The NOD/RIPK2 signaling pathway contributes to osteoarthritis susceptibility

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Abstract

Objectives—How inflammatory signaling contributes to OA susceptibility is undetermined. An allele encoding a hyperactive form of the RIPK2 proinflammatory signaling intermediate has been associated with familial OA. To test whether altered NOD/RIPK2 pathway activity causes heightened OA susceptibility, we investigated whether variants affecting additional pathway components are associated with familial OA. To determine whether the *Ripk2[104Asp]* disease allele is sufficient to account for the familial phenotype, we determined the effect of the allele on mice.

Methods—Genomic analysis of 150 independent families with dominant inheritance of OA affecting diverse joints was used to identify coding variants that segregated strictly with occurrence of OA. Genome editing was used to introduce the OA-associated *RIPK2* (p.Asn104Asp) allele into the genome of inbred mice. The consequences of the *Ripk2[104Asp]* disease allele on physiology and OA susceptibility in mice were measured by histology, immunohistochemistry, serum cytokine levels, and gene expression.

Results—We identified six novel variants affecting components of the NOD/RIPK2 inflammatory signaling pathway that are associated with familial OA affecting the hand, shoulder, or foot. The *Ripk2[104Asp]* allele acts dominantly to alter basal physiology and response to trauma in the mouse knee. Whereas the knees of uninjured *Ripk2[Asp104]* mice appear normal histologically, the joints exhibit a set of marked gene expression changes reminiscent of overt OA. Although the

Ripk2[104Asp] mice lack evidence of chronically elevated systemic inflammation, they do exhibit significantly increased susceptibility to PTOA.

Conclusions—Two types of data support the hypothesis that altered NOD/RIPK2 signaling confers susceptibility to OA.

Keywords

osteoarthritis genetics; osteoarthritis risk factors; osteoarthritis gene; RIPK2; NOD1; NOD2; RIP2; interphalangeal joint osteoarthritis; 1st MTP joint osteoarthritis; shoulder osteoarthritis

INTRODUCTION

The molecular pathways that are rate-limiting in the onset and progression of osteoarthritis (OA) are unknown, consistent with the complete lack of disease-modifying drugs currently available[1–4]. Knowledge of these pathways is required for identifying individuals at risk for disease, for understanding mechanisms that trigger or amplify disease processes, and for development of effective therapies. One proven approach toward identifying pathways and biological processes whose normal functions limit disease has been to identify gene variants responsible for highly penetrant familial forms of the disease. Increasing evidence demonstrates there are no/few differences between the genes contributing to "monogenic" disease and those contributing to complex disease[5–10]. Pathways that can be mutated to have determinate effects promoting OA will also be vulnerable to the modest genetic or environmental perturbations that underlie common spontaneous forms of OA[5]. Despite its promise, to date there have been relatively few studies of non-syndromic familial OA[11–14]. We have used a unique medical genetics resource, the Utah Population Database, to identify a large number of multigenerational families with dominantly inherited OA[15]. Here we employ genomic analyses of these families and functional analyses in mice to test the hypothesis that perturbation of the NOD/RIPK2 proinflammatory pathway is sufficient to significantly elevate susceptibility to OA.

In previous work, we identified a rare allele of the *Receptor Interacting Protein Kinase 2* (*RIPK2*) gene (p.Asn104Asp) that was associated with dominant inheritance of early-onset OA of the first metatarsophalangeal (1st MTP) joint in a single family[11]. The NOD/RIPK2 signaling pathway is a key arm of the innate immunity system, playing critical roles both in clearing bacterial infections and maintaining immune homeostasis[16]. The intracellular nucleotide-binding oligomerization domain (NOD) receptors are activated by bacterial cell wall breakdown products and additional damage-associated molecular patterns[16, 17]. Activated NOD receptors signal through the Receptor Interacting Protein Kinase 2 (RIPK2), stimulating the MAPK and NF-κB pathways to elicit tissue-specific responses, most notably inflammatory responses[18–21]. NOD/RIPK2 signaling is tightly regulated, as mutations that either abrogate or elevate signaling are associated with chronic inflammatory diseases, including Crohn"s, Blau syndrome, early-onset sarcoidosis, and Behcet"s disease[16, 22, 23]. Although chronic inflammatory diseases are often associated with arthritis, no single inflammatory pathway has yet been linked to classic non-syndromic forms of OA[4, 24].

Here we investigate the hypothesis that function of the NOD/RIPK2 signaling pathway limits susceptibility to OA. We find that individual gene variants affecting any of several components of the pathway appear sufficient to confer heightened susceptibility to OA in families. Significantly, variants affecting the NOD/RIPK2 signaling pathway are associated with divergent forms of familial OA. Last, we demonstrate that an OA-associated *RIPK2* gene variant encoding a single amino acid change in the kinase domain is sufficient to alter basal gene expression patterns in primary chondrocytes and bone marrow macrophages and confer increased susceptibility to post-traumatic OA when introduced into the mouse genome. Our data provide strong support for the hypothesis that modulation of the NOD/RIPK2 signaling pathway is a significant risk factor for OA.

METHODS

Study approval

Written informed consent was obtained under the guidance of the Institutional Review Boards of the University of Utah and Intermountain Healthcare, and the Resource for Genetic and Epidemiologic Research approved this study. Mice were maintained in accordance with approved institutional protocols at the University of Utah.

Identification of families with a dominant pattern of OA inheritance

Our study utilizes data drawn from the Utah Population Database (UPDB) (https:// uofuhealth.utah.edu/huntsman/utah-population-database/). The UPDB provides person-based interlinked records documenting genealogy, medical records, and vital statistics for over 11 million individuals from the late 18th century to the present. Medical records derive from the two largest healthcare providers in Utah (Intermountain Healthcare and University of Utah Health), Medicare claims, and the Utah and Idaho Cancer Registries. Vital records include statewide birth, death, and marriage certificates, as well as drivers" licenses. UPDB data are available for approved research projects. Privacy of individuals whose data is available through UPDB is strictly protected through the Utah Resource for Genetic and Epidemiological Research (https://rge.utah.edu), established by executive order of the Governor of Utah. We identified individuals with OA between 1996 and 2021 in the UPDB using the following diagnosis (ICD) and related procedure (CPT) codes: 1st MTP joint OA – ICD-9 735.2 or CPT 28289 and 28750; distal and proximal interphalangeal joint OA – ICD-9 715.14, ICD-10 M19.04x and CPT 26862, 26863, 26860, 26861, 26535, or 26536; and glenohumeral osteoarthritis OA – ICD-9 715.11, ICD-10 M19.011, M19.012, M19.019, Z96.611, Z96.612, or M19.0x and CPT 23472. Individuals with any of the following codes were excluded: ICD-9 714.0, 714.2, or 714.3 and ICD-10 M05.xxx, M06.xx, or M08.xxx. A detailed description of the ICD and CPT codes are provide in the online supplementary methods. Manual chart review was performed on affected individuals to verify our coding strategy to identify OA cases and determine if individuals had OA in additional joints. To determine if there was excess familial clustering of OA in each pedigree, we utilized the Familial Standardized Incidence Ratio (FSIR), with a threshold of 2.0. FSIR allows for the quantification of familial risk of a disease by comparing the incidence of a disease in a family to its expected incidence in the general population. See Kazmers, 2021 and Kazmers,

2020 for detailed methods[25, 26]. Pedigrees segregating a dominant pattern of OA inheritance were selected for genomic analysis.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism software. Tests performed and statistical significance are indicated in the figure legends. P-values <0.05 were considered statistically significant.

