ripk2*^{104Asp} variant allele in zebrafish embryos promoted a significant increase in the abundance of RNA encoding components of the NF- κ B pathway, including *nfk1b* ($P\leq 0.001$), *nfk2b* ($P\leq 0.05$), *nfkbiaa* ($P\leq 0.01$) and *nfkbiab* ($P\leq 0.01$), as compared with RNA expression in uninjected control embryos. Similar overexpression of the *ripk2*^{104Asn} common allele produced significant upregulation of only *nfk1b* ($P\leq 0.05$) (Fig. 4). Furthermore, expression levels of *nfk1b*, *nfkbiaa* and *nfkbiab* were significantly higher in *ripk2*^{104Asp} mRNA-injected embryos as compared with *ripk2*^{104Asn} mRNA-injected embryos (all $P\leq 0.01$) (Fig. 4).

NF- κ B signaling also directly stimulates expression of *il1b* and *il8*, genes that encode proinflammatory cytokines commonly associated with OA (51). Only *ripk2*^{104Asp} expression caused a dramatic increase of both *il1b* ($P\leq 0.01$) and *il8* ($P\leq 0.05$) as compared with control embryos. In addition, the induced levels of *il1b* RNA were significantly higher ($P\leq 0.01$) following expression of the variant *ripk2*^{104Asp} allele as compared with expression of the common *ripk2*^{104Asn} allele (Fig. 4). Marker genes commonly associated with the OA phenotype, such as *nos2* and *mmp13*, are expressed as a consequence of *il1b* and *il8* signaling (51). In contrast to the effects of ectopic expression of Ripk2^{104Asn} in zebrafish embryos, expression of Ripk2^{104Asp} led

Figure 4. The OA-associated Ripk2^{104Asp} variant has increased ability to stimulate the NF- κ B pathway. Wild-type zebrafish 1-cell zygotes were injected with 400–800 pg of *ripk2*^{104Asn} or *ripk2*^{104Asp} mRNA and the abundance of gene-specific RNA transcripts was measured 8–9 h later by qRT-PCR. Levels of gene expression relative to uninjected controls are depicted. Embryos with ectopic expression of the OA-associated Ripk2^{104Asp} variant (red bars) have increased abundance of NF- κ B-stimulated, innate immunity pathway and OA-associated gene transcripts compared with wild-type uninjected controls or to embryos that ectopically express the Ripk2^{104Asn} protein (blue bars). Data represent four biological replicates. Error bars represent $\pm$ SEM and statistically significant differences of $P\leq 0.05$ (*), $P\leq 0.01$ (**), $P\leq 0.001$ (***) were determined by one-way ANOVA with Tukey's multiple comparisons test.

to a significant upregulation of *nos2a* ($P\leq 0.05$) and *nos2b* ($P\leq 0.001$), while *mmp13a* levels were slightly, but not significantly upregulated (Fig. 4). Thus, expression of Ripk2 in the zebrafish embryo activates the NF- κ B pathway. Our quantitative tests indicate that the Ripk2^{104Asp} variant has increased activity relative to its Ripk2^{104Asn} counterpart in stimulating the innate immune response and stimulating NF- κ B pathway signaling, consistent with the interpretation that the first MTP OA-associated variant form of the RIPK2 protein is hyperactive in its signaling functions.

Arthritis is a common comorbidity of inflammatory diseases associated with perturbations of the NOD2/RIPK2/NF- κ B pathway (6,8,34,35). In particular, Behçet's disease has a high coincidence (5.3–93%) of articular manifestations ranging from arthralgia to polyarthritis (7). Recently, a coding variant in RIPK2 was identified as a susceptibility locus for Behçet's disease in Turkish and Japanese populations (34). The missense variant affects an evolutionarily conserved, invariant amino acid in the RIPK2 kinase domain (p.Ile259Thr); how the mutation might affect RIPK2 function is unknown. Based on the hyperinflammatory phenotype observed in Behçet's disease, we hypothesized that the p.Ile259Thr change might lead to hyperactivity of RIPK2.

