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Interleukin-1 Gene Cluster Variants With Innate Cytokine Production Profiles and Osteoarthritis in Subjects From the Genetics, Osteoarthritis and Progression Study

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Objective. **To assess whether genetic variation in the interleukin-1 (IL-1) gene cluster contributes to familial osteoarthritis (OA) by influencing innate ex vivo production of IL-1β or IL-1 receptor antagonist (IL-1Ra).**

Methods. **Innate ex vivo IL-1β and IL-1Ra production upon lipopolysaccharide (LPS) stimulation of whole blood cells was measured in subjects from the Genetics, Osteoarthritis and Progression (GARP) Study, which includes sibling pairs in which at least one sibling has symptomatic OA at multiple sites. Radiographic OA (ROA) was assessed by Kellgren/Lawrence score. Subjects from the GARP Study and controls from the Rotterdam Study were genotyped for 7 single-nucleotide polymorphisms (SNPs) encompassing the IL-1 gene cluster on chromosome 2q13. Linkage disequilibrium analysis and genotype and haplotype association analysis were performed to assess the relationship between the IL-1 gene cluster SNPs, innate ex vivo cytokine production, and OA.**

Results. **Among subjects in the GARP Study, the haplotype variable-number tandem repeat in intron 2/T+8006C/T+11100C 2/2/1 of the *IL1RN* gene was significantly associated with reduced innate ex vivo bioavailability of IL-1β upon LPS stimulation ($P = 0.026$) and with ROA at the highest number of joint locations.**

Conclusion. **These results show that genetic variation at the IL-1 gene cluster is associated with lower IL-1β bioavailability and with OA at a large number of joint locations. The data further indicate that, among subjects with OA affecting the highest number of joints, the innate immune system may be activated, thereby obscuring possible underlying mechanisms.**

Osteoarthritis (OA) is a common joint disease and an important cause of pain and disability in the general population. Genetic factors play an important role in the etiology of various subtypes of OA (1–5). There has been a large amount of interest in the role of cytokines as mediators of joint damage and inflammation in the pathogenesis of OA. Chondrocytes are known to respond to interleukin-1β (IL-1β) by reducing the synthesis of matrix components and increasing the synthesis of matrix metalloproteinases (MMPs) (6). MMPs degrade extracellular matrix components in articular cartilage. IL-1 receptor antagonist (IL-1Ra) is the natural competitive inhibitor of IL-1β, occupying the cell surface IL-1 receptor without triggering signal transduction, and its levels might be considered critical in determining IL-1β bioavailability (6).

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One way of investigating the influence of cytokine profiles on disease is by measuring innate ex vivo cytokine production upon lipopolysaccharide (LPS) stimulation of whole blood. Studies of twins have shown that ex vivo production of the cytokines IL-1β, IL-1Ra, tumor necrosis factor α (TNFα), and IL-10 varies by 60–70% based on heritability alone (7). Subjects can thus be characterized as high (proinflammatory) or low (antiinflammatory) producers based on these cytokine profiles (8,9). Such a characteristic may influence susceptibility to diseases with an inflammatory component (10–12). Data supporting this hypothesis in OA come from our previous investigations (13) in which we demonstrated that, in the Genetics, Osteoarthritis and Progression (GARP) Study (14), a proinflammatory profile, i.e., high innate ex vivo IL-1β and IL-1Ra levels and low IL-10 levels, occurred among subjects with familial OA at multiple sites, as compared with controls. The innate ex vivo production of TNFα, which was not associated with the onset of OA, was the only cytokine that predisposed to knee OA progression (15).

In recent years the concept that inflammation in OA contributes to symptoms and augments many pathologic changes has become generally accepted (16,17); however, it is unclear whether this is a causal association or marks the ongoing disease process. Furthermore, the interplay between secreted IL-1β and IL-1Ra levels must be taken into account, since together they influence IL-1β bioavailability (18).

