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## Genes and mediators of inflammation and development in osteoarthritis

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# 2.2

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The image is a composite of three main visual elements: a DNA double helix on the left side, a microscopic view of cells in the center, and a human pelvic X-ray on the right side. The background is a light gray with a repeating pattern of DNA base pairs (A, T, C, G) and the text '2.2'.

# **A genome wide linkage scan reveals *CD53* as an important regulator of innate TNF-alpha levels.**

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## Abstract

Cytokines are major immune system regulators. Previously, innate cytokine profiles determined by LPS stimulation were shown to be highly heritable. To identify regulating genes in innate immunity we analyzed data from a genome wide linkage scan using microsatellites in osteoarthritis patients (The GARP study) and their innate cytokine data on IL-1 $\beta$ , IL-1Ra, IL-10 and TNF $\alpha$ . A confirmation cohort consisted of the Leiden 85-Plus study.

In this study, a linkage analysis was followed by manual selection of candidate genes in linkage regions showing LOD scores over 2.5. A SNP gene tagging method was applied to select SNPs on the basis of highest level of gene tagging and possible functional effects. QTDT was used to identify the SNPs associated to innate cytokine production. Initial association signals were modelled by a linear mixed model. Through these analyses we identified 10 putative genes involved in the regulation of TNF $\alpha$ . SNP rs6679497 in gene *CD53* showed significant association to TNF $\alpha$  levels ( $P = 0.001$ ). No association of this SNP was observed to osteoarthritis. A novel gene involved in the innate immune response of TNF $\alpha$  is identified. Genetic variation in this gene may play a role in diseases and disorders in which TNF $\alpha$  is intimately involved.

## Introduction

The immune system is a complex network of interacting pathways and signaling proteins which enables organisms to respond to pathogens as well as to many other events that challenge homeostasis. The immune system is regulated through cytokines, which are mainly secreted by lymphocytes. The ability of lymphocytes to produce cytokines can be characterized by *ex vivo* stimulation with, for example, the bacterial surface molecule lipopolysaccharide (LPS). This ligand triggers *ex vivo* lymphocytes to produce a maximal pro-inflammatory cytokine response, subsequently followed by an anti-inflammatory response<sup>1</sup>. The as such determined maximal cytokine production profile has been shown to be stable<sup>2</sup> and to contain a significant heritable component estimated between 53% and 86%, indicating a strong genetic control<sup>3</sup>.

Based on innate *ex vivo* cytokine profiles, individuals can be characterized as pro- or anti-inflammatory and it has been shown that these profiles can be predisposing to diseases with an inflammatory component such as multiple sclerosis and cardiovascular events<sup>4,5</sup>. In osteoarthritis (OA), a pro-inflammatory profile may affect the articular cartilage homeostasis, which depends on a delicate balance of catabolic respectively anabolic activity induced by pro- (tumor necrosis factor (TNF) $\alpha$ , interleukin(IL)-1 $\beta$ ) and anti-inflammatory (IL-10 and IL-1 receptor antagonist(Ra)) cytokines<sup>6,7</sup>. In line with this hypothesis, Riyazi *et al.* showed that patients of the Genetics osteoARthritis and Progression (GARP) study with OA at multiple joints sites simultaneously, have high innate IL-1 $\beta$  and IL-1Ra and low innate IL-10 production as compared to controls<sup>8</sup>. Although a later study indicated that the mechanism underlying this association may be more complex, it confirmed the association of genetic variation associated to the innate cytokine levels to OA features<sup>9</sup>. We and others have shown that genetic variation of genes involved in the regulation of the immune system may be reflected by a specific profile of circulating plasma inflammatory markers<sup>10-12</sup>. Furthermore, it was shown that DNA variants

