

Genes and mediators of inflammation and development in osteoarthritis

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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 β , IL-1Ra, IL-10 and TNF α . 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 α . SNP rs6679497 in gene *CD53* showed significant association to TNF α levels (P = 0.001). No association of this SNP was observed to osteoarthritis. A novel gene involved in the innate immune response of TNF α is identified. Genetic variation in this gene may play a role in diseases and disorders in which TNF α 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 response¹. The as such determined maximal cytokine production profile has been shown to be stable² and to contain a significant heritable component estimated between 53% and 86%, indicating a strong genetic control³.

Based on innate *ex vivo* cytokine profiles, individuals can be characterized as pro- or antiinflammatory and it has been shown that these profiles can be predisposing to diseases with an inflammatory component such as multiple sclerosis and cardiovascular events^{4,5}. 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) α , interleukin(IL)-1B) and antiinflammatory (IL-10 and IL-1 receptor antagonist(Ra)) cytokines^{6,7}. 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 β and IL-1Ra and low innate IL-10 production as compared to controls⁸. 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 features⁹. 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 markers¹⁰⁻¹². Furthermore, it was shown that DNA variants

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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 stimulation¹³⁻¹⁶. 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 study¹⁷, as well as data on their *ex vivo* LPS stimulated production of IL-1 β , IL-1Ra, IL-10 and TNF α^8 . Confirmation by association analysis of innate cytokine levels was performed using 563 unrelated individuals of the Leiden 85-Plus study¹⁸. 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.*¹⁹. 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 study²⁰.

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⁻¹ LPS and after a 4 hours incubation the sample was centrifuged and the TNF α 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 β , IL-1Ra and IL-10 were determined^{1,19}. In concordance with previous studies^{3,4,5}, 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.*¹⁷. 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

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measured on the Sequenom MassARRAY system (Sequenom, San Diego, CA). PCR's were carried out in a final volume of 5 μ 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 loci²¹. 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 browser²², 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 browser²³. 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 selection²⁴. 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 QTDT²⁵. 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 effects²⁶. 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

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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 asses 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 β (P = 0.034), IL-1Ra (P < 0.01) and TNF α (P < 0.01). To check for age dependencies of the LPS stimulated cytokine profiles the correlation with age was analyzed. We observed no 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 α level.

Table 1. Characteristics GARP and Leiden 85-Plus study

	Ν	The GARP Study	Ν	Leiden 85-Plus Study
No.	-	382	-	563
Age, mean ± SD*	-	60.4 ± 7.6	-	85 ¹
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 ± 0.38 (0.142)
Mean Log(IL1Ra) ± SD* (variance)	369	4.37 ± 0.15 (0.023)	560	4.55 ± 0.20 (0.041)
Mean Log(IL10) ± SD (variance)	369	2.87 ± 0.19 (0.035)	560	2.85 ± 0.30 (0.090)
Mean Log(TNF α) ± SD* (variance)	368	3.87 ± 0.18 (0.032)	561	3.98 ± 0.21 (0.045)
* Significant difference between studies (t-test) ¹ All subjects of the Leiden 85-Plus study were 85 years old at sampling.				

IL-1β, IL-1Ra and IL-10 linkage and association analysis

The genome wide linkage analysis to find quantitative trait loci involved in innate IL-1 β , 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 β and IL-1Ra (2q13), IL-10 (1q32.1) or TNF α (6p21.33) (Figure 1 A-D).

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TNFα linkage and association analysis

Genome wide linkage analysis of innate TNF α 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•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 α levels for SNPs in CD53 and FAM19A3 (Table 2). We were unable to model the observed associations of FAM19A3 in a linear mixed model,

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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 α 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.