The genes encoding IL-1α, IL-1β, and IL-1Ra (*IL1A*, *IL1B*, and *IL1RN*, respectively) reside within a 430-kb region on chromosome 2q13. Although observed only in relatively small studies and not always consistently, it has been shown that several DNA variants within the genes of the IL-1 gene cluster may be responsible for the variation in heritable innate ex vivo cytokine production upon LPS stimulation (12). Furthermore, in vitro experiments have demonstrated functional ability of *IL1B* promoter single-nucleotide polymorphisms (SNPs) to enhance IL-1β production upon LPS stimulation (19). The role of the *IL1RN* variable-number tandem repeat (VNTR) allele 2 appears most consistent in affecting cytokine production in vivo (20) and may be considered most important for the fine-tuning of IL-1β bioavailability, as determined by the ratio of innate ex vivo cytokine production of IL-1β and IL-1Ra upon LPS stimulation (18).

Multiple genetic association studies have been undertaken to investigate whether these potential functional aspects of IL-1 gene cluster polymorphisms may in part explain genetic susceptibility to OA. Previously, we and others have reported associations of the IL-1 gene cluster with knee, hip, and hand OA (21–26), although others failed to confirm these associations (27,28). Taken together, the findings to date indicate that the effects of IL-1 gene cluster SNPs on innate ex vivo cytokine production and OA may be complex and may involve interactions among different polymorphic sites, and that they should therefore be investigated by studying independent haplotypes.

Combined investigation of ex vivo IL-1β bioavailability measures after LPS stimulation, genetic variation at the IL-1 gene cluster, and OA disease status in a single study population allows assessment of possible underlying relationships (29). In the present analysis, we tested for the influence and interaction of IL-1 gene cluster polymorphisms and haplotypes on IL-1β bioavailability in a relatively large number of subjects from the GARP Study. We further investigated whether the haplotypes relevant to IL-1β bioavailability correlate with a score of the number of joint locations with radiographically evident OA (ROA).

PATIENTS AND METHODS

The GARP Study. The ongoing GARP Study includes 191 Caucasian sibling pairs of Dutch ancestry, in which the proband is affected with symptomatic OA at multiple sites. Probands (ages 40–70 years) and their siblings were included in the GARP Study if the proband had OA at multiple joint sites of the hand according to the American College of Rheumatology criteria (30) or had symptomatic OA in 2 or more of the following joint sites: hand, spine (cervical or lumbar), knee, or hip (14). In the spine, knee, or hip, symptomatic OA was defined as the presence of symptoms of OA in addition to radiographic signs (31–34).

Conventional radiographs of the hands (dorsovolar), knees (posteroanterior with weight-bearing/semiflexed and lateral), hips (anteroposterior [AP]), lumbar (AP and lateral), and cervical spine (AP, lateral, and transoral) were obtained for all participants. Radiography was performed in a standardized manner with a fixed film-focus distance and a fixed joint position. Radiographic characteristics of OA were scored according to the Kellgren/Lawrence scale (35) by a single experienced and trained radiographer, according to an agreed-upon protocol as described in detail elsewhere (14). In the current study we used (the highest quartile of) the total ROA score. The total ROA score (0–10) represents a summed score proportional to radiographic cartilage abnormalities at each joint location in the knee (0–2), hip (0–2), hand (0–2), and facet joints (0–2) and spinal disc degeneration (0–2), as described previously in detail (36). The highest quartile of the total ROA score represents GARP subjects with the highest number of joint locations with radiographic abnormalities, who were compared with other subjects in the GARP Study and/or random subjects from the Rotterdam Study (37). We compared affected sibling pairs from the GARP Study with a

Table 1. Characteristics of the study population (n = 382)*

No. (%) female	311 (81)
No. (%) with ROA, by joint site	
Hip	107 (28)
Knee	150 (39)
Hand	213 (56)
Facet	235 (62)
Degenerative disc	256 (67)
Total ROA score, mean (range)	3.45 (0–9)
Age, mean (range) years	60.3 (43–79)
Body mass index, mean ± SD kg/m^2	27.0 ± 4.7
Mean IL-1β:IL-1Ra ratio, mean ± SEM	0.798 ± 0.003