within the *IL10* gene and genes of the *IL-1* cluster may be responsible for a part of the variation in the heritable innate *ex vivo* cytokine production upon LPS stimulation<sup>13-16</sup>. A large part of the heritability however, cannot be explained by the currently known genes. Characterisation of the genes that explain a considerable part of the individual variation in the innate cytokine profiles may shed more light on the regulatory elements designed to obtain or maintain proper balance of these cytokines. Through a better understanding of these elements more insight in underlying disease processes in diseases with an inflammatory component such as OA can be obtained, thereby enabling identification of putative therapeutic targets. In the present study we set off to discover such putative quantitative trait loci for innate cytokine levels by use of the available genome wide linkage data of subjects of the GARP study<sup>17</sup>, as well as data on their *ex vivo* LPS stimulated production of IL-1 $\beta$ , IL-1Ra, IL-10 and TNF $\alpha$ <sup>8</sup>. Confirmation by association analysis of innate cytokine levels was performed using 563 unrelated individuals of the Leiden 85-Plus study<sup>18</sup>. Identified genetic variation influencing the innate immunity profile was tested for association to OA and radiographic OA subtypes as assessed in the GARP study.

## Materials & methods

### Study subjects

The GARP study consists of 191 Caucasian sibling pairs affected predominantly by symptomatic OA at multiple joint sites. Characteristics of the GARP study are listed in Table 1. Details on description of the phenotype and data collection are described by Riyazi *et al.*<sup>19</sup>. As a confirmation cohort, we used the Leiden 85-Plus study which consists of inhabitants of Leiden (Netherlands), who were asked to participate in this study upon reaching the age of 85 years between September 1, 1997 and September 1, 1999. The response rate was 87% and in total 599 individuals were included in this study. OA data are not available for the Leiden 85-Plus study<sup>20</sup>.

### Phenotyping

In the GARP study and Leiden 85-Plus study, for most participants (N=370 and N=563 respectively) an *ex vivo* whole blood sample was stimulated with 10 ngml<sup>-1</sup> LPS and after a 4 hours incubation the sample was centrifuged and the TNF $\alpha$  levels were determined in the supernatant by use of an enzyme linked immunosorbent assay. In a second sample a similar protocol was performed with a 24 hour incubation after which the plasma levels of IL-1 $\beta$ , IL-1Ra and IL-10 were determined<sup>1,19</sup>. In concordance with previous studies<sup>3,4,5</sup>, the *ex vivo* LPS stimulated cytokine levels were not normally distributed and influenced by gender. In our analyses log transformed cytokine levels were used and analyses were adjusted for sex.

### Genotyping

Previously, a genome wide microsatellite scan was performed in the GARP study to identify new OA susceptibility loci, a detailed description of the genotyping methods and control policy has been described by Meulenbelt *et al.*<sup>17</sup>. In short, 417 microsatellite markers on an average spacing of 10 cM across the genome were measured. Initial linkage peaks were identified and two peaks were fine mapped by typing 3 additional microsatellite markers for each in the region of linkage (Supplementary Table 1). SNP multiplex genotyping assays were designed using Assay Designer software 3.1. iPLEX assays were

measured on the Sequenom MassARRAY system (Sequenom, San Diego, CA). PCR's were carried out in a final volume of 5  $\mu$ l and contained standard reagents and 5 ng of dried genomic DNA. Genotypes were called using the Genotyper v3.1 software (Sequenom, San Diego, CA). All SNPs were checked for deviations from Hardy-Weinberg equilibrium and approximately 8% of the subjects were genotyped twice as a check for genotyping and calling consistency. Of the 47 genotyped SNPs in this study, 3 SNPs failed quality check due to low amplification, bad cluster separation or low confidence in called genotypes and were excluded from further analysis.

### **Linkage analysis and candidate gene selection**

The GARP microsatellite genotype data and log transformed LPS stimulated levels were analyzed using the variance components option implemented in Merlin to assess linkage of the levels to genetic loci<sup>21</sup>. Merlin output files were modified to tab delimited files with LOD score per marker to facilitate uploading to a custom track in the UCSC genome browser genome graph function. The significance level of linkage peaks was assessed by use of random gene dropping simulations in Merlin using 5000 reruns. The regions showing LOD scores over 2.5 were explored for candidate genes by use of the UCSC genome browser<sup>22</sup>, where the individual markers' LOD scores were uploaded on a custom track. All UCSC annotated genes in the 1-LOD-drop region within the flanking areas of a linkage peak over 2.5 were considered for possible involvement in the cytokine response. This manual selection of genes was based on location within the linkage region, GO terms and Swissprot description provided in the UCSC genome graph function. Genes selected were genes which are described as being involved in immune system communication, antigen recognition and immune response. We selected 10 positional candidate genes (three linkage areas). Candidate genes were subsequently tagged using SNPs selected from the International HapMap Project genome browser<sup>23</sup>. SNP selection was based on genetic position and function as well as potential to tag genetic variation present within these genes. Tagger software implemented in the Haploview program with settings " $r^2 > 0.8$ " and "pair wise tagging" was used to optimize tagging SNP selection<sup>24</sup>. In the selection process we included only SNPs with minor allele frequencies over 0.05 in CEPH data. A prioritization was applied to SNPs in coding regions by forced inclusion of non-synonymous SNPs and lowest priority given to downstream SNPs. In total, 47 SNPs were selected for genotyping.