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Table 2. Genes and selected SNPs in Linkage Peak, TNFa

Chr	Gene (coverage ¹)	Short description ²	SNP	Position	QTDT add ³	QTDT dom ⁴
	CSF1 (58%)	Granulocyte/macrophage colony-stimulating factors are cytokines that act in hematopoissis by controlling the production, differentiation, and function of 2 related white cell populations of the blood, the granulocytes and the monocytes- reacrophenes	rs915357 rs333968 rs333970 rs3738760	Intron Intron Intron Coding Exon	0.055*	0.060*
1	CD53 (52%)	cell surface glycoprotein that is known to complex with integrins Familial deficiency of this gene has been linked to an immunodeficiency associated with recurrent infectious diseases caused by bacteria, fungi and viruses. Alternative splicing results in multiple transcript variants encoding the same protein	rs10494122 rs10857833 rs6679497 rs4839581 rs3790722	Intron Intron Intron Intron Intron	0.012*	0.009**
	FAM19A3 (100%)	Contains conserved cysteine residues at fixed positions, and are distantly related to MIP-lalpha, a member of the CC- chemokine family.	rs4450019 rs11102524	Intron Intron	0.019*	0.033*
	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 rs10501320 rs10501321 rs10838689 rs2290149 rs11039183 rs753993	Intron Intron Intron Intron Intron Intron		
11	SELH (100%)	This gene encodes a selenoprotein, which contains a selenocysteine (Sec) residue at its active site.	rs9420 rs3017889	Intron bound. Downstream		
	CD6 (30%)	CD6 is a monomeric 105- or 130-kD membrane glycoprotein that is involved in T-cell activation.	rs2905504 rs11230550 rs11230553 rs2283263 rs11230559 rs11230563 rs2074225 rs1050922	Intron Intron Intron Intron Coding exon* Coding exon* Coding exon		
	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.	rs3862667 rs572350 rs671444 rs12364244 rs637186	Intron Intron Intron Intron Coding exon*		
	GPS2 (100%)	This gene encodes a protein involved in G protein-mitogen-activated protein kinase (MAPK) signaling cascades.	rs2270981 rs2292064	Coding Exon Coding Exon		
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.	rs9899183 rs12937543 rs4968211 rs11552708 rs3803800 rs6608	Intron Promoter Promoter Coding Exon* Coding Exon* 3'UTR		
	CD68 (50%)	This gene encodes a 110-kD transmembrane glycoprotein that is highly expressed by human monocytes and tissue macrophagesThe 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.	rs9896688 rs9901673 rs9901675	Intron bound. Coding Exon* Coding Exon*		

* P < 0.1; * P <0.05; ** P < 0.01
 ¹ Gene coverage was based on genetic variation present in the HapMap database build 18.
 ² Short description adapted from gene ontology description provided in the UCSC genome graph tool.
 ³ P values reported by QTDT using command WEGA (additive model), tests for association given linkage on a specific locus.
 ⁴ P values reported by QTDT using command WEGD (dominant model), tests for association given linkage on a specific locus.

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

Study	$N(11)^{1}$	Log(TNFa)	N (12) ¹	Log(TNFa)	N(22) ¹	Log(TNFa)	P LMM ²	P LMM ³
GARP	86	3.90	178	3.86	99	3.85	0.013*	0.001**
85-Plus	312	4.01	564	3.97	238	3.97	0.032*	0.001

 * P < 0.1; * P <0.05; ** P < 0.01

¹11=homozygote common allele; 12=heterozygote; 22=homozygote rare allele

² Modelling dominant effect, corrected for familial relationship, age, sex and BMI

³Modelling 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 α 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 α levels. It can be hypothesized that the specific genotype of rs6679497 predisposes or protect its carriers from diseases and disorders in which TNF α plays a substantial role. Previously, it was shown that TNF α does not play a major role in the onset of OA⁸ 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 viruses²⁷ 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 conditions²⁸. Furthermore, treatment of neutrophils with TNF α down-regulates the presence of the CD53 antigens on the cell surface through a proteolytic mechanism²⁹. 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 death³⁰. 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 control³¹ 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 surfaces³². Investigating the role if this gene in relation to this and other TNF α 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

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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-1B bio-availability^{33,34}, 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-1β, 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 study³³, particularly the estimate of innate IL-1ß 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 activity³⁵ 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-1β, IL-10 and TNFα 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-Regress²¹ 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 α 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 α level independent of age and sex effects. In diseases with a large TNF α 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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References

