Genes and mediators of inflammation and development in osteoarthritis

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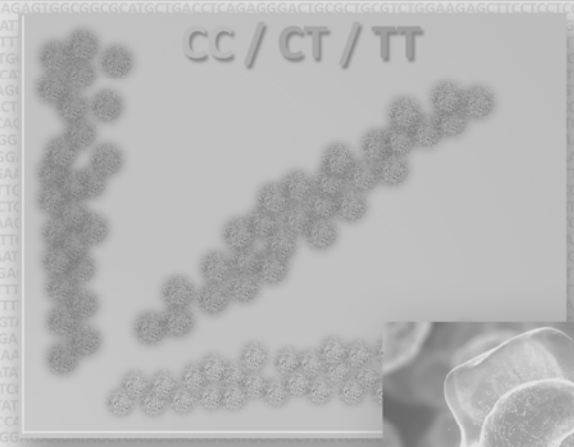

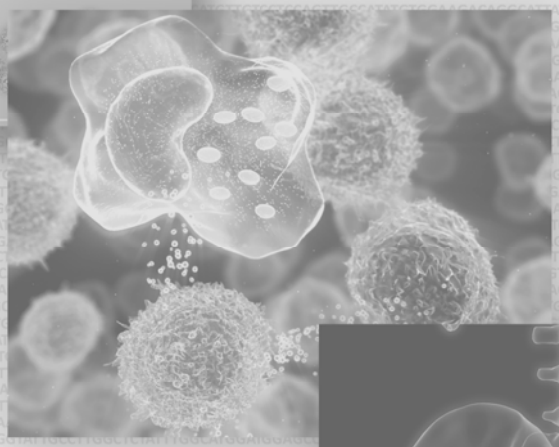

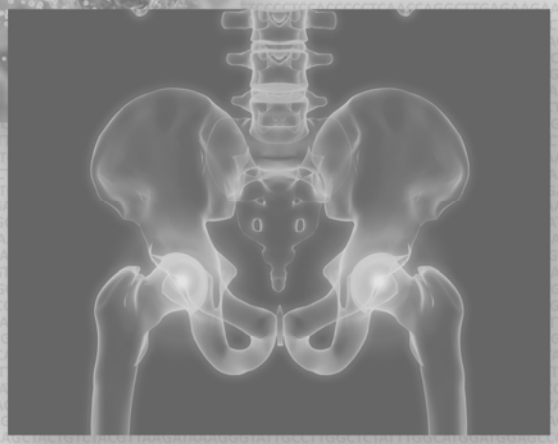

Genetic association of the interleukin-1 gene cluster with innate cytokine production profiles and osteoarthritis in subjects of the GARP study.

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Abstract

Objective: To assess whether genetic variation in the interleukin (IL)-1 gene cluster contributes to familial osteoarthritis (OA) by influencing the innate *ex vivo* IL-1ß or IL-1Ra cytokine production.
Methods: Innate *ex vivo* IL-1β and IL-1Ra production upon LPS stimulation (10 ng/ml) of whole blood cells was measured in the GARP study which consists of sibling pairs predominantly with symptomatic OA at multiple sites. Radiographic characteristics of OA (ROA) were assessed by Kellgren and Lawrence score. Subjects of GARP and controls of the Rotterdam study were genotyped for 7 single nucleotide polymorphisms (SNPs) encompassing the IL-1 gene cluster on chromosome 2q13. Linkage disequilibrium (LD) analysis, genotype and haplotype association analysis were performed in order to assess the relationship between the IL-1 gene cluster SNPs, innate *ex vivo* cytokine production and OA.
Results: Haplotype VNTR, +8006 and +11100 2-2-1 of the *IL1RN* gene was within the GARP study significantly associated to lower innate *ex vivo* bio-availability of IL-1ß upon LPS stimulation (P-value = 0.026) and to subjects with ROA at the highest number of joint locations.
Conclusion: We show that genetic variation at the IL-1 gene cluster associates to lower IL1ß bio-availability and to OA at a large number of joint locations. Furthermore, our data also indicates that among subjects with OA at the highest number of joints the innate immune system may be activated thereby obscuring possible underlying mechanisms.