### **Association analysis of quantitative innate cytokine levels**

The GARP data were analyzed for association of LPS levels to SNPs in the candidate genes using QTDT<sup>25</sup>. Initially, the -WEGA and -WEGD commands were applied to test for association given linkage on a specific locus under an additive and dominant model respectively. To test for possible population stratification we used the -AP -WEGA or -AP -WEGD command. Furthermore, a linear mixed model was used to model the association with the SNP in the GARP and Leiden 85-Plus data including sex, age, BMI as covariates. Here, the random family effect models correlation between siblings of the GARP study due to shared genetic and environmental effects<sup>26</sup>. In the combined GARP and Leiden 85-Plus analysis, in addition to family numbers and covariates sex, age and BMI, we included study identifiers in the model to correct for putative batch differences. Genotypes were coded as 0 (homozygote common allele), 1 (heterozygote) and 2 (homozygote rare allele) to test an additive model and 0, 1 and 1 respectively for testing a dominant model with one degree of

freedom. All reported p-values are nominal p-values uncorrected for multiple testing for the reader's interpretation of the results, unless mentioned otherwise.

### Qualitative association analysis of OA status and SNPs

To assess association of SNPs to OA at multiple joint sites as defined in the GARP study, a logistic regression was performed in STATA/SE 8.0, using the Leiden 85-Plus sample as a reference sample, whereby we used family numbers in the GARP study as a random effect variable to model familial dependencies. Dominant effects for the rare allele were tested by pooling heterozygotes with homozygotes for the rare allele.

## Results

### Characteristics

The characteristics of GARP and the study sample of the Leiden 85-Plus study where both innate cytokine production levels and genotypes were available are shown in Table 1. The participants of the Leiden 85-Plus study were significantly older as compared to the GARP participants ( $P < 0.01$ ) and we observed significant differences in the transformed LPS stimulated levels between the GARP and Leiden 85-Plus participants for IL-1 $\beta$  ( $P = 0.034$ ), IL-1Ra ( $P < 0.01$ ) and TNF $\alpha$  ( $P < 0.01$ ). To check for age dependencies of the LPS stimulated cytokine profiles the correlation with age was analyzed. We observed no significant correlations of age and IL-1 $\beta$ , IL-10 or IL-1Ra. However, TNF $\alpha$  showed a significant ( $P=0.037$ ) correlation with age with a Pearson correlation coefficient of 0.11 (data not shown). The older subjects of the GARP study had on average a higher LPS stimulated TNF $\alpha$  level.