* The study population consisted of subjects from the Genetics, Osteoarthritis and Progression Study who had symptomatic osteoarthritis (OA) at multiple joint locations including subjects with unilateral and/or bilateral joint replacement (n = 38 for hip and n = 8 for knee). ROA = radiographic OA; IL-1β = interleukin-1β; IL-1Ra = IL-1 receptor antagonist.

random sample of unrelated subjects ages 55–65 years (n = 809) from the Rotterdam Study as a reference group representing the general population. Both study populations comprise Caucasian subjects from the western areas of The Netherlands (mean age 60.3 years) and may represent the same genetic background. In this sample, symptomatic OA has not been assessed previously.

Whole blood stimulation system. Whole blood stimulation was performed as previously described (38). Briefly, blood samples were collected in pyrogen-free heparinized tubes (Endotube; Chromogenix, Mölndal, Sweden). Eight-milliliter whole blood samples were diluted 1:1 with RPMI 1640 (Gibco Life Technologies, Paisley, UK) and stimulated with 10 ng/ml *Escherichia coli* LPS (Difco, Detroit, MI). To minimize the influence of circadian rhythms and measurement errors, blood samples were obtained between 8:00 AM and 11:00 AM, the time frame between blood collection and stimulation was <90 minutes, and all stimulations were performed with the same endotoxin batch. One medium-diluted blood sample without LPS was used as a negative control. After 24-hour incubation, samples were centrifuged twice (600*g*) and the supernatants stored at −70°C. Production of IL-1β and IL-1Ra was measured in one batch by enzyme-linked immunosorbent assay, according to the instructions of the manufacturer (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Nine patients were excluded from these analyses because either whole blood samples had not been obtained (n = 5) or data on levels of IL-1Ra or IL-1β were missing (n = 4).

Genotyping measurements. Genomic DNA was isolated from blood samples from subjects in the GARP Study; DNA was missing for 1 subject. In total, 809 subjects from the Rotterdam Study and 381 subjects from the GARP Study were genotyped for 7 SNPs encompassing the IL-1 gene cluster on chromosome 2q13: 1 SNP located in the *IL1A* gene (C−889T [rs1800587]), 3 SNPs in the *IL1B* gene (C+3953T [rs1143634], T–31C [rs1143627], and C–511T [rs16944]), and 3 SNPs in the *IL1RN* gene (VNTR in intron 2, T+8006C [rs419598], and T+11100C [rs315952]). The genotypes of C+3953T, C–511T, and VNTR in the Rotterdam Study had been assessed previously (23). The genotypes of the SNP were determined by mass spectrometry (homogeneous MassArray system; Sequenom, San Diego, CA), under standard conditions. Genotypes were analyzed using Genotyper 3.0 software (Sequenom). Throughout this report the common alleles of the SNPs are designated as 1 and the rare alleles as 2. As controls, genotype data were available on 788 subjects from the Rotterdam Study.

Statistical analysis. The contribution of the individual genotypes of the SNPs of the IL-1 gene cluster to innate ex vivo cytokine production upon LPS stimulation was estimated using a mixed-model regression analysis performed with the logarithmically transformed cytokine levels as the dependent variable and the genotypes of the SNPs and sex as covariables. In the mixed-model analyses, random effects modeled the familial dependencies that might occur for the cytokine levels. These analyses were carried out with SPSS version 14 (SPSS,