Introduction

Osteoarthritis (OA) is a common joint disease and is 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 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 (IL)–1β by decreasing synthesis of matrix components and increasing the synthesis of metalloproteinases (MMPs)[6]. MMPs degrade extracellular matrix (ECM) components in articular cartilage. IL-1Ra is the natural competitive inhibitor of IL1ß, occupying the cell surface IL-1 receptor without triggering signal transduction and its levels might be considered critical in determining the IL-1β bioavailability[6].
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 samples. Studies of twins have shown that *ex vivo* production of cytokines IL-1β, IL-1Ra, TNFα, IL-10 varies by 60-70% based on heritability alone[7]. Subjects can thus be characterized as high (pro-inflammatory) or low (anti-inflammatory) producers based on these cytokines profiles[8,9]. Such a characteristic may influence susceptibility to diseases with an inflammatory component[10-12]. Data supporting this hypothesis in OA comes from our previous studies[13] in which we demonstrated that a pro-inflammatory profile, high innate *ex vivo* cytokine IL-1β and IL-1Ra and low IL-10, occurs among subjects with familial OA at multiple sites of the GARP study as compared to controls. The innate *ex vivo* production of TNFα which did not associate to the onset of OA, was the only cytokine that predisposed to knee OA progression[14]. In recent years the concept that inflammation in OA

contributes to symptoms and augments many pathological changes has become generally accepted[15,16], however, it is unclear whether this is a causal association or marks the ongoing disease process. Furthermore, the interplay between secreted IL-1ß and IL1-Ra levels should be taken into account since together they influence the IL-1ß bio-availability[17].

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 not always consistently and determined in relative small studies, it has been shown that several DNA variants within the genes of the IL-1 gene cluster may be responsible for the variation in the heritable innate *ex vivo* cytokine production upon LPS stimulation[12]. Furthermore, *in vitro* experiments have shown functional ability of IL1B promoter SNPs to enhance IL-1ß production upon LPS stimulation[18]. The role of the *IL1RN* VNTR allele 2 appears most consistent in affecting of cytokine production *in vivo*[19] and may be considered most important for the fine tuning of the IL-1ß bio-availability as determined by the ratio of the innate *ex vivo* cytokine production upon LPS stimulation of IL-1ß and IL-1Ra[17]. Multiple genetic association studies, tried to investigate whether these potential functional aspects of the IL-1 gene cluster polymorphisms may in part explain genetic susceptibility of OA. Previously, we and others have reported associations of the IL-1 gene cluster for knee, hip and hand OA[20-25], although others failed to confirm these associations[26,27]. Together the effects of the IL-1 gene cluster SNPs on innate *ex vivo* cytokine production and OA may be complex and involve interactions among different polymorphic sites and should therefore be investigated by means of independent haplotypes.

Combining *ex vivo* IL-1ß bio-availability measures upon LPS stimulation, genetic variation at the IL-1 gene cluster and OA disease status in a single study population allows to assess possible underlying relationships[28]. We have tested for the influence and interaction of the IL-1 gene cluster polymorphisms and haplotypes on the IL-1ß bio-availability in a relatively large number of individuals of the GARP study. In the same study it was investigated whether the haplotypes relevant for IL-1ß bio-availability correlate to a proportioned score of the number of ROA affected joint locations.

Patients and methods

The GARP study (Genetics, osteoARthritis and Progression)

The ongoing GARP study, which consists of 191 Caucasian sibling pairs of Dutch ancestry affected with symptomatic OA at multiple sites. Probands (aged 40-70 year) and their siblings were included in the GARP study with OA at multiple joint sites of the hand according to the American College of Rheumatology criteria or with symptomatic OA in two or more of the following joint sites: hand, spine (cervical or lumbar), knee or hip[29]. In the spine, knee or hip symptomatic OA was defined as having symptoms of OA in addition to radiographic signs[30-33].