RESULTS

Rare alleles of NOD-RIPK2 pathway genes are associated with multiple types of familial OA

We took an unbiased genetic approach to determine if the NOD/RIPK2 signaling pathway has a strong effect on OA susceptibility. We assembled a cohort of 150 independent OA families with a dominant inheritance pattern of OA. Each family is characterized by disease that primarily affects a distinct subset of joints: distal and proximal interphalangeal OA[27], glenohumeral OA[28], or 1st MTP joint OA[29, 30]. While all affected individuals have OA in the primary joint used for identification, two families contain a subset of individuals with OA in additional joints. The proband in MTP25 was diagnosed with triscaphe and thumb OA and had a total knee and hip arthroplasty. His daughter was also diagnosed with spine OA. The proband"s sister in SA735 had surgery for thumb OA and bilateral total knee and hip arthroplasty (online supplemental table 1). Whole exome sequence (WES) analysis was performed on informative members of families and coding variants that invariably segregated with OA were identified[11]. Variants were prioritized using the pedigree Variant Annotation, Analysis & Search Tool (pVAAST)[31], which identifies the most likely causal variants in a pedigree based on gene tolerance to mutation, variant frequency, phylogenetic conservation and biological function. We next used the PHEnotype-driven Variant Ontological Re-ranking (PHEVOR)[32] tool with the Human Phenotype Ontology (HPO) search term 'Osteoarthritis' to identify high-priority candidate genes in each family.

In addition to the previously identified OA-associated *RIPK2* allele, six novel alleles affecting five NOD/RIPK2 pathway genes were found associated with OA in the cohort of families (table 1 and online supplemental figure 1). Consistent with a dominant pattern of inheritance with strong penetrance, the variants are rare in human populations. Three variants affected the *NOD1* (FIJ744 - pVAAST: P-value = 0.00809; LOD = 0.6; PHEVOR: score 3.26, final rank = 33) and *NOD2* genes (UUHR2 - pVAAST: P -value = 0.0592; LOD = 7.195; PHEVOR: score 3.42, final rank = 6 and FIJ7 - pVAAST: P -value = 0.00333; LOD = 15.556; PHEVOR: score 4.7, final rank = 1), which encode intracellular receptors that function as upstream activators of RIPK2. The amino acid substitutions encoded by two of the variants reside within the autoinhibitory domain of the respective NOD protein[33]. Candidate variants in three additional families affected genes known to modify activity of the NOD/RIPK2 pathway, *CARD9* (FIJ9 - pVAAST: P -value = 0.00106; LOD = 16.838; PHEVOR: score 3.08, final rank = 19), *CHUK* (MTP25 - pVAAST: P -value = 0.00102; LOD = 12.55; PHEVOR: score 4.55, final rank = 3), and *IKBKB* (SA735 - pVAAST: P -value = 0.000743; LOD = 16.15; PHEVOR: score 4.23, final rank = 10)[34–37]. The family studies indicate a striking correlation between inheritance of variants that alter conserved

sites within proteins of the NOD/RIPK2 signaling pathway and the occurrence of disease within families exhibiting OA of the hand, 1st MTP joint, or shoulder.

Generation of the *Ripk2*[104Asp] mouse

Nod1/2 and *Ripk2* are expressed in uninjured joints of mice, and the pathway is activated following injury (online supplemental figure 2). To determine whether altered pathway signaling is sufficient to confer increased susceptibility to OA, we used precise genome editing to introduce the human *RIPK2*[104Asp] variant into the C57BL/6J inbred strain of mice, thereby creating an isogenic pair of mouse lines: the parental strain, which encodes the mammalian lineage-conserved Asn at position 104 (WT), and a derived line whose *Ripk2* allele encodes Asp at position 104 (*Ripk2*[104Asp]) (figure 1A). The modified allele is expressed at WT levels (figure 1B and online supplemental figure 3). Homozygous and heterozygous mice carrying the *Ripk2*[104Asp] allele are viable and display no overt phenotypes. To recapitulate the dominant human phenotype[11], heterozygous *Ripk2*[104Asp] mice were used for all subsequent analyses.

A single copy of the *Ripk2*[104Asp] allele is sufficient to alter gene expression in primary chondrocytes

To determine if the *Ripk2*[104Asp] allele perturbed NOD/RIPK2 signaling, cultured primary chondrocytes[38] were stimulated with the Nod2 agonist, muramyldipeptide (MDP). Both WT and *Ripk2*[104Asp] chondrocytes responded to MDP by upregulating genes associated with proinflammatory signaling (online supplemental figure 4). However, the transcriptional response of *Ripk2*[104Asp] chondrocytes was significantly amplified as compared with that of WT controls, consistent with previous functional assays of the *Ripk2*[104Asp] allele[11] (figure 1C). MDP-stimulated gene expression was indeed dependent on Ripk2 activity as co-incubation of chondrocytes with the Ripk2 inhibitor WEHI-345[39] significantly reduced expression of many genes, including those whose expression is directly associated with OA (*Mmp13*, *Col1a1*, *Col1a2*, and *Ccna2*) (figure 1D and E). These data indicate the *Ripk2*[104Asp] allele confers heightened gene expression upon activation of the NOD2 receptor.

The *Ripk2*[104Asp] allele acts dominantly and is sufficient to confer increased susceptibility to OA

The joints of mature *Ripk2*[104Asp] animals appear structurally similar to those of WT mice with no histological evidence of joint degeneration (figure 2 A-D, I and J). Nevertheless, *Ripk2*[104Asp] mice displayed increased sensitivity to experimentally induced OA, initiated by destabilization of the medial meniscus (DMM) of the stifle (knee) joint[40]. Eight weeks after DMM surgery *Ripk2*[104Asp] mice exhibited a significant increase in the extent and severity of cartilage damage on the medial (both femoral condyle and tibial plateau) and lateral (femoral condyle) faces of the knee as compared to operated WT controls (figure 2A-J and online supplemental figure 5). Blinded histological scoring of the entire joint or individual quadrants revealed highly significant differences in the average and maximal OARSI scores[40] of DMM-treated WT and *Ripk2*[104Asp] mice (figure 2I and J). In contrast, no significant difference was evident in the degree of synovitis observed in the operated joints of the two groups of mice (figure 2K). As a non-invasive alternate method for

inducing PTOA in 16-week-old mice, we employed mechanical rupture of the anterior cruciate ligament (ACL)[41]. Again, *Ripk2^104Asp* mice showed a significant increase in cartilage damage compared to WT controls when examined 5 weeks post-rupture (online supplemental figure 6).