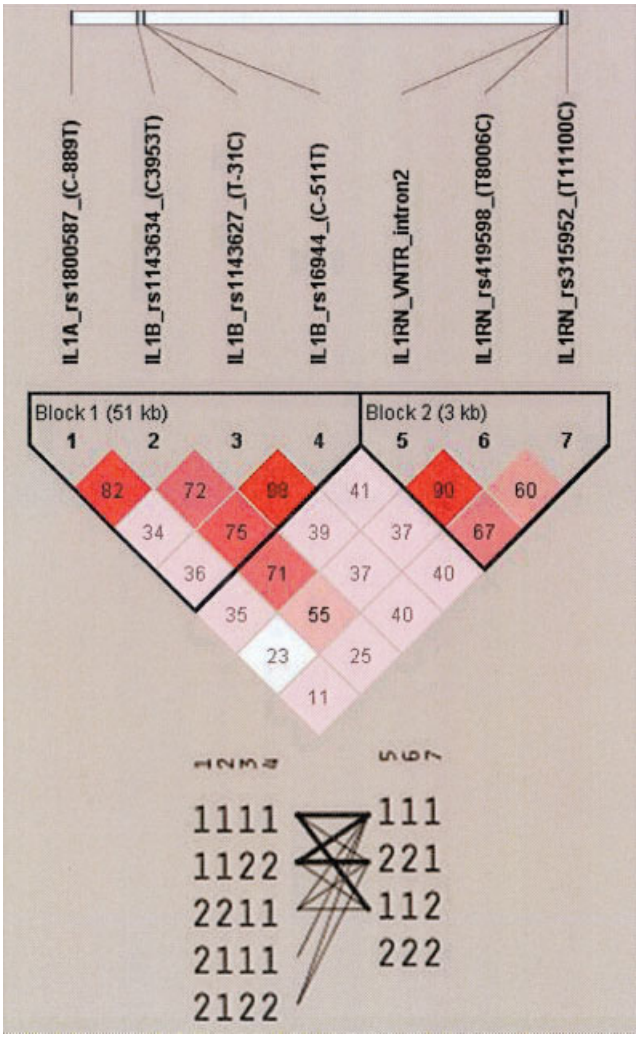

Figure 1. Pairwise linkage disequilibrium across the interleukin-1 (IL-1) cluster single-nucleotide polymorphisms as visualized with the Haploview program and expressed by the linkage disequilibrium coefficient D′.

Table 2. Haplotype association analysis of the association of block 1, consisting of the *IL1A* (C−889T) and *IL1B* (C+3953T, T−31C, and C−511T) genes, and block 2, consisting of 3 *IL1RN* genes (VNTR/T+8006C/+T11100C) with innate ex vivo IL-1β and IL-1Ra production upon LPS stimulation of whole blood cells*

Haplotype block (%)	Haplotypic mean (95% CI)†		
	Log IL-1β	Log IL-1Ra	Log IL-1β:log IL-1Ra
Block 1			
1/1/1/1 (40)	1.72 (1.69–1.75)	2.14 (2.08–2.20)	0.39 (0.36–0.41)
1/1/2/2 (27)	1.73 (1.69–1.77)	2.12 (2.07–2.17)	0.40 (0.37–0.42)
2/2/1/1 (22)	1.77 (1.72–1.81)	2.14 (2.09–2.20)	0.40 (0.37–0.43)
2/1/2/2 (4)	1.66 (1.57–1.75)	2.17 (2.08–2.26)	0.38 (0.35–0.41)
2/1/1/1 (4)	1.71 (1.61–1.81)	2.11 (1.99–2.22)	0.38 (0.35–0.42)
Others (3)			
Block 2			
1/1/1 (44)	1.75 (1.72–1.78)	2.14 (2.09–2.19)	0.40 (0.37–0.42)
1/1/2 (31)	1.74 (1.71–1.78)	2.14 (2.09–2.19)	0.39 (0.36–0.42)
2/2/1 (22)	1.66 (1.62–1.71)‡	2.11 (2.06–2.16)	0.38 (0.35–0.40)§
2/2/2 (2)	1.83 (1.58–2.08)	2.27 (2.19–2.34)¶	0.40 (0.35–0.46)
Others (1)			

