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Common variants at *TRAF3IP2* are associated with susceptibility to psoriatic arthritis and psoriasis

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Psoriatic arthritis (PsA) is an inflammatory joint disease distinct from other chronic arthritides and frequently accompanied by psoriasis vulgaris (PsV) and seronegativity for rheumatoid factor. In a SNP array based genome wide association study (GWAS) of a German case/control collective, we confirmed *HLA-C* and *IL12B* as PsA susceptibility genes and found and replicated association to intragenic variants of *TRAF3IP2* in various European study groups ($1.39\times10^{-12} > p > 8.56\times10^{-17}$). These SNPs were also associated in

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Author contributions H.B., A.B. and A.R. designed the study and worked out its concept. U.H., A.B.E., M.A. and J.B. planed and performed genotyping with genome-wide arrays and/ or assays for single SNPs, and U.H., S.U., A.B.E., J.B., M.A., C.H., M.S., T.F.W. and A.R. analyzed genetic data. B.B. and H.B. performed functional studies. U.H., J.B., E.G., E.K., K.J., R.McM., P.H., I.N.B., A.W.R., F.B., J.La., H.T., J.Lo., C.G., H.E.W., O.F., G.M.A., N.McH., H.B. and A.B. recruited patients and control individuals and collected the phenotypic data. U.H., S.U., H.B. and A.R. wrote a first draft of the manuscript. A.B. edited the manuscript in a major way. All authors reviewed and approved the manuscript.

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a German psoriasis vulgaris cohort. Sequencing revealed coding variant p.D10N (rs33980500) as strongly associated ($p = 1.13\times10^{-20}$, OR = 1.95) and the only variant present on all risk haplotypes. Association with PsV was weaker ($p = 1.35\times10^{-4}$), although we are currently unable to establish its primary association with PsA. Functional assays for interaction showed reduced binding of this but not other TRAF3IP2 variants to TRAF6 suggesting altered modulation of immunoregulatory signals via altered TRAF-interactions as a novel pathway for PsA and PsV.

Psoriasis vulgaris (PsV) is a common chronic inflammatory disease that affects the skin and is mediated by T-cells. The more than coincidental occurrence of PsV with an inflammatory arthritis in up to 15 % of PsV patients [1] led to the recognition of psoriatic arthritis (PsA) as a separate disease entity. In order to distinguish PsA from other forms of inflammatory arthritis, consensus classification criteria (CASPAR) were recently established [2]. A strong genetic component has been suggested by several studies, indicated for example by a relative risk of up to 40 for first degree and 12 for second degree relatives [3], which suggests a stronger heritability than PsV [4]. However, knowledge of the genetic components contributing to PsV is more advanced than in PsA. Common established susceptibility factors for PsV and PsA are an *HLA-C* risk allele [5,6] as well as variants in/ near the *IL23R* and *IL12B* genes [7,8], while a frequent deletion within the epidermal differentiation complex contributes to skin (PsV) [9], but not to joint involvement (PsA) [10].

In order to identify additional susceptibility genes for PsA, we performed a genome wide association study (GWAS) in 609 German PsA patients and 990 control individuals using Affymetrix Genome-Wide Human SNP Array 6.0. After genotyping, stringent quality control and imputation of SNPs in linkage disequilibrium (LD), 1,585,307 SNPs in 572 cases and 888 controls were used in the analysis. P-values corrected for inflation showed a final $\lambda_{GC} = 1.055$ (see online methods). This post-quality-control (QC) sample size had 80% power to detect an OR of 1.63 for an allele frequency of 0.5 or an OR of 2.20 for an allele frequency of 0.1 at genome-wide significance level, taking into account the final λ_{GC}. A quantile-quantile plot of the initial GWAS data showed substantial deviation from the expected distribution of genome-wide p-values for an unusually high number of SNPs (Supplementary Fig. 1a) which was considerably reduced upon removal of SNPs within the MHC region (Supplementary Fig. 1b).

We initially identified genome-wide significant association ($p < 5\times10^{-8}$) at two well established susceptibility loci (Fig. 1, Table 1): *HLA-C* indicated by the upstream variant rs13191343 ($p = 2.63\times10^{-23}$) and *IL12B* indicated by the upstream variant rs12188300 ($p = 5.60\times10^{-13}$). At a third locus, two intronic variants (rs13196377, rs13210247) as well as one coding variant (rs13190932) of the *TRAF3IP2* gene, not previously reported to be involved in PsA susceptibility, were significantly associated ($5.76\times10^{-7} < p < 9.36\times10^{-7}$) . In the replication phase, genome-wide significant association ($p = 4.53\times10^{-10}$) was also observed at this locus.