Injured knee joints of *Ripk2^104Asp* mice exhibited gene expression signatures associated with classical OA, but indicative of a more advanced disease state as compared with WT. Genes upregulated in the injured *Ripk2^104Asp* knee joints soon after ACL rupture are involved in both the innate and adaptive immune response (*Prg2*, *Trpc6*, *Bank1*, *Siglecf*, *Clca3a1*, *Isg15*, *Ighg1*, *Ighg2b*, and *Rsad2*) and include genes linked to OA (*B2m*, *A2m*, *Il17r*, *Ctsk*, *Angpt4*, *Aldh1a2*, and *Alox15*) (figure 3A). Conversely, many ECM genes whose depletion is associated with OA pathogenesis were significantly downregulated in *Ripk2^104Asp* joints, including *Acan*, *Col2a1*, *Col3a1*, *Comp*, *Prg4*, *Fn1*, *Hspg2*, *Matn2*, and *Cspg4* (figure 3A). Thus, whereas the transcriptional response of *Ripk2^104Asp* mice to injury closely parallels that of WT, as indicated by the altered expression of OA-associated genes, it is exaggerated, with increased expression of inflammatory and catabolic factors and increased down-regulation of many ECM genes.

The *Ripk2^104Asp* allele alters the basal physiology of knees joints and the response to PTOA

In the absence of evident tissue remodeling that might indicate emergent OA in the knee joints of *Ripk2^104Asp* mice (figure 2A-D, I and J), we asked whether the joints exhibited altered signs of gene expression and/or inflammatory state. Analysis of primary chondrocytes revealed that gene expression was significantly altered in *Ripk2^104Asp* cells as compared with those isolated from WT mice (figure 3B). Genes upregulated included well-known markers of OA, including those associated with hypertrophic chondrocytes and ECM remodeling (*Mmp13*, *Mmp14*, *Timp1*, *Timp2*, *Loxl4*, and *Col10a1*), growth factor signaling (*Ctnnb1*, *Ckap4*, and *Pdgfrb*), leptin signaling (*Lepr*), PI3K/Akt/mTOR signaling (*Akt1*), as well as genes involved in inflammatory signaling (*Lpcat1*, *Rbx1*, *Ccn2*, *Fasn*, *Cfhr2*, *F5*, *Elovl6*, Hc, and *Thbs1*) (figure 3B).

The striking linkage between the altered gene expression profile of cultured *Ripk2^104Asp* chondrocytes and markers of mature OA led us to investigate if altered marker expression presaged the response to injury in the whole joint. The joints of both sham-operated and DMM mice revealed substantive effects of the *Ripk2^104Asp* allele on Nod/Ripk2 activity, matrix components, and markers of inflammation (figure 4 and online supplemental figure 7). Although the Ripk2 protein is present at low levels in tibial chondrocytes of WT and *Ripk2^104Asp* animals subjected to sham surgery (figure 4A), pathway activity appears elevated in the *Ripk2^104Asp* joint, as reflected in higher levels of activated phospho-NF-κB (pNF-κB) compared with that of WT sham controls (figure 4B and online supplemental figure 7). Following DMM surgery, pathway activity differences are enhanced, as both the level of Ripk2 expression and the number of chondrocytes expressing NF-κB are considerably elevated in the operated joints of *Ripk2^104Asp* mice relative to WT (figure 4A, B, C, and online supplemental figure 7). Similarly, expression of matrix markers is altered in sham-operated *Ripk2^104Asp* joints in a manner that parallels the gene expression changes

associated with overt OA. As compared with WT sham controls, knee joints of sham surgery *Ripk2^{104Asp}* mice express elevated levels and have increased numbers of chondrocytes expressing Mmp13, a metalloproteinase that targets collagen for degradation (figure 4C and E). Mmp13 expression in the *Ripk2^{104Asp}* mice extends beyond the superficial layer of cartilage into deeper layers relative to WT controls (brackets in figure 4C and online supplemental figure 7). Consistent with this finding, collagen deposition scored by the presence of Col2 appears relatively deficient in the joints of sham-operated *Ripk2^{104Asp}* mice (figure 4D, C, and online supplemental figure 7). The differences between WT and *Ripk2^{104Asp}* mice in the levels of expression and numbers of chondrocytes expressing Mmp13 and Col2 between WT and *Ripk2^{104Asp}* mice become even more exaggerated following DMM surgery (figure 4C, D, E and online supplemental figure 7). Altogether these data indicate the *Ripk2^{104Asp}* allele alters the basal physiological state of the joint so that it expresses features normally associated with overt OA.

As *Ripk2^{104Asp}* chondrocytes have elevated expression of proinflammatory genes, and as local inflammation can sensitize joints to PTOA[42], knees were examined in situ for the expression of inflammatory markers. iNos is a major inflammatory mediator that is increased in OA. It is expressed in many tissues of the joint, including chondrocytes and macrophages[43], both of which contribute directly to homeostatic maintenance of the joint[44, 45]. Whereas sham-operated WT mice have uniformly low levels of iNos expression throughout the joint, following DMM surgery iNos is highly induced in the joints, especially in the meniscus and osteophyte (figure 5A). In contrast, even sham-operated *Ripk2^{104Asp}* mice exhibit a striking elevation of proinflammatory iNos signal in cartilage, meniscus, and synovial tissue (figure 5A). Following DMM surgery, elevated iNos expression is also evident in the cartilage, meniscus, and osteophytes (figure 5A and B).

In the normal response to inflammation, CD206+ macrophages accrue in joints and are involved in resolving inflammation and tissue repair. Despite elevated iNos expression in the knees of sham-operated *Ripk2^{104Asp}* mice, they did not have increased numbers of CD206+ cells relative to WT joints (figure 5C and D). Moreover, *Ripk2^{104Asp}* mice had a clear deficit in the recruitment of anti-inflammatory CD206+ cells into the cartilage, meniscus, and synovium of operated joints as compared with joints of WT mice (figure 5C and D). In sum, prior to overt injury, knee joints of *Ripk2^{104Asp}* mice exhibit higher than normal levels of inflammatory gene expression; following injury, *Ripk2^{104Asp}* mice are relatively poor at recruiting factors to resolve acute inflammation.