* Data on quantitative phenotypes for interleukin-1β (IL-1β) and IL-1 receptor antagonist (IL-1Ra) levels (logarithmically transformed) were analyzed using the Thesias program. *P* values were determined by comparing the specific haplotypic mean with the haplotypic mean for all other haplotypes. VNTR = variable-number tandem repeat; LPS = lipopolysaccharide; 95% CI = 95% confidence interval.
† Values for IL-1β were adjusted for sex; values for IL-1Ra and IL-1β:IL-1Ra were adjusted for sex and body mass index.
‡ $P < 0.002$.
§ $P < 0.026$.
¶ $P < 1.1 \times 10^{-4}$.

Chicago, IL). Haplotypic effects on quantitative innate ex vivo cytokine production and/or total ROA scores were assessed with the Thesias 3.1 program (39) and adjusted for sex and/or body mass index (BMI) where indicated. When interpreting the Thesias results it should be taken into account that siblings in the GARP Study were assessed as independent individuals in these analyses. To assess the strength (odds ratio) of the haplotypic effect in the GARP subjects with the highest number of affected joints, logistic regression with robust standard errors to adjust for family relationship (40) was used, with Stata SE8 software (StataCorp, College Station, TX). In this case the haplotypes of individuals were estimated by the expectation maximization algorithm implemented in SNPHap version 1.3, and posterior haplotype probabilities were used as sampling weight in the analysis. Instead of adjusting *P* values a priori for multiple testing, nominal *P* values are provided, in order to allow the reader to interpret the level of significance.

RESULTS

Characteristics of the 382 patients with symptomatic OA at multiple sites who were included in the GARP Study are shown in Table 1. The study population consisted predominantly of women (81%). Innate ex vivo production of IL-1β and IL-1Ra in whole blood upon LPS stimulation had been previously measured in all subjects in the GARP Study (13). Because the IL-1β and IL-1Ra levels were significantly lower in female subjects as compared with male subjects ($P = 1.6 \times 10^{-5}$ and $P = 0.002$, respectively) and the IL-1Ra levels were significantly associated with BMI ($P = 0.01$), all analyses concerning these levels were adjusted for sex and BMI. We did not detect an effect of age on these levels. To take into account the interaction between IL-1β and IL-1Ra levels, we examined the effect of IL-1β bioavailability as expressed by the ratio between ex-vivo IL-1β and IL-1Ra production upon stimulation with LPS.

In total, 7 SNPs encompassing the IL-1 cluster on chromosome 2q13 were measured in both the GARP Study subjects and the controls. All SNPs were in Hardy-Weinberg equilibrium. Figure 1 shows the linkage disequilibrium (LD) pattern of the SNPs in the case and control groups together across the region, as visualized with the Haploview program of the HapMap project (41). Notably, low LD between the *IL1B* C−511T and *IL1RN* VNTR SNPs was observed, with $D' = 0.41$ and $r^2 = 0.1$, dividing the region into 2 separate blocks; the first block consisted of *IL1A* C–889T and *IL1B* C+3953T, T−31C, and C–511T and

the second block consisted of *IL1RN* VNTR, T+8006C, and T+11100C, which were used for the haplotype association analysis.

Association analysis of IL-1 cluster haplotypes and IL-1β bioavailability based on ex vivo production levels. As shown in Table 2, there were 2 haplotypes in the second block (VNTR/T+8006C/T+11100C) covering the *IL1RN* gene that were significantly associated with cytokine production levels. Haplotype 2/2/1 (frequency 0.22) was associated with lower IL-1β production levels ($P = 0.002$), whereas haplotype 2/2/2 (frequency 0.02) was associated with higher IL-1Ra production levels ($P = 1.1 \times 10^{-4}$). These haplotypes are not tagged by one of the individual SNPs, and the associations appeared more significant than, but were consistent with the results of, the genotype analysis (Table 3).