**Table 1.** Characteristics GARP and Leiden 85-Plus study

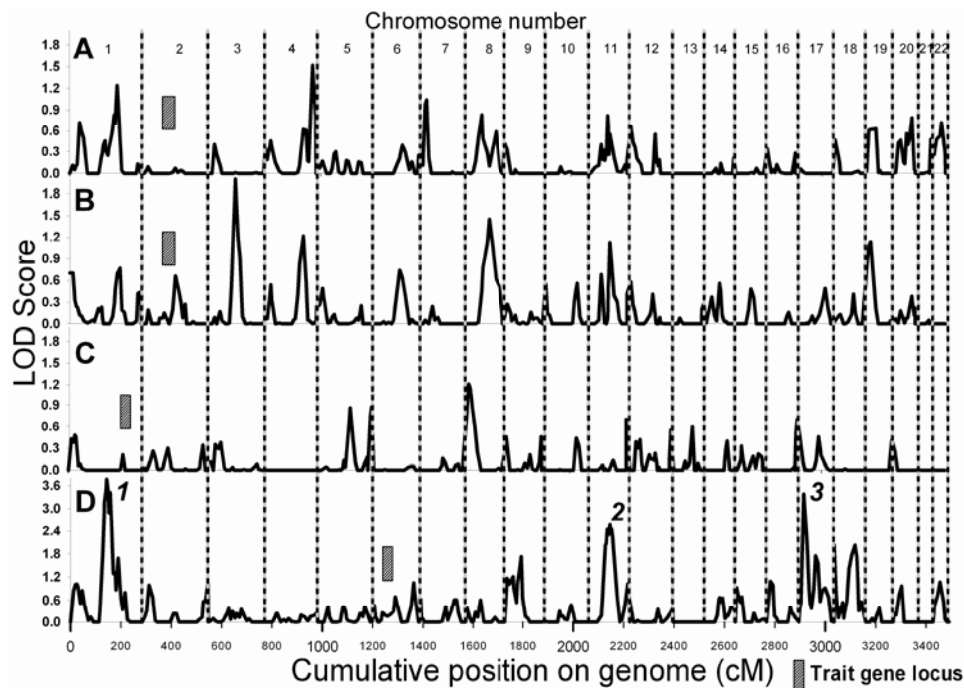
	N	The GARP Study	N	Leiden 85-Plus Study
No.	-	382	-	563
Age, mean $\pm$ SD*	-	60.4 $\pm$ 7.6	-	85 <sup>1</sup>
No. women (frequency)*	-	301 (0.81)	-	375 (0.67)
Mean Log(IL1 $\beta$ ) $\pm$ SD* (variance)	370	3.49 $\pm$ 0.30 (0.088)	559	3.54 $\pm$ 0.38 (0.142)
Mean Log(IL1Ra) $\pm$ SD* (variance)	369	4.37 $\pm$ 0.15 (0.023)	560	4.55 $\pm$ 0.20 (0.041)
Mean Log(IL10) $\pm$ SD (variance)	369	2.87 $\pm$ 0.19 (0.035)	560	2.85 $\pm$ 0.30 (0.090)
Mean Log(TNF $\alpha$ ) $\pm$ SD* (variance)	368	3.87 $\pm$ 0.18 (0.032)	561	3.98 $\pm$ 0.21 (0.045)

\* Significant difference between studies (t-test) <sup>1</sup>All subjects of the Leiden 85-Plus study were 85 years old at sampling.

### IL-1 $\beta$ , IL-1Ra and IL-10 linkage and association analysis

The genome wide linkage analysis to find quantitative trait loci involved in innate IL-1 $\beta$ , IL-1Ra and IL-10 analyses using variance components did not reveal any evidence for linkage above a LOD score of 2.5 (Figure 1A, 1B and 1C respectively). We did not select candidate genes and single nucleotide polymorphism(SNP)s for follow up analysis of these traits. No substantial evidence for linkage was observed at the loci encoding cytokines IL-1 $\beta$  and IL-1Ra (2q13), IL-10 (1q32.1) or TNF $\alpha$  (6p21.33) (Figure 1 A-D).



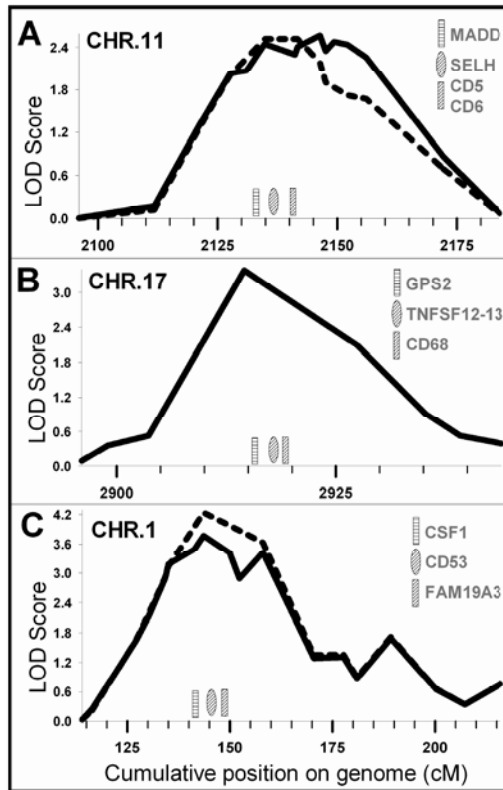


**Figure 1.** LOD scores for genome wide linkage analyses for QTL's of (A) IL-1 $\beta$ , (B) IL-1Ra, (C) IL-10 and (D) TNF $\alpha$ .