The *Ripk2^{104Asp}* mice do not exhibit an elevated inflammatory phenotype

The NOD/RIPK2 signaling pathway operates broadly and the heightened expression of OA-associated markers in the knee joints of *Ripk2^{104Asp}* mice may reflect widespread elevation of inflammation. We examined the ability of cultured bone marrow-derived macrophages (BMM) from WT and *Ripk2^{104Asp}* mice to respond to MDP. In contrast, to the effects of the *Ripk2^{104Asp}* allele on the expression profile of chondrocytes, relatively few genes were differentially expressed upon stimulation of the BMM cells (online supplemental figure 8). To assess systemic differences in the inflammatory states of the mice, serum cytokine levels pre- or post-DMM surgery were measured. There was no difference in

the serum concentration of any of 13 inflammatory cytokines sampled from 16-week old pre-surgery WT and $Ripk2^{104Asp}$ mice (figure 6), indicating unoperated $Ripk2^{104Asp}$ mice do not have a measurably elevated systemic phenotype. In contrast, in response to localized joint injury, $Ripk2^{104Asp}$ mice mount a highly augmented systemic response. At 4 weeks following DMM surgery both WT and $Ripk2^{104Asp}$ responded with raised serum levels of the proinflammatory cytokines IL1β, INFβ, and the anti-inflammatory IL10, but the degree of increase and the levels of these cytokines were significantly elevated in the $Ripk2^{104Asp}$ mice (figure 6). The differences in serum cytokine response are transient, as they are resolved by 8 weeks post-surgery (figure 6).

DISCUSSION

Animals carrying the single amino acid change encoded by the $Ripk2^{104Asp}$ variant have a magnified response to joint injury that leads to a predisposition to develop OA. The allele creates a chronically hyperactive inflammatory state in the joint with early signs of defective joint maintenance, as evidenced by gene expression in chondrocytes isolated from young mice and altered expression of pNF-κB, iNos, Mmp13, and Col2 in mature animals. Nevertheless, the elevated activity of the NOD/RIPK2/NF-κB pathway caused by the variant allele has a very modest effect on tissue remodeling under normal laboratory conditions. The $Ripk2^{104Asp}$ allele does not alter which signaling pathways and genes are activated in response to joint injury. Rather, consistent with the elevated signaling activity of the variant protein[11], it simply leads mice to mount an accelerated and elevated response to injury, characterized locally in the joint by amplified Ripk2 and pNF-κB expression, exaggerated changes in the expression of genes indicative of OA progression, including Mmp13 and Col2, and histologically recognizable deterioration. Both local as well as systemic inflammatory responses are amplified following acute injury, seen by the deficit of CD206+ cells in the joint, altered gene expression in BMM, and the transient rise in serum cytokines.

Multiple sources of inflammation have been proposed as potential drivers of OA, including mechanosensory signaling and responses of chondrocytes or other joint resident cells to damage-associated molecular patterns (DAMPs)[4, 46–48]. Our work helps identify which of the potential inflammatory signaling pathways functions to limit or promote the occurrence of OA and which cells are essential for promoting the inflammatory response that leads to the tissue remodeling seen in OA[24]. Here we demonstrate the NOD/RIPK2 is an important inflammatory pathway in the development of OA. Mice that constitutively express the $Ripk2^{Asp104}$ allele exhibit a set of marked changes in their knee joints without chronic systemic inflammation, reflecting alteration in normal local homeostatic mechanisms in the joint. Although NOD/RIPK2 signaling is known to activate multiple downstream pathways (e.g., NF-κB, p38, etc.), we do not yet know which of the targets of the activated Ripk2Asp104 protein are particularly significant for OA susceptibility. Finally, even though our results suggest $Ripk2^{Asp104}$ activity functions in the knee joint, we have not defined the specific cells and tissues of the joint that require $Ripk2^{Asp104}$ activity for OA development. Conditional spatial and temporal activation of $Ripk2^{Asp104}$ will allow us to test the necessity of the NOD/RIPK2 pathway in OA development.

Understanding the ligands that activate the NOD/RIPK2 pathway is important for determining risk factors. The NODs were initially described as activated by bacterial peptidoglycan fragments[49, 50], yet there is increasing evidence they can be activated by non-pathogen associated molecules, including DAMPs[51]. One possible DAMP activator of NODs in the synovial joint is the pro-catabolic Fibronectin fragment (FN-f), which is produced by cleavage of the Fibronectin protein in response to injury[52, 53]. *NOD1* and *NOD2* expression is upregulated in human chondrocytes treated with the 29-kDa amino-terminal FN-f, and activation NF-κB pathway is dependent on NOD2/RIPK2 activity[17]. Thus, local hyperactivity of the NOD/RIPK2 pathway may augment the response to FN-f release after injury to the joint and lead to increased OA susceptibility. Interestingly, two of the OA-associated NOD variants we have identified affect the Leucine Rich Repeat (LRR) domain, which functions in autoinhibition of NOD-mediated signaling activity and ligand binding. In the absence of ligand, the LLR domain interacts with the NOD domain to keep NOD1/2 in an inactive state and prevent NOD:NOD protein interactions that promote signaling[18, 33, 54]. Thus, the NOD variant proteins may have heightened basal activity or may be hyperresponsive to ligand binding. Functional analyses of the NOD variants will allow us determine if the variants alter signaling activity and if the altered activity is dependent on FN-f or other ligands.

Our study is focused on the NOD/RIPK2 pathway in OA of the 1st MTP joint, hand, and shoulder. We selected these joints to minimize the phenotypic heterogeneity to increase our power of identifying causal variants in these families. We have excluded families with knee and hip OA from our study due to confounding factors often present in affected individuals (e.g., traumatic injury, ligament tear, developmental dysplasia of the hip, Perthes disease, and avascular necrosis of the hip). Given that we have identified NOD/RIPK2 pathway variants in multiple joints, we predict that alteration of the pathway is also a risk factor for knee and hip OA. It is possible the NOD/RIPK2 pathway is not a major risk factor for these joints as no NOD/RIPK2 pathway genes have been identified in GWAS studies to date[55]. Identification and analysis of knee and hip OA families free from confounding factors will allow us to test this hypothesis. Further, we only address the role of *Ripk2[104Asp]* in PTOA and do not test if the allele leads to increased OA onset or severity in aged animals. Given the *Ripk2[104Asp]* allele creates a chronically hyperactive inflammatory state in the joint, the most parsimonious hypothesis is that aged animals carrying the disease allele may have increased prevalence of OA. Alternatively, in the absence of traumatic injury to the joint, aged *Ripk2[104Asp]* mice may not have elevated OA susceptibility.

In sum, we propose modulation of the NOD/RIPK2 signaling pathway is a general vulnerability factor for OA. Supporting our hypothesis, we have found rare variants altering conserved positions in NOD/RIPK2 pathway proteins in 7 of the 151 (5%) families we have examined with non-syndromic OA. Consistent with a causal connection between pathway activity and OA, hyperactivity of the NOD/RIPK2 pathway is associated with several human inflammatory syndromes that include arthritis as a comorbidity[22, 23, 56]. Our data indicate that modification of the NOD/RIPK2 pathway can render multiple joints (both weight and non-weight bearing) susceptible to OA. While the initiating factor (injury, repetitive use, diabetes, obesity, etc.) and activating ligand may differ between joints and individuals, our work has shown that elevated NOD/RIPK2 signaling is a predictive indicator of

susceptibility to OA. Further pursuit of this signaling pathway and the spatiotemporal requirement for its activity may lead to assays for detection of early stages of disease and have therapeutic potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key messages

What is already known about this subject?