To take into account the interaction between IL-1β and IL-1Ra levels, we examined the effect of the haplotypes on bioavailability as expressed by the ratio between IL-1β and IL-1Ra levels. As can be seen in Table 2, only the *IL1RN* haplotype VNTR/T+8006C/T+11100C 2/2/1 showed significantly lower bioavailability of IL-1β upon LPS stimulation ($P = 0.026$).

Association analysis of IL-1 cluster haplotype and OA. Next, we assessed whether these haplotypes also contributed to the degree of cartilage abnormalities in the GARP subjects, as expressed by the summed ROA score for all joint locations. For haplotype *IL1RN* VNTR/T+8006C/T+11100C 2/2/1 of the second block, which was associated significantly and consistently with lower IL-1β availability, we could not detect an association among GARP subjects as compared with random controls from the Rotterdam Study. However, when we explored the quantitative association with the total ROA score for all joint locations among subjects of the GARP Study with Thesias, a trend toward a higher mean summed ROA score was observed for haplotype 2/2/1 ($P = 0.07$) as compared with the other haplotypes. Upon further investigation it was shown that among subjects whose total ROA score was in the highest quartile (ROA score >5; n = 64) there was significant association with this *IL1RN* haplotype, with an odds ratio (OR) of 1.76 (95% confidence interval [95% CI] 1.14–2.76, $P = 0.011$) compared with the subjects from the Rotterdam Study (n = 788) and an OR of 1.91 (95% CI 1.21–3.02, $P = 0.006$) compared with the remaining GARP subjects (n = 317). Adjustment for age, sex, or BMI did not considerably affect this haplotypic association. None of the other haplotypes was associated with OA (subtypes).

Table 3. Genotype association analysis of logarithmically transformed innate ex vivo cytokine production upon LPS stimulation, as measured in subjects in the Genetics, Osteoarthritis and Progression Study*

Genotype (n)	Mean log IL-1β	Mean log IL-1Ra
Overall (368)	3.49	4.37
IL1A C−889T		
0 alleles (172)	3.47	4.37
1 allele (147)	3.50	3.36
2 alleles (38)	3.50	4.40
IL1B C+3953T		
0 alleles (203)	3.46	4.37
1 allele (144)	3.51	4.36
2 alleles (18)	3.58	4.44
IL1B T−31C		
0 alleles (160)	3.49	4.39
1 allele (169)	3.49	4.35
2 alleles (34)	3.45	4.36
IL1B C−511T		
0 alleles (162)	3.50	4.39†
1 allele (154)	3.48	4.35
2 alleles (29)	3.42	4.36
IL1RN VNTR		
0 alleles (205)	3.53‡	4.38
1 allele (137)	3.43	4.37
2 alleles (20)	3.44	4.32
IL1RN T+8006C		
0 alleles (201)	3.53§	4.37
1 allele (127)	3.42	4.38
2 alleles (24)	3.47	4.33
IL1RN T+11000C		
0 alleles (166)	3.47	4.35¶
1 allele (161)	3.49	4.39
2 alleles (39)	3.55	4.39

* Data were analyzed using mixed-model regression analyses with logarithmically transformed IL-1β and IL-1Ra levels as dependent variables and the genotypes coded as 0, 1, or 2 carriers of the rare allele as covariables. Values for IL-1β were adjusted for sex; values for IL-1Ra were adjusted for sex and body mass index. Family numbers were used as random-effect variables to adjust for the family relationship between siblings. See Table 2 for definitions.
† $P = 0.047$.
‡ $P = 0.004$.
§ $P = 0.005$.
¶ $P = 0.056$.

In a combined analysis of the effect of haplotype *IL1RN* VNTR/T+8006C/T+11100C 2/2/1 on both IL-1β availability and the total ROA score in GARP subjects, the haplotype was found to be independently and significantly associated with lower IL-1β availability ($P = 0.007$) and with the highest number of joint locations with ROA (25% of the subjects) ($P = 0.006$). In contrast, we did not observe lower innate ex vivo IL-1β availability among the subjects with the highest number of joint locations with ROA in the GARP Study.