#### TNF $\alpha$ linkage and association analysis

Genome wide linkage analysis of innate TNF $\alpha$  levels revealed three regions with positive evidence for linkage with LOD scores over a LOD score of over 2.5 (Figure 1D), of which 1 peak reached a genome wide linkage significance level. The linkage peak on chromosome 11q12.1 (Figure 1D, peak 2) was fine mapped using three microsatellite markers and after fine mapping showed a maximum LOD score of 2.57 (marker D11S1314  $P = 3.0 \cdot 10^{-4}$ , genome wide p-value 0.497) between markers D11S935 and D11S901 (width 53 megabases (Mb), Figure 2A). Furthermore on chromosome 17p13.2 (Figure 1D, peak 3) we observed a narrow linkage peak with maximum a LOD score of 3.38 (marker D17S938  $P = 4.0 \cdot 10^{-5}$ , genome wide p-value 0.064) between markers D17S831 and D17S799 (width 5Mb, Figure 2B) which was not fine mapped. The largest peak on chromosome 1p13.3 (Figure 1D, peak 1) was fine mapped using three additional microsatellite markers and after fine mapping showed a maximum LOD score of 3.77 (marker D1S2726  $P = 3.0 \cdot 10^{-4}$ , genome wide p-value 0.018) between markers D1S2868 and D1S484 (width 52Mb, Figure 2C). Within these linkage peaks we selected 10 candidate genes (Table 2). On chromosome 11 we selected candidate genes *MADD*, *SELH*, *CD6* and *CD5*, on chromosome 17 *GPS2*, *TNFA-SF12/13* and *CD68* and on chromosome 1 *CSF1*, *CD53* and *FAM19A3*. The genes were tagged using 44 haplotype tagging SNPs, thereby tagging from 30-100% of the genotypic variation recorded in the HapMap database (Table 2). QTDT analysis indicated significant associations to TNF $\alpha$  levels for SNPs in *CD53* and *FAM19A3* (Table 2). We were unable to model the observed associations of *FAM19A3* in a linear mixed model,

however, when a dominant linear mixed model was fitted for *CD53* rs6679497 we again observed a significant association in both the GARP and Leiden 85-Plus separately ( $P = 0.013$  and  $0.032$  respectively, Table 3). When we combined the data of both studies in a linear mixed model using a dominant model we observed a highly significant association of rs6679497 ( $P=0.001$ , Table 3). The association remains significant after a Bonferroni correction to account for the 11 SNPs tested on the locus (corrected p-value =  $0.012$ ), or accounting for all 44 SNPs selected upon the linkage analysis (corrected p-value =  $0.047$ ). The minor allele of this intron SNP (frequency 0.48) associated with significantly lower innate TNF $\alpha$  levels.



**Figure 2.** Detailed view of initial and fine mapped linkage peaks identified on chromosome 11 (Pane A, peak 2), chromosome 17 (Pane B, peak 3) and chromosome 1 (Pane C, peak 1). Schematically represented are the tested genes positions in the linkage area. The dotted lines represent the initial linkage signal, whereas the solid lines represent the finemapped linkage signal.