A coding variant that elevates the proinflammatory activity of the RIPK2 signal transducer has been associated with familial early-onset OA, raising the possibility that perturbations of NOD/RIPK2 signaling may confer susceptibility to OA.

What does this study add?

We discover alleles affecting several components of the NOD/RIPK2 signaling pathway are associated with multiple forms of familial OA, supporting the novel hypothesis that the pathway is a common vulnerability factor for OA.

Introduction of the OA-associated hyperactive $Ripk2^{104Asp}$ allele into the mouse genome causes changes in the basal physiological status of the joint in ways that presage a definitive OA state.

Although $Ripk2^{104Asp}$ mice display no evidence of systemic inflammation or histological evidence of joint degeneration, they displayed significantly increased sensitivity to experimentally induced OA.

How might this impact on clinical practice or future developments?

By showing that altered NOD/RIPK2 signaling is predictive of susceptibility to multiple forms of OA, the work brings new focus to the functions of the signaling pathway in maintaining joint homeostasis, may guide development of assays to detect early stages of OA, and may indicate new therapeutic approaches to disease intervention.
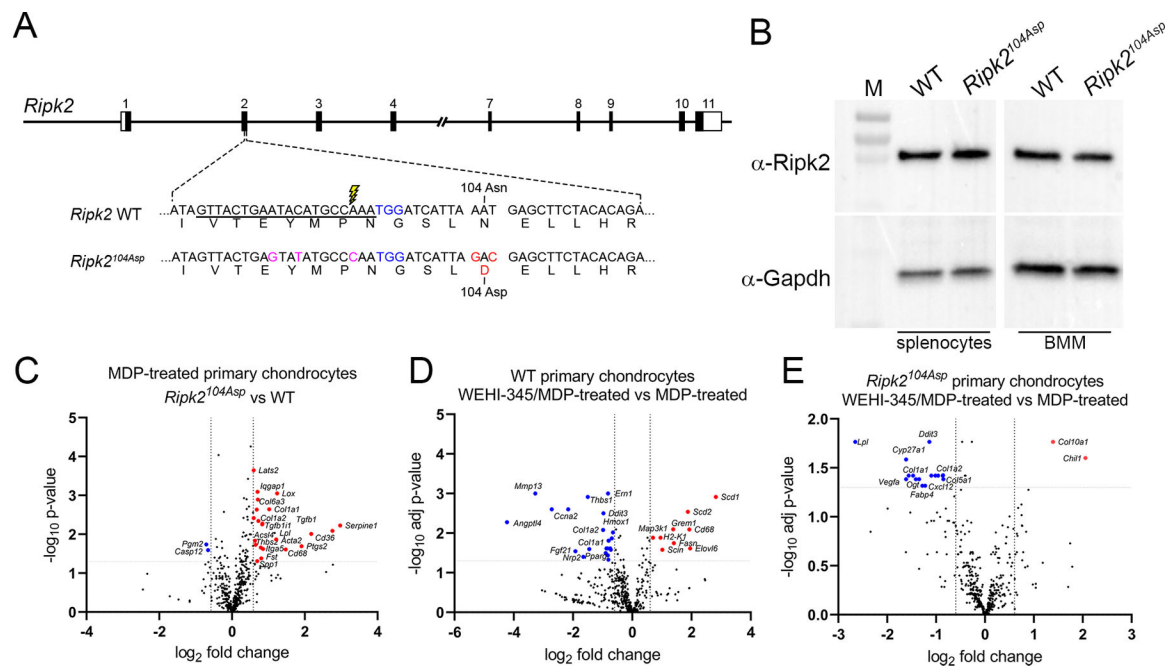
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Figure 1.