Conventional radiographs of the hands (dorso-volar), knees (Posterior-Anterior in weight bearing / semi flexed and lateral), hips (AP), lumbar (AP and lateral) and cervical spine (AP, lateral and transoral) were obtained of all participants. This was performed in a standard manner with a fixed film-focus distance and a fixed joint position. Radiographic characteristics of OA were defined according to Kellgren and Lawrence[34] by a single, experienced and trained radiographer according to an agreed protocol as described in detail

elsewhere[29]. In the current paper we used (the highest quartile of) the total ROA score. The total ROA score (0-10) represents a summed score proportional to radiological cartilage abnormalities at each joint location in knee (0-2), hip (0-2), hand (0-2), facet joints (0-2), and spinal discus degeneration DD (0-2) as described previously in detail[35]. The highest quartile of the total ROA score represents GARP subjects with the highest number of joint locations with radiographic abnormalities which were compared to other subjects of the GARP study and/or random subjects of the Rotterdam study. We compared affected sibling pairs from the GARP study to a random sample of unrelated subjects aged 55-65 years (n = 809) of the Rotterdam study as reference group representing the general population[36]. Both studies comprise of Caucasian subjects from the western areas of the Netherlands with a mean age of 60.3 years and may represent the same genetic background. In this sample symptomatic OA has not been assessed.

Whole-blood stimulation system

Whole-blood sample stimulation was performed as previously described[37]. In short, blood samples were collected in pyrogen-free heparinized tubes (Endotube®, Chromogenix, Mölndal, Sweden). Eight-millilitre whole-blood samples were diluted 1:1 with RPMI 1640 (Gibco Life Technologies, Paisely, United Kingdom) and stimulated with 10 ng/mL *Escheria coli* LPS (Difco Laboratories, Detroit, Mich). To minimize the influence of circadian rhythms and measurement errors, blood samples were taken between 8 AM and 11 AM, the time frame between blood collection and stimulation was less than one hour and a half, and all stimuli were performed with the same endotoxin batch. One medium-diluted blood without LPS was used as negative control. After 24-hour incubation, samples were centrifuged twice (600g) and the supernatants stored at -70ºC. IL-1β and IL-1Ra production were measured in one batch by enzyme-linked immunosorbent assays (ELISA) according to manufacturer's guidelines (Central Laboratory of the Blood Transfusion Service, Amsterdam, the Netherlands). Nine patients were excluded from the analyses because either whole-blood samples had not been obtained (N = 5) or levels of IL-1Ra or IL-1β were missing (N=4).

Genotyping measurements

Genomic DNA was isolated from blood samples from the GARP study, for 1 subject DNA was missing. In total 809 subjects of Rotterdam and 381 subjects of 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 C3953T (rs1143634), T-31C (rs1143627) and C–511T (rs16944) and 3 SNPs in the *IL1RN* gene VNTR in intron 2, T8006C (rs419598) and T+11100C (rs315952). The genotypes of the C3953T C–511T and VNTR in the Rotterdam study were assessed previously[22]. The genotypes of the SNP were determined by mass spectrometry (homogeneous Mass ARRAY system; Sequenom Inc., San Diego, CA), using standard conditions. Genotypes were analyzed by using Genotyper 3.0 software (Sequenom Inc.). Throughout the paper the common alleles of the SNPs are designated 1 and the rare alleles as 2. For the controls genotypes from the Rotterdam study were available of 788 subjects.

Statistical analysis

The contribution of the individual genotypes of the SNPs of the IL-1 gene cluster to the innate *ex vivo* cytokine production upon LPS stimulation was estimated using a mixed model regression analyses performed with the logarithmically transformed cytokine levels as dependent variable and as co-variable the genotypes of the SNPs and sex. 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 software (SPSS, Chicago, Illinois, USA). Haplotypic effects on the quantitative innate *ex vivo* cytokine production and/or total ROA scores were assessed by the THESIAS 3.1 program[38] and adjusted for sex and/or BMI where indicated. When interpreting the THESIAS results it should be taken into account that in these analysis siblings of the GARP study were used as independent individuals. To assess the strength (odds ratio) of the haplotypic effect in the GARP subjects with the highest number of joints logistic regression with robust standard errors to adjust for family relationship[39] was used in Stata SE8 software (Stata Corporation, USA). 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