**Table 2. Genes and selected SNPs in Linkage Peak, TNF $\alpha$** 

Chr	Gene (coverage <sup>1</sup> )	Short description <sup>2</sup>	SNP	Position	QTD add <sup>3</sup>	QTD dom <sup>4</sup>					
1	CSF1 (58%)	Granulocyte/macrophage colony-stimulating factors are cytokines that act in hematopoiesis by controlling the production, differentiation, and function of 2 related white cell populations of the blood, the granulocytes and the monocytes-macrophages	rs915357	Intron	0.055 <sup>+</sup>	0.060 <sup>+</sup>					
			rs333968	Intron							
			rs333970	Intron							
11	CD6 (30%)	CD6 is a monomeric 105- or 130-kD membrane glycoprotein that is involved in T-cell activation.	rs3738760	Coding Exon	0.012 <sup>*</sup>	0.009 <sup>**</sup>					
			rs10494122	Intron							
			rs10857833	Intron							
			rs6679497	Intron							
			rs4839581	Intron							
17	FAM19A3 (100%)	Contains conserved cysteine residues at fixed positions, and are distantly related to MIP-1alpha, a member of the CC-chemokine family.	rs3790722	Intron	0.019 <sup>*</sup>	0.033 <sup>*</sup>					
			rs4450019	Intron							
			rs11102524	Intron							
11	MADD (68%)	Tumor necrosis factor alpha (TNF-alpha) is a signaling molecule that interacts with one of two receptors on cells targeted for apoptosis. The protein encoded by this gene is a death domain-containing adaptor protein that interacts with the death domain of TNF-alpha receptor 1 to activate mitogen-activated protein kinase (MAPK) and propagate the apoptotic signal	rs7114704	Intron	0.019 <sup>*</sup>	0.033 <sup>*</sup>					
			rs10501320	Intron							
			rs10501321	Intron							
			rs10838689	Intron							
			rs2290149	Intron							
			rs11039183	Intron							
			rs753993	Intron							
			11	SELH (100%)			This gene encodes a selenoprotein, which contains a selenocysteine (Sec) residue at its active site.	rs9420	Intron bound.	0.019 <sup>*</sup>	0.033 <sup>*</sup>
								rs3017889	Downstream		
			11	CD5 (68%)			Human T-cell surface glycoprotein of relative molecular mass (Mr) 67,000, has been implicated in the proliferative response of activated T cells and in T-cell helper function.	rs2905504	Intron	0.019 <sup>*</sup>	0.033 <sup>*</sup>
rs11230550	Intron										
rs11230553	Intron										
rs2283263	Intron										
rs11230559	Intron										
rs11230563	Coding exon*										
17	GPS2 (100%)	This gene encodes a protein involved in G protein-mitogen-activated protein kinase (MAPK) signaling cascades.	rs2074225	Coding exon*	0.019 <sup>*</sup>	0.033 <sup>*</sup>					
			rs1050922	Coding exon							
			rs3862667	Intron							
			rs572350	Intron							
17	TNFA-SF (80%)	This gene encodes a member of the tumor necrosis factor superfamily. It encodes a hybrid protein composed of the cytoplasmic and transmembrane domains of family member 12 fused to the C-terminal domain of family member 13. The hybrid protein is membrane anchored and presents the receptor-binding domain of family member 13 at the cell surface. It stimulates cycling in T- and B-lymphoma cell lines.	rs671444	Intron	0.019 <sup>*</sup>	0.033 <sup>*</sup>					
			rs12364244	Intron							
			rs637186	Coding exon*							
			rs2270981	Coding Exon							
			rs2292064	Coding Exon							
			rs9899183	Intron							
17	CD68 (50%)	This gene encodes a 110-kD transmembrane glycoprotein that is highly expressed by human monocytes and tissue macrophages. The protein is also a member of the scavenger receptor family. Scavenger receptors typically function to clear cellular debris, promote phagocytosis, and mediate the recruitment and activation of macrophages. Alternative splicing results in multiple transcripts encoding different isoforms.	rs12937543	Promoter	0.019 <sup>*</sup>	0.033 <sup>*</sup>					
			rs4968211	Promoter							
			rs11552708	Coding Exon*							
17	CD68 (50%)	This gene encodes a 110-kD transmembrane glycoprotein that is highly expressed by human monocytes and tissue macrophages. The protein is also a member of the scavenger receptor family. Scavenger receptors typically function to clear cellular debris, promote phagocytosis, and mediate the recruitment and activation of macrophages. Alternative splicing results in multiple transcripts encoding different isoforms.	rs3803800	Coding Exon*	0.019 <sup>*</sup>	0.033 <sup>*</sup>					
			rs6608	3' UTR							
			rs9896688	Intron bound.							
17	CD68 (50%)	This gene encodes a 110-kD transmembrane glycoprotein that is highly expressed by human monocytes and tissue macrophages. The protein is also a member of the scavenger receptor family. Scavenger receptors typically function to clear cellular debris, promote phagocytosis, and mediate the recruitment and activation of macrophages. Alternative splicing results in multiple transcripts encoding different isoforms.	rs9901673	Coding Exon*	0.019 <sup>*</sup>	0.033 <sup>*</sup>					
			rs9901675	Coding Exon*							