Generation and validation of the *Ripk2^104Asp* mouse. (A) Schematic illustration of the mouse *Ripk2* locus and detailed view of exon 2. CRISPR/Cas9-stimulated homology directed repair was used to edit sequences of C57Bl/6J mice (WT) encoding the *Ripk2^104Asn* protein to generate an isogenic line that expressed the OA-associated Ripk2^104Asp protein from the native locus. The guide RNA target sequence is underlined, the PAM site is highlighted in blue, and the Cas9 cleavage site is denoted with lightning bolt. An oligonucleotide donor was used as a template to create the mutations to generate Asp 104 (red) as well as silent mutations (magenta) to prevent targeting of the modified locus. (B) Immunoblot analysis indicates similar Ripk2 protein present in WT and *Ripk2^104Asp* splenocytes or bone marrow derived macrophages (BMM). Gapdh is used as a loading control. M = protein mass standards in kDa. (C) A single copy of *Ripk2^104Asp* is sufficient to alter the gene expression response of primary chondrocytes to MDP treatment. Volcano plots indicate genes significantly upregulated (red) or downregulated (blue) in MDP-treated *Ripk2^104Asp* as compared to MDP-treated WT primary chondrocytes. (D and E) Increased gene expression in response to MDP stimulation is dependent on Ripk2 activity. D) WT or E) *Ripk2^104Asp* primary chondrocytes were stimulated with MDP in the presence or absence of the Ripk2 inhibitor, WEHI-345. Volcano plots indicate genes significantly upregulated (red) or downregulated (blue) upon MDP-stimulation in the presence of the inhibitor.
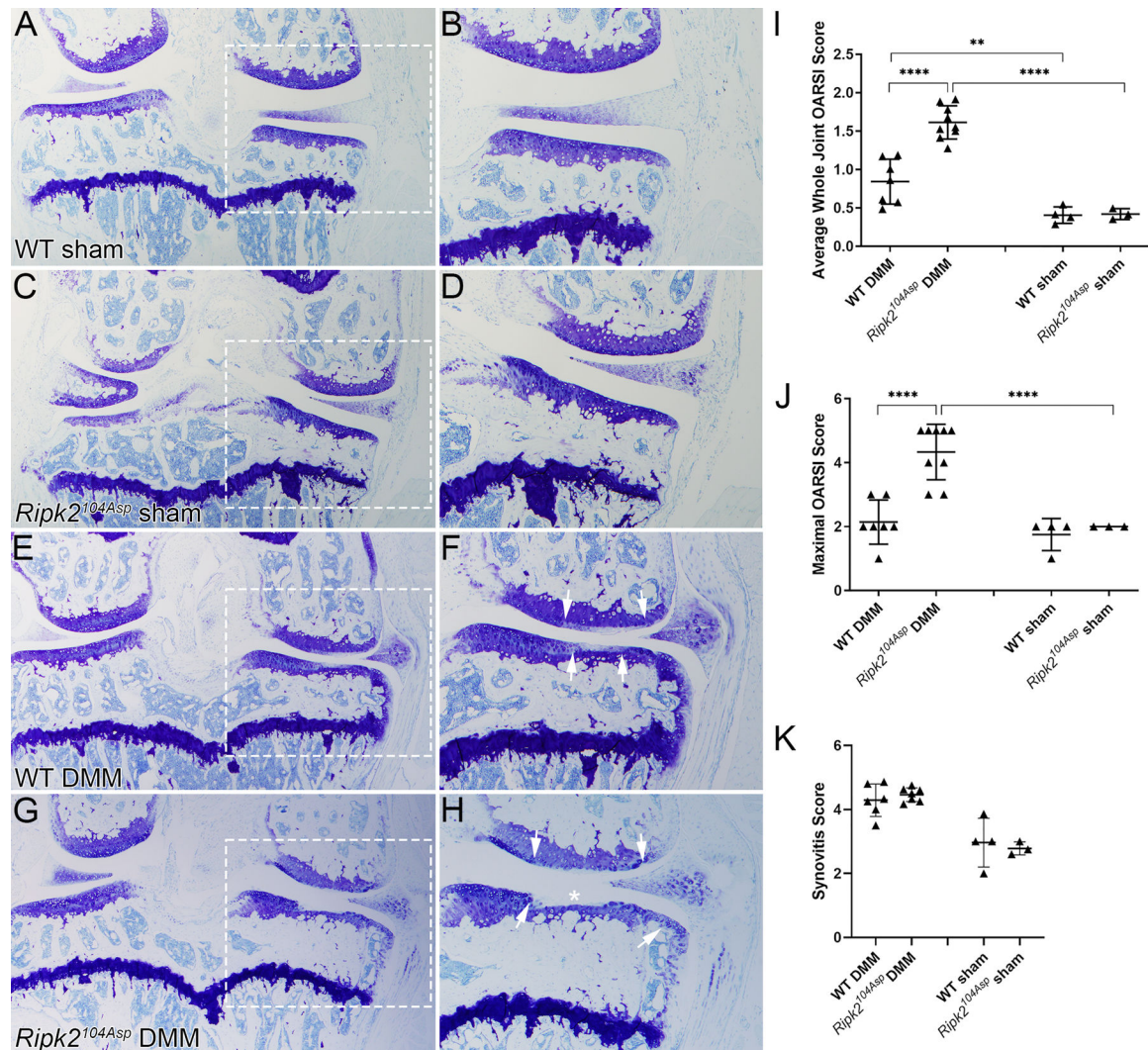
Figure 2.
The *Ripk2^{104Asp}* allele acts dominantly and is sufficient to confer increased susceptibility to post-traumatic osteoarthritis. (A-D) Knee joints of WT and *Ripk2^{104Asp}* mice that underwent sham surgery are similar histologically, with no indication of an OA phenotype. (E, F) Following DMM surgery, WT knees displayed mild/moderate loss of proteoglycan content in the articular cartilage on the medial side of the knee (indicated by loss of toluidine blue staining). The extent of the damage is indicated by the arrows in F, G, H. Following DMM surgery, joints of *Ripk2^{104Asp}* mice displayed moderate/severe loss of proteoglycan content (arrows in H), cartilage fibrillation, and complete loss of articular cartilage in the medial tibial plateau (asterisk in H). (I) Average whole joint and (J) maximal OARSI scores of sham-operated and DMM-operated knee joints. (K) There was no difference in the degree of synovitis between different genotypes. A, C, G, E are images of the entire knee joint; dashed boxes were magnified in B, D, F, H to focus on degradation on the medial side of the joint. Femur is up and medial is to the right in all images. WT sham (n=4), *Ripk2^{104Asp}* sham (n=3), WT DMM (n=7), *Ripk2^{104Asp}* DMM (n=9). All animals were analyzed 8 weeks post-surgery. Error bars represent ±SD and statistically significant differences of P ≤ 0.01

(**), P   0.001 (***), and P   0.0001 (****) were determined by two-way ANOVA with Tukey"s multiple comparisons test.
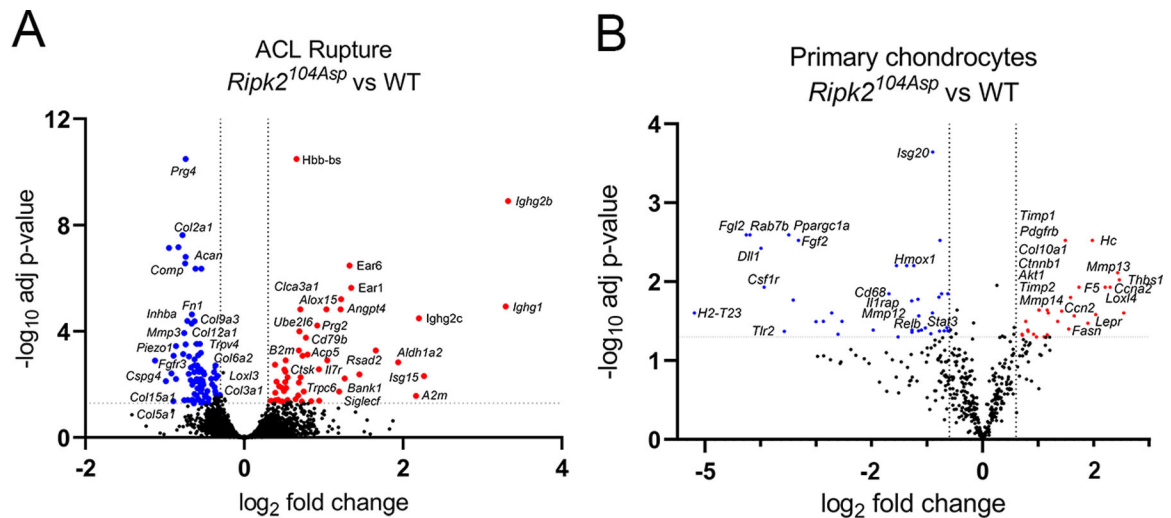
Figure 3.