In Table 1 the characteristics of the 382 patients with symptomatic OA at multiple sites who were included in the GARP study are provided. The study consists predominantly of women (82%). Whole blood innate *ex vivo* productions of IL-1β and IL-1Ra upon LPS stimulation were previously measured in all subjects of the GARP study[13]. As the IL-1ß and IL-1Ra levels were significantly lower in females as compared to males (P-value = 1.6 x 10^{-5} and P-value = 0.002, respectively) and the IL-1Ra levels were significantly associated to body mass index (BMI, P-value = 0.01), all analyses concerning these levels were adjusted for sex and BMI. We could not detect an effect of age to these levels. To take into account the interaction between IL-1ß and IL-1Ra levels, we examined the effect of the IL-1ß bio-availability as expressed by the ratio between *ex-vivo* IL-1ß 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 and controls. All SNPs were in Hardy Weinberg equilibrium. In Figure 1, the linkage disequilibrium (LD) pattern of the SNPs in the case and control group together across the region is visualized by the HAPLOVIEW program of the Hapmap project[40]. Notably a low LD occurs between the -511 *IL1B* and VNTR *IL1RN* SNPs with D' = 0.41 and r^2 = 0.1 dividing the region in two separate blocks, the first block consisting of *IL1A* –889, and *IL1B* +3953, -31, and –511 and the second block consisting of *IL1RN* gene VNTR, +8006 and +11100 which were used for the haplotype association analysis.

Association analysis of IL-1 cluster haplotypes and IL-1ß bio-availability based on *ex vivo* production levels

As shown in Table 2 there were 2 haplotypes in the second block (VNTR, +8006 and +11100) covering the IL1RN gene that associated significantly to the cytokine production

levels. Haplotype 2-2-1 (frequency 0.22) was associated to lower IL-1β production levels (P = 0.002) whereas haplotype 2-2-2 (frequency 0.02) to higher IL-1Ra production level (P = $1.1x10^{-4}$).

Table 1: Characteristics of study populations

	GARP study
Total number of subjects[1]	382
Number of females (%)	311 (82)
ROA scores (> 0) of subjects within (N=382):	
Hip (%)	107 (28)
Knee (%)	150 (39)
Hand (%)	213 (56)
Facet (%)	235 (62)
DD (%)	256 (67)
Mean total ROA score (range)	3.45 (0-9)
Mean age in years (range)	60.3 (43-79)
Mean body mass index in kg/m^2 (SD)	27.0 (4.7)
Mean IL1-ß /IL-1Ra ratio, IL1ß bio-availability (SE)	0.798 (0.003)

[1]Numbers represent GARP subjects with symptomatic OA at multiple joint locations including subjects with uni and/or bilateral joint replacement (N=38 for hip and N = 8 for knee). DD = spinal disc degeneration SD = standard deviation IQR = interquartile range.

These haplotypes are not tagged by one of the individual SNPs and the associations appear more significant but are in agreement with the results obtained for the genotype analysis (Supplementary Table 1).

To take into account the interaction between IL-1ß and IL-1Ra levels we examined the effect of the haplotypes to the bio-availability as expressed by the ratio between IL-1ß and IL-1Ra (Table 2). As can be seen only *IL1RN* haplotype VNTR, +8006 and +11100 2-2-1 showed significant lower bio-availability of IL-1ß upon LPS stimulation (P-value = 0.026). Next we assessed whether these haplotypes also contributed to the degree of cartilage abnormalities in the GARP subjects expressed by the summed ROA score of all joint locations.

Association analysis of IL-1 cluster haplotype and OA

For haplotype *IL1RN* VNTR, +8006 and +11100 2-2-1 of the second block, which associated significantly and consistently to lower IL-1ß availability, we could not detect an association among GARP subjects as compared to random controls of 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 in THESIAS, a trend towards a higher mean summed ROA score was observed for haplotype 2-2-1 (P = 0.07) as compared to the other haplotypes. Upon further investigation it was shown that subjects that reside within the highest quartile of the total ROA score (ROA score > 5, N = 64) showed significant

association with this *IL1RN* haplotype with an OR of 1.76, 95%CI 1.14-2.76, P-value = 0.011 when compared to the subjects of the Rotterdam study (N = 788) and an OR of 1.91, 95% CI 1.21-3.02, P-value = 0.006 when compared to the remaining GARP subjects (N = 317). Adjusting for age, sex or BMI did not considerably affect this haplotypic association. None of the other haplotypes was associated to OA (subtypes).