- 1. Ohlsson, K., Linder, C., Lundberg, E., Axelsson, L.: Release of cytokines and proteases from human peripheral blood mononuclear and polymorphonuclear cells following phagocytosis and LPS stimulation. *Scand.J.Clin.Lab Invest* 1996; **56**: 461-470.
- van der Linden, M. W., Huizinga, T. W., Stoeken, D. J., Sturk, A., Westendorp, R. G.: Determination of tumour necrosis factor-alpha and interleukin-10 production in a whole blood stimulation system: assessment of laboratory error and individual variation. *J.Immunol.Methods* 1998; **218**: 63-71.
- 3. de Craen, A. J., Posthuma, D., Remarque, E. J., van den Biggelaar, A. H., Westendorp, R. G., Boomsma, D. I.: Heritability estimates of innate immunity: an extended twin study. *Genes Immun.* 2005; **6**: 167-170.
- 4. de Jong, B. A., Schrijver, H. M., Huizinga, T. W.*et al*: Innate production of interleukin-10 and tumor necrosis factor affects the risk of multiple sclerosis. *Ann.Neurol.* 2000; **48**: 641-646.
- 5. McGeer, P. L., McGeer, E. G.: Innate immunity, local inflammation, and degenerative disease. *Sci.Aging Knowledge.Environ.* 2002; **29**: re3-
- 6. Goldring, M. B.: Update on the biology of the chondrocyte and new approaches to treating cartilage diseases. *Best.Pract.Res.Clin.Rheumatol.* 2006; **20**: 1003-1025.
- Goldring, M. B., Otero, M., Tsuchimochi, K., Ijiri, K., Li, Y.: Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism. *Ann.Rheum.Dis.* 2008; 67 Suppl 3: iii75-iii82.
- 8. Riyazi, N., Slagboom, E., de Craen, A. J.et al: Association of the risk of osteoarthritis with high innate production of interleukin-1beta and low innate

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production of interleukin-10 *ex vivo*, upon lipopolysaccharide stimulation. *Arthritis Rheum.* 2005; **52**: 1443-1450.

- Chapman, K., Loughlin, J.: Association of the interleukin-1 gene cluster with osteoarthritis of the hip: comment on the article by Meulenbelt et al and the letter by Smith *et al. Arthritis Rheum* 2006; 54: 3722-3723.
- Bos, S. D., Suchiman, H. E., Kloppenburg, M.*et al*: Allelic variation at the Creactive protein gene associates to both hand osteoarthritis severity and serum high sensitive CRP levels in the GARP study. *Ann.Rheum.Dis.* 2007; 67: 877-879.
- Curran, J. E., Jowett, J. B., Elliott, K. S.*et al*: Genetic variation in selenoprotein S influences inflammatory response. *Nat.Genet.* 2005; 37: 1234-1241.
- 12. Rafiq, S., Frayling, T. M., Murray, A.*et al*: A common variant of the interleukin 6 receptor (IL-6r) gene increases IL-6r and IL-6 levels, without other inflammatory effects. *Genes Immun.* 2007; **8**: 552-559.
- 13. Haukim, N., Bidwell, J. L., Smith, A. J.*et al*: Cytokine gene polymorphism in human disease: on-line databases, supplement 2. *Genes Immun.* 2002; **3**: 313-330.
- Danis, V. A., Millington, M., Hyland, V. J., Grennan, D.: Cytokine production by normal human monocytes: inter-subject variation and relationship to an IL-1 receptor antagonist (IL-1Ra) gene polymorphism. *Clin.Exp.Immunol.* 1995; **99**: 303-310.
- Wen, A. Q., Wang, J., Feng, K., Zhu, P. F., Wang, Z. G., Jiang, J. X.: Effects of haplotypes in the interleukin 1beta promoter on lipopolysaccharide-induced interleukin 1beta expression. *Shock* 2006; 26: 25-30.
- Temple, S. E., Lim, E., Cheong, K. Y.*et al*: Alleles carried at positions -819 and -592 of the IL10 promoter affect transcription following stimulation of peripheral blood cells with Streptococcus pneumoniae. *Immunogenetics* 2003; 55: 629-632.
- Meulenbelt, I., Min, J. L., Bos, S.*et al*: Identification of DIO2 as new susceptibility locus for symptomatic Osteoarthritis. *Hum.Mol.Genet.* 2008; .17: 1867-1875.
- 18. van Exel, E., Gussekloo, J., Houx, P.*et al*: Atherosclerosis and cognitive impairment are linked in the elderly. The Leiden 85-plus Study. *Atherosclerosis* 2002; **165**: 353-359.
- 19. Riyazi, N., Meulenbelt, I., Kroon, H. M.*et al*: Evidence for familial aggregation of hand, hip, and spine but not knee osteoarthritis in siblings with multiple joint involvement: the GARP study. *Ann.Rheum.Dis.* 2005; **64**: 438-443.
- van Exel, E., Gussekloo, J., de Craen, A. J., Bootsma-van der, W. A., Frolich, M., Westendorp, R. G.: Inflammation and stroke: the Leiden 85-Plus Study. *Stroke* 2002; **33**: 1135-1138.
- Abecasis, G. R., Cherny, S. S., Cookson, W. O., Cardon, L. R.: Merlin--rapid analysis of dense genetic maps using sparse gene flow trees. *Nat.Genet.* 2002; 30: 97-101.
- 22. Kuhn, R. M., Karolchik, D., Zweig, A. S.*et al*: The UCSC Genome Browser Database: update 2009. *Nucleic Acids Res.* 2009; **37**: D755-D761.
- Frazer, K. A., Ballinger, D. G., Cox, D. R.*et al*: A second generation human haplotype map of over 3.1 million SNPs. *Nature* 2007; 449: 851-861.
- Goode, E. L., Fridley, B. L., Sun, Z.et al: Comparison of tagging single-nucleotide polymorphism methods in association analyses. BMC.Proc. 2007; 1 Suppl 1: S6-