Ripk2[104Asp] enhances expression of OA-associated markers in the surgically injured joint as well as primary chondrocytes. (A) Comparative analysis of RNA-seq performed on whole joints isolated from WT or *Ripk2[104Asp]* mice 10 days post ACL rupture. The volcano plot indicates genes significantly upregulated (red) or downregulated (blue) in *Ripk2[104Asp]* as compared to WT joints. (B) The nCounter Fibrosis panel was used to measure gene expression in AC cultured from WT or *Ripk2[104Asp]* mice. Volcano plot indicates genes significantly upregulated (red) or downregulated (blue) in *Ripk2[104Asp]* primary chondrocytes compared to WT primary chondrocytes.
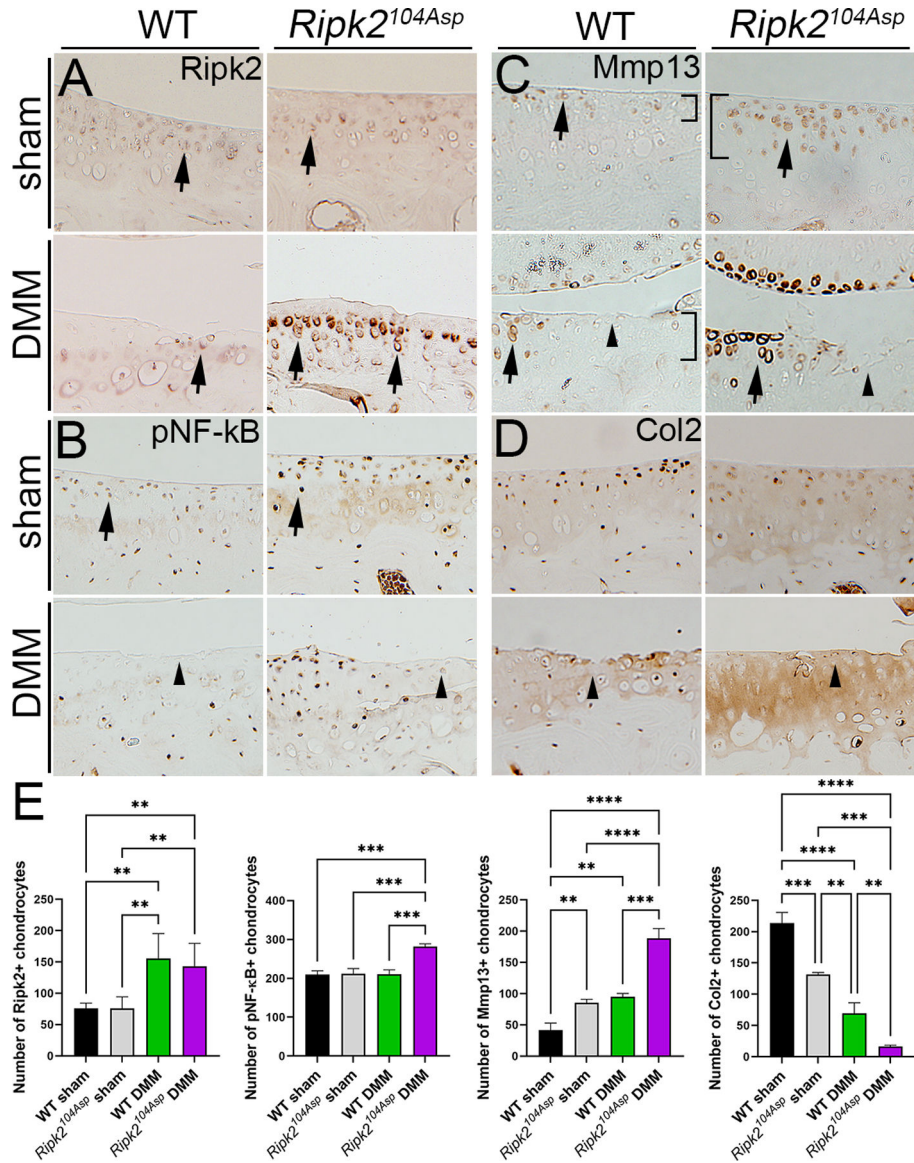
Figure 4.

Ripk2^104Asp enhances NOD/RIPK2 signaling as well as expression of OA-associated markers of matrix remodeling in uninjured joints and joints with PTOA. Immunohistochemical detection of (A) Ripk2, (B) pNF-κB, (C) Mmp13, or (D) Col2 in WT and *Ripk2^104Asp* mice 8 weeks following sham or DMM surgery. (A) Ripk2 is expressed at low levels in chondrocytes (arrows) of WT sham-, WT DMM-, and *Ripk2^104Asp* sham-operated joints. In contrast, Ripk2 expression is highly elevated in chondrocytes in *Ripk2^104Asp* knees following DMM surgery (arrows). (B) Activated NF-κB (pNF-κB) expression levels are higher in *Ripk2^104Asp* sham-operated joints as compared to WT controls (arrows). The relatively increased Ripk2 expression is maintained in *Ripk2^104Asp* joints following DMM surgery. (C) Mmp13 expression is elevated in chondrocytes of sham-operated *Ripk2^104Asp* mice as compared to WT controls (arrows); the relatively increased expression Ripk2 is maintained in *Ripk2^104Asp* mice following DMM surgery (arrows).

Furthermore, in the unoperated *Ripk2^{104Asp}* joint, Mmp13 expression extends into deeper layers of cartilage, an expression domain normally only seen in WT joints following DMM surgery (brackets in C). (D) Col2 expression is reduced in *Ripk2^{104Asp}* sham surgery mice relative to WT controls, and this loss is further exacerbated by DMM surgery (arrows). In regions with severe cartilage damage (arrowheads), pNF-κB, Mmp13, and Col2 expression is low in both WT and *Ripk2^{104Asp}* DMM-operated joints. Images are of selected regions of the medial tibial condyle (see online supplemental figure 7). (E) Quantification of the number of Ripk2, pNF-κB, Mmp13, and Col2 positive chondrocytes in the medial knee joint of WT sham, *Ripk2^{Asp104}* sham, WT DMM, and *Ripk2^{Asp104}* DMM mice. n = 3 independent animals for each experimental condition. Error bars represent ±SD and statistically significant differences of P 0.01 (**), P 0.001 (***), and P 0.0001 (****) were determined by two-way ANOVA with Tukey's multiple comparisons test.
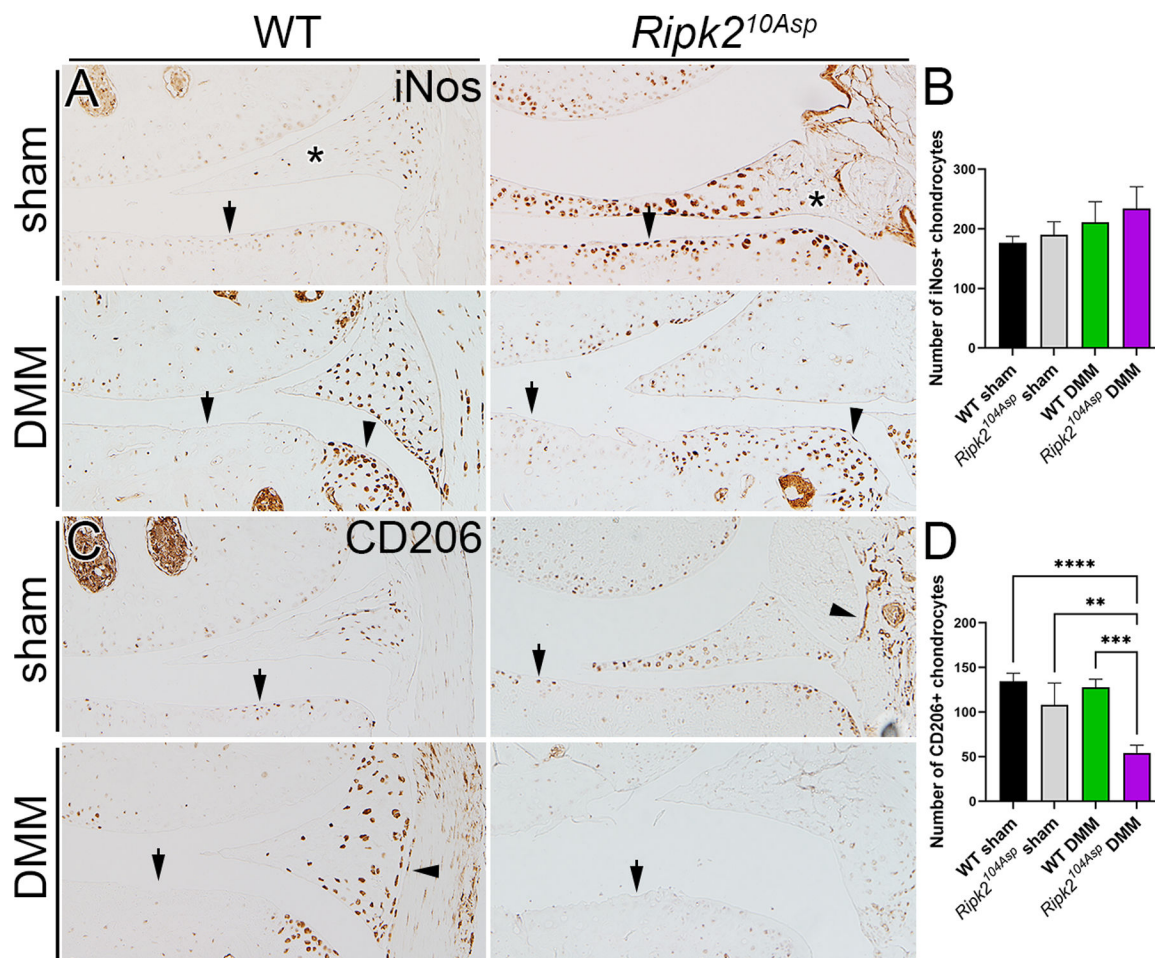
Figure 5.