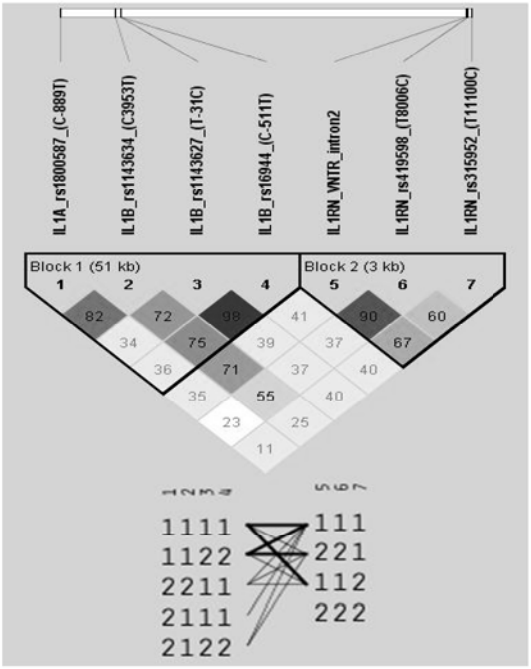

Figure 1. Pairwise linkage disequilibrium across the IL-1 cluster single nucleotide polymorphisms (SNPs) as visualized by the Haploview program and expressed by the linkage disequilibrium coefficient D'.

When combining the effect of haplotype *IL1RN* VNTR, +8006 and +11100 2-2-1 to both IL-1ß availability and the total ROA score within GARP subjects, the haplotype was independently and significantly associated to lower IL-1ß availability (P-value = 0.007) and to subjects (25%) with highest number of joint locations with ROA (P-value = 0.006). In contrast, we did not observe lower innate *ex vivo* IL-1ß availability among the subjects with the highest number of joint locations with ROA within the GARP study.

Discussion

It was 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 symptomatic OA at multiple joint sites as assessed in the GARP study.
Haplotype 2-2-1 (frequency 0.22) of the *IL1RN* block showed significant lower bio-availability calculated by the ratio of IL-1ß and IL-1Ra as compared to the other *IL1RN*

Table 2. Haplotype association analysis of block 1 consisting of the *IL1A* (–889) and *IL1B* (3953, -31, and -511) gene and block 2 consisting of 3 the *IL1RN* gene;VNTR_T8006C_T11100C with the innate *ex vivo* IL-1β and IL-1Ra production upon LPS stimulation of whole blood cells.

Haplotypes IL1 block 1	%	Haplotypic mean (95% CIL)[1]		
		Log IL-1β[1]	Log IL-1Ra	Log IL-1ß / IL-1Ra
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			
Haplotypes IL1 block 2	%	Haplotypic mean (95% CIL)[1]		
		Log IL-1β	Log IL-1Ra	Log IL-1ß / IL-1Ra
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 was analyzed using the THESIAS program for quantitative phenotypes for IL1β and IL1Ra levels logarithmically transformed. P-value is determined by comparing the specific haplotypic mean as compared to the haplotypic mean of all other haplotypes. [1]Values for IL1ß were adjusted for sex whereas values for IL1Ra and IL1ß/IL1Ra were adjusted for sex and BMI. HAP = haplotype allele frequency. *P<0.05, **P<0.005 and ***P<0.0002

haplotypes (P-value = 0.026) and to subjects (25%) with highest number of joint locations with ROA (P-value = 0.006). Our result of this *IL1RN* haplotype with IL-1ß bio-availability are in line with the results of Vamvakopoulos *et al.*[17] who showed that the *IL1RN* VNTR allele 2 was significantly associated to lower IL-1β production levels upon stimulation with LPS. Together our results confirm that genetic variation within the *IL1RN* gene exert their influence on the IL-1β bio-availability possibly via a functional difference of the IL-1Ra protein. As antagonist of IL-1β to the IL-1 receptor, aberrant IL-1Ra may hamper a correct regulation of the biological IL-1β level. In normal cartilage lower IL-1ß bio-availability, as result of genetic variation at the IL-1 gene cluster, may cause an inefficient repair of damaged cartilage and thereby influence the propensity to develop OA at various joint sites. To this end the association with the *IL1RN* VNTR, +8006 and +11100 haplotype 2-2-1 to lower IL-1ß availability and to ROA at the highest number of joint locations in our dataset appear consistent. The subsequent absence of low IL-1ß availability among this severe ROA subtype is, however, more difficult (final sentence of the result section). Moreover, we previously showed higher IL-1ß, IL-1Ra and lower IL-10 cytokine production levels upon LPS stimulation occur among subjects of the GARP study as compared to controls[13] whereas these levels did not predispose to knee OA progression[14]. A possible explanation