<sup>+</sup> P < 0.1; <sup>\*</sup> P < 0.05; <sup>\*\*</sup> P < 0.01

<sup>1</sup> Gene coverage was based on genetic variation present in the HapMap database build 18.

<sup>2</sup> Short description adapted from gene ontology description provided in the UCSC genome graph tool.

<sup>3</sup> P values reported by QTD using command WEGA (additive model), tests for association given linkage on a specific locus.

<sup>4</sup> P values reported by QTD using command WEGD (dominant model), tests for association given linkage on a specific locus.

**Table 3.** Genotype analysis and linear mixed model for CD53 SNP rs6679497, assuming a dominant model of association

Study	N (11) <sup>1</sup>	Log(TNF $\alpha$ )	N (12) <sup>1</sup>	Log(TNF $\alpha$ )	N(22) <sup>1</sup>	Log(TNF $\alpha$ )	P LMM <sup>2</sup>	P LMM <sup>3</sup>
GARP	86	3.90	178	3.86	99	3.85	0.013*	
85-Plus	312	4.01	564	3.97	238	3.97	0.032*	0.001**

\* P < 0.1; † P < 0.05; \*\* P < 0.01

<sup>1</sup> 11=homozygote common allele; 12=heterozygote; 22=homozygote rare allele

<sup>2</sup> Modelling dominant effect, corrected for familial relationship, age, sex and BMI

<sup>3</sup> Modelling dominant effect combining studies, corrected for study differences in level, familial relationship, age, sex and BMI

### Association analysis of rs6679497 to OA

No significant association was observed for *CD53* rs6679497 when GARP subjects (cases) were compared to subjects of the Leiden 85-Plus study as controls using the dominant model (adjusted for age, sex and BMI,  $P=0.142$ ), indicating that TNF $\alpha$  QTL locus did not confer susceptibility to OA.

### Discussion and conclusion

Through a genome wide linkage scan we were able to identify SNP rs6679497 in *CD53* of which the minor allele associates to lower innate TNF $\alpha$  levels. It can be hypothesized that the specific genotype of rs6679497 predisposes or protect its carriers from diseases and disorders in which TNF $\alpha$  plays a substantial role. Previously, it was shown that TNF $\alpha$  does not play a major role in the onset of OA<sup>8</sup> and in line with this hypothesis we were unable to show associations of rs6679497 to OA as defined in the GARP study.

*CD53* codes for cluster of differentiation 53, a leukocyte surface antigen. The protein family which this cell surface glycoprotein belongs to is known to complex with integrins, cellular components involved in cell-cell and cell-matrix interactions. *CD53* deficiency has been linked to recurring infectious diseases caused by bacteria, fungi and viruses<sup>27</sup>, susceptibility to these might be increased for carriers of the minor allele of rs6679497. The protein is implicated in elevated cellular glutathione in response to LPS activation and may increase cell survival under UV-B and oxidative conditions<sup>28</sup>. Furthermore, treatment of neutrophils with TNF $\alpha$  down-regulates the presence of the *CD53* antigens on the cell surface through a proteolytic mechanism<sup>29</sup>. This indicates that the protein may play a substantial role in cellular stability and the inflammatory response to adverse conditions. Furthermore, the protective effect of ligated *CD53* on the cellular surface may help specific tumors to escape from programmed cell death<sup>30</sup>. Although rs6679497 or any of the SNPs in its LD block are investigated for effects on the expression levels or protein function, the gene is under strong genetic control<sup>31</sup> and genetic variation might have a role in tumor biology or other diseases. Such a relation can readily be elucidated through investigation of this marker SNP in cancer cohorts. Furthermore, in a study which characterizes leukocytes from normal and rheumatoid arthritis (RA) patients, *CD53* was found to be elevated on the RA lymphocytes surfaces<sup>32</sup>. Investigating the role if this gene in relation to this and other TNF $\alpha$  driven diseases later in life may show protective effects of the rs6679497 minor allele.