In order to confirm association to these loci, validation groups comprising a total of 1,761 patients and 3,727 control subjects of European descent from the Psoriatic Arthritis Genetics in Europe (PAGE) Consortium were genotyped for the best SNPs at *HLA-C* and *IL12B* as well as for the three most significantly associated variants at *TRAF3IP2*. As expected, the most significant association was observed with the HLA region: rs13191343, located 1.2 kB upstream of the gene *HLA-C*, had the strongest evidence for association both in the initial GWAS and in the validation study (Table 1, Fig. 1b). Taking into account the differing European background of our study groups, we used the Cochran-Mantel-Haenszel test in order to determine the level of association in the GWAS data set, the validation groups and the combined data set. rs12191877 has previously been described as the most significant

associated PsV variant in a GWAS of individuals of European origin [11] and is located further upstream (13kB) of *HLA-C*. Analysis of this SNP (data not shown) revealed strong LD with rs13191343 (r^2=0.95) in German PsA cases, suggesting that these two signals are not independent. Similarly, we observed strong LD (r^2 = 0.95) to the most significantly associated variant rs10484554 described in a combined study for PsA and PsV [12] that is even further upstream.

Association to rs12188300 at *IL12B* (Table 1, Supplementary Fig. 2c) was confirmed in the validation groups. This locus is a known PsV susceptibility locus and previous studies have identified two SNPs that contribute to risk to PsV, independently of each other (rs3212227 and rs6887695) [7]. Association of both variants with PsA has also been reported previously [8,13]. However, a so far unreported third SNP, rs12188300, located 72kB upstream of the gene, showed the most significant association in our PsA GWAS (Table 1 and Supplementary Table 1). This variant may represent an independent risk factor as its LD with rs2082412 was negligible (r^2 = 0.02). It is located in a moderately conserved sequence that might modify expression of *IL12B*, but this will require experimental confirmation.

Association with the *TRAF3IP2* gene was also confirmed in the independent validation groups and in a combined analysis the evidence for association was considerable (Table 1). This gene has not previously been implicated in any immunological disease. Two intronic variants (rs13210247, rs13196377) as well as one coding variant (rs13190932, p.R74W [NM_147686]) were convincingly associated with odds ratios (ORs) of up to 1.8 (Table 1). Comparative analyses of the three SNPs in German individuals with either PsA or an independent group with PsV suggested a weaker association with PsV than with PsA, even more so when compared to a subset of 788 PsV patients with disease duration of more than 15 years who are unlikely to develop PsA (Supplementary Table 2). Although ORs were higher for PsA, the 95% confidence intervals overlapped with those observed in PsV, so it remains possible that the different effect sizes between psoriasis phenotypes are due to chance and not because of a primary association with PsA. Interestingly, a GWAS of psoriasis samples published in this issue shows lower OR for a highly correlated SNP mapping to the locus [14], but further detailed analyses are necessary to determine whether it is indeed primarily associated with PsA. All three variants tag a haplotype present in 2-10 % of Europeans as well as individuals of African and Asian (Han Chinese / Japanese) descent. Further sequencing of all exons of *TRAF3IP2* in 24 PsA patients revealed a further coding variant (rs33980500, p.D10N [NM_147686]). Genotyping in single individuals showed an even stronger association with PsA ($p_{combined} = 1.13\times10^{-20}$, OR = 1.95) as well as association to PsV ($p=1.35^{-4}$, OR=1.52). SNP rs33980500 (p.D10N) but not rs13190932 (p.R74W) is present also on an additional rarer haplotype (3.8%) associated with PsA and PsV (Fig. 2a and Suppl. Table 3), indicating that this is the true causative variant. Comparison with sequences of Chimpanzee, other mammals as well as Neanderthal indicates that the non-risk allele (D) is the ancestral one. Both coding variants lead to an amino acid substitution in the N-terminal TRAF binding site.

TRAF3IP2 codes for Act1, a signalling adaptor involved in the regulation of adaptive immunity. Studies of TRAF3IP2-deficient mice suggest that Act1 is a negative regulator of humoral immunity via its inhibitory effect on CD40- and BAFFR-mediated signalling [15]. This B-cell-specific negative regulatory impact on the CD40 pathway by TRAF3IP2 [15] as a newly proposed PsA-associated gene is consistent with the lack of an autoantibody signature in PsA contrary to rheumatoid arthritis (RA). On the other hand, Act1 concomitantly operates as a positive signalling adaptor in IL-17-mediated cellular immune responses [15,16]. IL-17 is a dominant ‘signature’ cytokine of TH-17 cells and upregulates neutrophil-mobilizing cytokines, chemokines, and tissue-degrading matrix-metalloproteases [17]. IL-17-dependent receptor ligation induces Act1-recruitment to the cytoplasmic tail of the IL-17R