Ripk2^{104Asp} joints have elevated expression of proinflammatory markers. (A) In WT mice, the proinflammatory marker iNos is normally expressed at low levels in the joint, and is markedly elevated following DMM surgery. In contrast, knee joints of *Ripk2^{104Asp}* have chronically high levels of iNos expression, independent of injury. In A arrows indicate chondrocytes, asterisks mark the meniscus, and arrowheads indicate osteophytes in DMM-operated joints. (B) There is no difference in expression of the anti-inflammatory marker, CD206, between sham-operated surgery WT and *Ripk2^{104Asp}* joints. Following DMM surgery, CD206 is prominently expressed in WT joints whereas it is almost absent in the joints of *Ripk2^{104Asp}* mice. In B arrows indicate chondrocytes and arrowheads indicate synovium. All joints are 8 weeks post-surgery. C) Quantification of the number of iNos and CD206 positive chondrocytes in the medial knee joint of WT sham, *Ripk2^{Asp104}* sham, WT DMM, and *Ripk2^{Asp104}* DMM mice. n = 3 independent animals for each experimental condition. Error bars represent ±SD and statistically significant differences of P　0.01 (**), P　0.001 (***), and P　0.0001 (****) were determined by two-way ANOVA with Tukey"s multiple comparisons test.
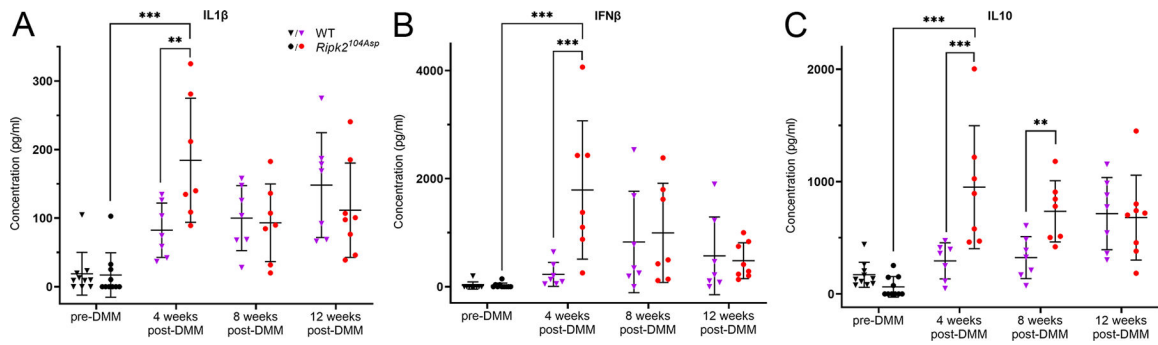
Figure 6.
DMM surgery induces an acute systemic inflammatory response in *Ripk2^104Asp* mice.
Quantification of serum A) IL1β, B) IFNβ, and C) IL10 levels from 16-week-old WT and
Ripk2^104Asp mice just prior to DMM surgery (black diamonds and triangles) and at 4, 8,
and 12 weeks post-surgery (magenta triangles and red circles). Error bars represent ±SD and
statistically significant differences of P 0.01 (**) and P 0.001 (***) were determined by
one-way ANOVA with Tukey"s multiple comparisons test (4-week post-DMM group) and a
two-tailed unpaired t-test (8 and 12 week groups).

Table 1.

NOD/RIPK2 Pathway Variants Identified in Independent Osteoarthritis Families

Gene	OA Phenotype (Family)	Variant	Minor Allele Frequency	Protein Domain Affected by Variant
NOD1	Finger Interphalangeal Joint OA (FIJ744)	c.G2114A:p.R705Q	0.0008	Leucine Rich Repeat Domain
NOD2	1st MTP Joint OA (UUHR2)	c.C2465T:p.A822V	0.00007	Leucine Rich Repeat Domain
NOD2	Finger Interphalangeal Joint OA (FIJ7)	c.G247A:p.A83T	0.00008	Caspase Activation and Recruitment Domain
IKBKB	Glenohumeral OA (SA735)	c.G1663A:p.G555R	0.00008	Scaffold Dimerization Domain
CARD9	Finger Interphalangeal Joint OA (FIJ9)	c.G722A:p.R241Q	0.00005	Structural Maintenance of Chromosomes
CHUK	1st MTP Joint OA (MTP25)	c.A376T:p.S126C	0.0008	Kinase Domain
*RIPK2**	1st MTP Joint OA (UUHR1)	c.A310G:pN104D	0.0004	Kinase Domain

*
- Previously described in Jurynec, 2018[11].