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- Abecasis, G. R., Cardon, L. R., Cookson, W. O.: A general test of association for quantitative traits in nuclear families. *Am.J.Hum.Genet.* 2000; 66: 279-292.
- 26. Diggle P.J., Liang K.Y., Zeger S.L.: Analysis of longitudinal data. 1994;
- Mollinedo, F., Fontan, G., Barasoain, I., Lazo, P. A.: Recurrent infectious diseases in human CD53 deficiency. *Clin.Diagn.Lab Immunol.* 1997; 4: 229-231.
- Kim, T. R., Yoon, J. H., Kim, Y. C.*et al*: LPS-induced CD53 expression: a protection mechanism against oxidative and radiation stress. *Mol.Cells* 2004; 17: 125-131.
- Mollinedo, F., Martin-Martin, B., Gajate, C., Lazo, P. A.: Physiological activation of human neutrophils down-regulates CD53 cell surface antigen. *J.Leukoc.Biol.* 1998; 63: 699-706.
- Yunta, M., Lazo, P. A.: Apoptosis protection and survival signal by the CD53 tetraspanin antigen. *Oncogene* 2003; 22: 1219-1224.
- Hernandez-Torres, J., Yunta, M., Lazo, P. A.: Differential cooperation between regulatory sequences required for human CD53 gene expression. *J Biol.Chem.* 2001; 276: 35405-35413.
- Pedersen-Lane, J. H., Zurier, R. B., Lawrence, D. A.: Analysis of the thiol status of peripheral blood leukocytes in rheumatoid arthritis patients. *J Leukoc.Biol.* 2007; 81: 934-941.
- 33. Smith,A.J. *et al.* Extended haplotypes and linkage disequilibrium in the *IL1R1-IL1A-IL1B-IL1RN* gene cluster: association with knee osteoarthritis. *Genes Immun.* **5**, 451-460 (2004).
- 34. Moxley, G., Han, J., Stern, A. G., Riley, B. P.: Potential influence of *IL1B* haplotype and *IL1A-IL1B-IL1RN* extended haplotype on hand osteoarthritis risk. *Osteoarthritis.Cartilage*. 2007; **15**: 1106-1112.
- Scanzello, C. R., Plaas, A., Crow, M. K.: Innate immune system activation in osteoarthritis: is osteoarthritis a chronic wound? *Curr.Opin.Rheumatol.* 2008; 20: 565-572.

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

Inte mapping of	initiai niikage peaks.
Marker	Position (cM)
D1S2626	135.42
D1S2778	141.321
D1S2696	152.317
D11S986	68.014
D11S1889	78.155
D11S4081	86.331

CD53 as an important regulator of innate TNFa