could be that the actual whole blood *ex vivo* cytokine production measurement is not entirely independent on disease status, but brings about increased sensitivity to LPS activation in subjects with severe OA disease pathology. In our own dataset, this explanation is substantiated by the observation that although the *IL1RN* 2-2-1 haplotype is more frequent among subjects with OA at the highest number of joints, the association of the haplotype with low IL-1ß availability gets lost in this particular group (results not shown) possibly due to an activated innate immune system. Similar to the relation between plasma CRP and ischemic events as discussed by others[41], the association observed in epidemiological studies between high innate *ex vivo* cytokine IL-1ß and IL-1Ra with OA[13] may not reflect causality but rather a marker of the ongoing disease process that affects an individual's sensitivity for LPS stimulation. As elegantly outlined in a review of Scanzello *et al.*[42] OA may indeed be considered as a chronic wound in which the innate immune response (via up regulation of Toll like receptors) may be activated by molecular signals of tissue damage. The fact that we observe such an effect mainly in subjects with a high number of OA affected joints may indicate that this is particularly true in subjects with advanced disease. To validate these effects further it should be investigated whether indeed an individuals cytokine production capacity upon LPS stimulation changes in the course of OA onset or progression and/or whether healthy subjects with a specific inflammatory cytokine production profile are prone to develop OA (at multiple joint locations).
Our data show a common *IL1RN* haplotype that is significantly associated to lower IL-1ß availability and to subjects with highest number of joint locations with ROA. The fact that this association is counter intuitive to the concept that inflammation in OA contributes to symptoms and augments many pathological changes underlines the complex interplay between cytokines and the OA disease process.

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Supplementary Table 1. Genotype association analysis of logarithmically transformed innate *ex vivo* cytokine production upon LPS stimulation measured in the GARP study.

Genotypes (N)	Log IL-1β		Log IL-1Ra	
	Mean	P-value[1]	Mean	P-value[1]
Overall (368)	3.49		4.37	
*IL1A*_-889 0 (172)	3.47		4.37	
*IL1A*_-889 1 (147)	3.50		3.36	
*IL1A*_-889 2 (38)	3.50		4.40	
*IL1B*_3953 0 (203)	3.46		4.37	
*IL1B*_3953 1 (144)	3.51		4.36	
*IL1B*_3953 2 (18)	3.58		4.44	
*IL1B*_-31 0 (160)	3.49		4.39	
*IL1B*_-31 1 (169)	3.49		4.35	
*IL1B*_-31 2 (34)	3.45		4.36	
*IL1B*_-511 0 (162)	3.50		4.39	
*IL1B*_-511 1 (154)	3.48		4.35	0.047
*IL1B*_-511 2 (29)	3.42		4.36	
*IL1RN*_VNT 0 (205)	3.53		4.38	
*IL1RN*_VNT 1 (137)	3.43	0.004	4.37	
*IL1RN*_VNT 2 (20)	3.44		4.32	
*IL1RN*_8006 0 (201)	3.53		4.37	
*IL1RN*_8006 1 (127)	3.42	0.005	4.38	
*IL1RN*_8006 2 (24)	3.47		4.33	
*IL1RN*_11000 (166)	3.47		4.35	
IL1RN 11100 1 (161)	3.49		4.39	0.056
*IL1RN*_11100 2 (39)	3.55		4.39	

[1]Data was analyzed using mixed model regression analyses with IL1β, and IL1Ra levels logarithmically transformed as dependent variable and as co-variables the genotypes coded as 0, 1, 2 carriers of the rare allele and for IL1ß adjusted for sex and for IL1Ra adjusted for sex and BMI. Family numbers were used as random effect variables to adjust for the family relationship between siblings.