DISCUSSION

In this investigation, using subjects from the GARP Study as the study population, we investigated whether genetic variation at the IL-1 cluster contributes to innate ex vivo cytokine production upon LPS stimulation and whether the relevant haplotypes contribute to the development of symptomatic OA at multiple joint sites. Compared with the other *IL1RN* haplotypes, haplotype 2/2/1 of the *IL1RN* block (frequency 0.22) was significantly associated with lower IL-1β bioavailability as calculated by the ratio of IL-1β and IL-1Ra levels ($P = 0.026$) and with the highest number of joint locations affected with ROA ($P = 0.006$). Our findings with regard to the relationship between this *IL1RN* haplotype and IL-1β bioavailability are in accordance with the results of Vamvakopoulos et al (18), who showed that *IL1RN* VNTR allele 2 was significantly associated with lower IL-1β production levels upon stimulation with LPS. Taken together, these results confirm that genetic variation within the *IL1RN* gene exerts its influence on IL-1β bioavailability possibly via a functional difference in the IL-1Ra protein. As an IL-1β antagonist to the IL-1 receptor, aberrant IL-1Ra may hamper correct regulation of biologic IL-1β levels. In normal cartilage, lower IL-1β bioavailability, as result of genetic variation at the IL-1 gene cluster, may cause inefficient repair of damaged cartilage and may thereby influence the propensity to develop OA at various joint sites.

The associations of *IL1RN* VNTR/T+8006C/T+11100C haplotype 2/2/1 with lower IL-1β availability and with ROA at the highest number of joint locations in our data set appear consistent with the above proposal. The subsequent finding of an absence of low IL-1β availability among subjects with this severe ROA subtype is, however, more challenging. Moreover, although we previously showed that higher IL-1β and IL-1Ra levels and lower IL-10 levels occurred upon LPS stimulation among subjects in the GARP Study as compared with controls (13), these levels did not predispose to knee OA progression (15). A possible explanation could be that the actual whole blood ex vivo cytokine production measurement is not entirely independent of disease status, but brings about increased sensitivity to LPS activation in subjects with severe OA pathology. In our data set, this explanation is substantiated by the observation that, although the *IL1RN* 2/2/1 haplotype was more frequent among subjects who had OA in the highest number of joints, the association of the haplotype with low IL-1β availability gets lost in this particular group, possibly due to an activated innate immune system.

Similar to the relationship between plasma C-reactive protein levels and ischemic events as discussed by others (42), the association of high innate ex vivo cytokine IL-1β and IL-1Ra with OA as observed in epidemiologic studies (13) may not reflect causality, but may rather be a marker of the ongoing disease process that affects an individual's sensitivity to LPS stimulation. As elegantly outlined in a review by Scanzello et al (17), OA may indeed be considered as a chronic wound in which the innate immune response may, via upregulation of Toll-like receptors, be activated by molecular signals of tissue damage. The fact that we observed such an effect mainly in subjects with a high number of OA-affected joints may indicate that this is particularly true among those with advanced disease. To validate these effects further, investigations must be conducted to determine whether an individual's cytokine production capacity upon LPS stimulation does indeed change during the course of OA and/or whether healthy subjects with a specific inflammatory cytokine production profile are prone to develop OA (at multiple joint locations).

We have identified a common *IL1RN* haplotype that is significantly associated with lower IL-1β availability and with the presence of ROA at the highest number of joint locations. The fact that this association is counterintuitive to the concept that inflammation in OA contributes to symptoms and augments many pathologic changes highlights the complex interplay between cytokines and the OA disease process.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Meulenbelt had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Meulenbelt, Kloppenburg, de Craen, Slagboom.

Acquisition of data. Meulenbelt, Kloppenburg, Lakenberg, Watt, de Craen, Slagboom.

Analysis and interpretation of data. Meulenbelt, Bos, Houwing-Duistermaat, de Craen.

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