The SNP *CD53* rs6679497 resides within the intron of the gene, which shows low levels of conservation across species, and it is in an LD-block across several introns and exons

encompassing at least 23 other intron SNPs (recorded in the HapMap phase 1 & 2 data). Several transcripts are known as recorded in the UCSC database (accession number ENSG00000143119), however, only a proportion of these will actually be translated into a protein. Given the current level of total variation tagging (52%) it is likely the SNP is only a proxy marker in LD with a causal (functional) polymorphism which could have a more obvious implication to the gene regulation or protein stability and functioning. This is substantiated by the fact that the *CD53* SNP explains only part of the linkage as determined in QTDT analyses (results not shown). To find the true functional variant, a more detailed analysis of this gene by sequencing or SNP saturation is necessary. Alternatively, in our candidate gene approach we may have missed additional genetic variation at the loci of interest because of a knowledge bias on both the presence and role of genes at these loci.

Although it was shown previously that the *IL1* cluster haplotypes were associated to IL-1 $\beta$  bio-availability<sup>33,34</sup>, we observed no evidence of linkage on the genetic loci for the respective cytokines' genes, the *IL-1* gene cluster on chromosome 2, *IL-10* on chromosome 1 or *TNF* on chromosome 6. Possibly, the genetic variation in LPS stimulated cytokine levels explained by these loci is not readily detected by linkage analysis, which is known to be most suitable to detect loci that explain a major part of genetic variation. Furthermore, our linkage analyses of innate IL-1 $\beta$ , IL-1Ra and IL-10 levels in general revealed only moderate linkage peaks up to a LOD score of 2.5. More likely as was shown in a previous study<sup>33</sup>, particularly the estimate of innate IL-1 $\beta$  production upon LPS stimulation may not be entirely independent of the OA disease status, possibly by sensitization of the Toll-like receptor pathways as a result of disease activity<sup>35</sup> or otherwise sensitization of the response by lymphocytes, which may have interfered with the current linkage analysis by introducing cohort heterogeneity or bias. In the linkage analyses, we checked whether the levels in the GARP study sample were normally distributed to facilitate powerful linkage analysis using the variance component option. The innate levels of IL-1 $\beta$ , IL-10 and TNF $\alpha$  were normally distributed, whereas innate IL-1Ra levels were normally distributed after removal of 1 extreme value, which did not alter the linkage analysis results (data not shown). The use of Merlin-Regress<sup>21</sup> which may be more appropriate for use in highly selected samples showed a similar pattern of LOD scores for all traits, with slightly lower maximum LOD scores. It remains possible that the observed association only occurs in middle aged and elderly, therefore, further searches for genetic loci that influence the *ex vivo* innate cytokine profiles may benefit from the use of healthy young subjects in these searches.

Following a genome wide linkage analysis, association analysis of positional candidate gene SNPs within the 1-LOD-drop interval of a linkage peak, we show a consistent association of SNP rs6679497 in *CD53* to innate TNF $\alpha$  levels in both the GARP study ( $P = 0.013$ ) and the confirmation cohort consisting of Leiden 85-Plus participants ( $P = 0.032$ ). A dominant linear mixed model analysis on combined data from the GARP and Leiden 85-Plus study showed that the minor allele of this SNP associated to a highly significantly lower innate TNF $\alpha$  level independent of age and sex effects. In diseases with a large TNF $\alpha$  component such as inflammatory bowel disease or rheumatoid disorders, the minor allele of rs6679497 might exert a protective effect in susceptibility or severity.

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**Supplementary Table 1.** Markers used in fine mapping of initial linkage peaks.

Marker	Position (cM)
D1S2626	135.42
D1S2778	141.321
D1S2696	152.317
D11S986	68.014
D11S1889	78.155
D11S4081	86.331