Utilizing induced gene expression in zebrafish embryos as a measure of the relative activities of RIPK2 alleles, we tested whether the p.Ile259Thr variant form of RIPK2 might have altered ability to stimulate NF- κ B-dependent gene expression. We generated a mutant form of the zebrafish *ripk2* cDNA that

Figure 5. The Behçet's disease-associated *Ripk2*^{269Thr} variant and the OA-associated *Ripk2*^{104Asp} variant have similar augmented abilities to activate the NF-κB pathway. Wild-type zebrafish 1-cell zygotes were injected with 400–800 pg mRNA encoding zebrafish *Ripk2*^{104Asp}, *Ripk2*^{104Asn} or *Ripk2*^{269Thr} proteins, and gene-specific transcript levels were measured 8–9 h later by qRT-PCR. Levels of gene expression relative to embryos ectopically expressing *Ripk2*^{104Asn} protein are depicted. Embryos expressing the Behçet's disease associated *Ripk2*^{269Thr} variant (magenta bars) or the OA-associated *Ripk2*^{104Asp} variant (red bars) have similarly increased activation of the transcription of NF-κB-stimulated, innate immunity pathway and OA-associated genes compared with *Ripk2*^{104Asn} expressing embryos. Data represent three biological replicates. Error bars represent ± SEM and statistically significant differences of $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) were determined by one-way ANOVA with Tukey's multiple comparisons test.

encoded a p.Ile269Thr variant, a change homologous to that present in the Behçet's disease-associated protein. mRNAs encoding zebrafish *Ripk2* proteins with 104Asn and 269Ile (corresponding to the human WT form), 104Asn and 269Thr (corresponding to the Behçet's disease form) or 104Asp (corresponding to the first MTP OA form) were injected into 1-cell stage zygotes and RT-qPCR gene expression analysis was performed as above. Levels of target gene expression relative to *eef1a1l1* RNA were calculated, and normalized to the amount of ectopic *ripk2* mRNA present in injected embryos. To illustrate the relative activities of the *ripk2* alleles, data are presented as gene expression in embryos expressing the *Ripk2*^{104Asn269Thr} or *Ripk2*^{104Asp} protein relative to that of embryos ectopically expressing *Ripk2*^{104Asn} protein. Very similar to the effects of ectopic expression of the zebrafish *Ripk2*^{104Asp} protein (Figs 4 and 5), overexpression of the zebrafish *Ripk2*^{269Thr} mutant protein resulted in significant upregulation of genes encoding NF-κB components, proinflammatory cytokines and genes downstream of proinflammatory cytokine signaling (Fig. 5). The only significant differences between the effects of ectopic expression of the zebrafish *ripk2*^{104Asp} or *ripk2*^{269Thr} alleles were in their abilities to stimulate *nfkb1a* ($P < 0.05$) and *tnfa* ($P < 0.01$) expression (Fig. 5). These data indicate that the human *RIPK2*^{259Thr} variant allele associated with Behçet's disease, like the first MTP OA-associated *RIPK2*^{104Asp} allele, encodes a hyperactive form of *RIPK2* with enhanced ability to stimulate NF-κB pathway signaling.

Discussion

Here we report identification of a rare missense variant in *RIPK2* (rs200818100, c.310A>G, p.Asn104Asp) in affected members of a family with an early-onset, dominantly inherited OA of the first MTP joint. A potential link between *RIPK2* and OA had been

previously noted in experiments in which *RIPK2* was identified as a top hit in an unbiased screen to identify factors that induce expression of OA markers in chondrocytes (52). Our studies indicate that the disease-associated allele discovered here has heightened biological activity relative to the common WT allele. We utilized zebrafish as an *in vivo* system to measure *Ripk2* signaling activity, assessing the effects of ectopic expression on embryogenesis, response to bacterial infection, and induction of gene expression. Our data support the interpretation that the variant *RIPK2*^{104Asp} allele is hyperactive and promotes a heightened or prolonged inflammatory response by stimulating proinflammatory gene expression.

The interpretation that the first MTP OA-associated *RIPK2*^{104Asp} allele has enhanced proinflammatory activity is consistent with studies demonstrating that loss of *Ripk2* or *Nod2* function in null mutant mice protects against experimentally induced arthritis and reduces proinflammatory cytokine expression in the joint (39). Furthermore, *in vivo* pharmacological inhibition of *RIPK2* kinase function alleviates the inflammatory response in mouse models of inflammatory disease (36). Given the recent interest in, but lack of strong genetic evidence for, the potential role of chronic inflammation in the initiation and progression OA, our findings are highly significant in that they demonstrate augmented proinflammatory signaling via the *RIPK2*-NF-κB pathway is likely sufficient to significantly increase susceptibility to early-onset OA.

Inflammation and arthritis are commonly linked, but whether the association is primary or due to secondary effects is unclear. Osteoarthritic joints are characterized both by loss of cartilage and by heightened expression of cytokines and inflammatory genes (5). Conversely, inflammatory diseases are often associated with some form of arthritis. The NF-κB pathway plays a central role in the proinflammatory response, and mutations that inappropriately activate NF-κB signaling, such as activating mutations in *NOD2* (Blau Syndrome) or loss-of-function mutations in the negative regulator of NF-κB signaling, *TNFAIP3/A20* (early-onset autoinflammatory disease phenotypically similar to Behçet's disease), result in severe inflammatory diseases that are often accompanied by arthritis (6,8,34,35,51,53–55).

Given the severity and number of tissues affected in the inflammatory diseases resulting from elevated NF-κB signaling, it is notable that systemic disease is absent in our first MTP joint OA family harboring a hyperactive *RIPK2*^{104Asp} allele. Based on the absence of general inflammation phenotypes in the family, and our functional studies in zebrafish, which uncovered rather modest dose dependent but reproducible differences between the common and variant forms of the *RIPK2* protein, we conclude that the first MTP OA-associated *RIPK2* allele is mildly hypermorphic, although we cannot rule out the possibility that the OA-associated allele has neomorphic function. The simplest explanation is that modest perturbation of inflammatory homeostasis is a driving factor in susceptibility to OA.

An apparent conundrum arises from our studies indicating the *RIPK2*^{259Thr} protein associated with Behçet's disease also appears to have only modestly elevated signaling capacity and yet it has been linked to a severe inflammatory disorder. In fact it appears the *RIPK2*^{Ile259Thr} allele is not sufficient to account for Behçet's disease and is likely to be only a minor contributor to the overall genetic burden underlying the condition in the population where it was discovered. The Behçet's disease allele of *RIPK2* (rs2230801) is relatively common [MAF in the ExAC database ranges from 13.19% (Finnish) to 0.93% (South Asian) with an average MAF of 6.8%], and thus it is not surprising that we find it has only a modest effect on *RIPK2*-NF-κB pathway signaling.

As the OA-associated allele of *RIPK2* is mildly hyperactive in our assays, the OA phenotype in individuals with the p.Asn104Asp variant may only arise after repetitive, non-severe injury to the first MTP joint resulting in a mild, yet chronic, inflammatory state. It is unclear how this hyperactivity is translated by cells or which cell types are responding to the increased *RIPK2*-NF- κ B activity. There are several possibilities including a low-grade, yet chronic inflammatory response that may be systemic or localized to the synovial joint, enhanced signaling response to stimuli or a prolonged signaling response that fails to be resolved in a normal manner. We will resolve these questions by generating a mouse model using the OA-associated *RIPK2* variant allele and determining the spatial and temporal consequences of hyperactive *RIPK2* signaling on the initiation and progression of OA.

Identifying the causative variants in early-onset forms of OA will likely reveal diverse pathways that lead to OA and provide targets for therapeutic intervention. For example, whereas our work identified a novel variant of *RIPK2* with a marked proinflammatory effect, a recent study of another family with early-onset first MTP joint OA identified a likely causal variant of the *TUFT1* gene, which is thought to affect chondrocyte differentiation and cartilage mineralization (29). In addition, since the entire disease spectrum of OA is represented by rare early-onset forms that cluster in families (25), family studies will uncover genes that contribute to a range of OA types. As an example, the OA-associated gene *COMP*, which was initially discovered in a family study, has also been identified in a large GWAS to have a strong effect on hip OA (56,57). Hence further studies of severe early-onset forms of OA will uncover genes and molecular and cellular pathways that are likely to influence the initiation and/or progression of common forms of OA.

Materials and Methods

Whole exome sequencing and Sanger sequencing

Whole exome sequencing was performed by the University of Utah High-Throughput Genomics Core on genomic DNA isolated from whole blood. Libraries were prepared using the Agilent SureSelect XT Human All Exon+UTR (v5) kit followed by Illumina HiSeq2500 125 cycle paired end sequencing. We followed best practices established by the Broad Institute GATK for variant discovery (<https://software.broadinstitute.org/gatk/>; date last accessed April 18, 2018). Analysis of variants was performed with ANNOVAR (<http://annovar.openbioinformatics.org/en/latest/>; date last accessed April 18, 2018) and pVAAS (http://www.hufflab.org/software/pvaas/; date last accessed April 18, 2018) in concert with PHEVOR (<http://weatherby.genetics.utah.edu/phevor2/index.html>; date last accessed April 18, 2018). Verification of candidate variants was performed by direct Sanger sequencing of PCR products amplified from genomic DNA.

Hair follicle genomic DNA isolation and amplification

Hair follicle genomic DNA was isolated according to the methods described in (58) and the isolated genomic DNA was amplified using the PicoPLEX WGA Kit (NEB). Selected genomic locations containing the candidate variants were PCR amplified and directly sequenced by Sanger sequencing.

Plasmids and mRNA generation

We cloned the cDNA corresponding to the zebrafish *Ripk2* gene (NM_194411) into the pCS2+ vector and generated, using *in vitro* mutagenesis (NEB Q5 Site-Directed Mutagenesis Kit), two forms

of the gene: one encoding *Ripk2* with the common human Asn at position 104 (*Ripk2*^{104Asn}) and the other encoding *Ripk2* with the OA-associated human variant Asp at position 104 (*Ripk2*^{104Asp}). We also generated a mutant form of the zebrafish *ripk2* cDNA that encoded a p.Ile269Thr variant, a change homologous to that present in the Behçet's disease-associated protein. mRNA was generated by *in vitro* transcription of pCS2+-*Ripk2* plasmids linearized with NotI (mMESSAGE mMACHINE SP6 kit; Life Technologies). mRNA was injected into the cytoplasm of 1-cell stage zebrafish zygotes.

Generation of *Ripk2* mutant zebrafish

sgRNAs were synthesized to target exon 3 or exon 10 of *Ripk2* (Ensembl Zebrafish GRCv10—ENSDBG00000104290) (exon 3 sgRNA sequence—AGATCGTGGTGTAGAAGTGG and exon 10 sgRNA sequence—GGAGAGCAGTTGTCCATAAG) and coinjected with Cas9 protein (IDT) into the cytoplasm of 1-cell stage zebrafish zygotes. Germline transmitted mutations were isolated and fully sequenced, and three mutant lines were established (49).

Bacterial infection

We used a modification of an established bacterial infection assay (49). *Pseudomonas alcaligenes* (PA) were grown in LB media at 28.5°C for 24 h, collected, washed in 1/10× PBS and resuspended in 1/10× PBS for microinjection. Approximately 300 bacteria were injected into the yolk cell of 48 hpf wild-type and *ripk2*^{-/-} zebrafish larvae.

Quantitative real-time PCR

mRNA injected and control embryos (50 embryos per condition) were collected into TRIzol (Life Technologies) at 8–9 h post injection, RNA was isolated using the Direct-zol RNA MiniPrep Kit (Zymo) and cDNA was generated using Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Fisher). qPCR was performed using Luna Universal qPCR Master Mix (NEB). Levels of induced gene expression were determined in relation to expression of the housekeeping gene *eef1a1l1*. Relative expression was calculated using the 2^{-ΔΔCt} method and normalized to the amount of injected *ripk2* mRNA, which was determined by qRT-PCR that specifically detected injected mRNA sequences. Primers used for qRT-PCR analysis are as follows: *ripk2*qF—tgatggatggggagtaccat, *ripk2*qR—cctcccatcagcaggtt, *mmp13*aqF—catgatctcttcgccactg, *mmp13*aqR—cgccctcatatggagatag, *mmp13*bqF—aatggctcgtgtggagtt, *mmp13*bqR—agatcagga gtgtagttcagaatcc, *nos2*aqF—agttccctgctttgttcacc, *nos2*aqR—gaaa ggagcaatccactgc, *nos2*bqF—aacggcatcatgaactgttg, *nos2*bqR—ta cattgtagtctccatgcaaa. Primers used to amplify *eef1a1l1*, *nfk1b1*, *nfk1b2*, *nfk1biaa* and *nfk1biab* are from (59) and *il1b*, *il8* and *tnfa* are from (60).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Tests performed and statistical significance are indicated in the figure legends.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Study Approval

Written informed consent was obtained under the guidance of the Institutional Review Board at the University of Utah. Zebrafish were maintained in accordance with approved institutional protocols at the University of Utah. Wild-type zebrafish were from the Tübingen strain. Embryos and adults were maintained under standard conditions (61).

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Conflict of Interest statement. None declared.

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