[15]. This in turn allows the incorporation of TNF receptor-associated factors (TRAF) TRAF3 and TRAF6 into the signaling complex and the subsequent 'downstream' activation of the MAPK- and NF-κB pathway [15,16,18]. Accordingly, Act1 is not only involved in pathways balancing humoral and cellular immunity, but also represents a chief link between IL-17-mediated adaptive immune responses and NF-κB as the master regulator of innate immunity [19] controlling the inducible transcription of various pro-inflammatory cytokines. Interestingly, recent GWAS for PsV and RA have identified different genes involved in NF-κB regulation as influencing susceptibility: *TNFAIP3* for both diseases [11,20], *TNIP1* for PsV [11] and *PRKCQ* and *TNFRSF14* for RA [21,22]. None of these, though, was significantly associated in our PsA study.

Given the position of the two coding *TRAF3IP2*-associated SNPs, we hypothesized that the associated alleles may affect interaction with TRAF3 and/or TRAF6. We therefore investigated binding of the alleles using a mammalian-two-hydrid dual-luciferase reporter assay [23] (Fig. 2b). In our investigations we detected a strong and specific firefly luciferase reporter induction upon the interaction between the cotransfected TRAF6-bait and wildtype TRAF3IP2-prey constructs (up to 15-40 fold of respective negative controls, Fig. 2b). Since the bait samples containing TRAF3 instead of TRAF6 remained negative with all cotransfected TRAF3IP2 prey variants (results not shown) in agreement with earlier mammalian two hydrid studies [23], we focused in our further functional analysis on the interaction between TRAF6 and wildtype as well as mutant TRAF3IP2. The introduction of the N-coding risk allele of rs33980500 (p.D10N) in the amino terminal domain of Act1 resulted in a nearly complete loss of ability to interact with TRAF6 as indicated by a luciferase reporter activity in the range of negative control. (Fig. 2b). By contrast, the PsA-associated TRAF3IP2-allele coding for W at rs13190932 (p.R74W) did not cause any detectable change of TRAF6-binding as indicated by luminescence signals not significantly different from those determined for TRAF6-interaction with wild type *TRAF3IP2*. When both PsA-risk alleles (p.D10N+p.R74W) were present in the same TRAF3IP2-construct, the negative effect of the p.D10N-mutation on TRAF6 interaction was dominant (Fig. 2b). One-way analysis of variance (ANOVA) showed that differences of the mean normalized luciferase responses are highly significant ($p = 3.17\times10^{-11}$ for the interaction with TRAF6). The p.D10N mutation of Act1 identified as critical for TRAF6 interaction is also present on both risk-conferring haplotypes of TRAF3IP2 (Fig. 2a, Supplementary Table 3). Accordingly, our results suggest that p.D10N may modulate downstream signals of different crucial immunoreceptors via altered TRAF-interactions. This may ultimately lead to a shift in balance between adaptive B- and T-cell-responses and innate immunity into an arthritogenic disequilibrium. The functional data, however, should be viewed as preliminary and in need of further confirmation. The delineation of the involved pathways and how that predisposes to PsA will require further functional studies.

In summary, our study provides new insights into the pathophysiology of PsA, highlighting a novel susceptibility gene and identifying a *TRAF3IP2* coding variant which may affect binding of interacting proteins. Likely, many more variants of weaker effect are involved in PsA susceptibility but our study is underpowered to detect them. Further studies in larger cohorts and meta-analyses will be required to unravel these as well as studies on endophenotypes which might have stronger genetic determinants.

URLs

PLINK (vs. 1.07) available at: http://pngu.mgh.harvard.edu/purcell/plink/

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Online Methods

Study groups

The cases and controls consisted of a variety of different collections from Europe. Detailed clinical data is provided as a supplementary note. The studies were approved by the local ethical committees. Written informed consent was obtained from each patient and control subject prior to enrollment. The investigations were conducted according to the Declaration of Helsinki Principles.

Genotyping and primary data analysis

609 German PsA samples and 990 control samples from the population based study of the area of Augsburg, Germany (kooperative Gesundheitsforschung in der Region Augsburg = KORA) were genotyped on the Affymetrix Genome-Wide Human SNP Array 6.0. Genotype calling was performed with Affymetrix Power Tools version 1.10.2 using the birdseed-v2 algorithm. Samples with an overall call rate of less than 97% were excluded from further analysis, bringing the sample numbers to 603 PsA and 899 KORA. 718,347 SNPs had an overall call rate of at least 95% in both PsA and KORA and a minor allele frequency of ≥ 1%.

Imputation

For imputation, SNPs passing QC were extracted into an initial data set. From this set, 300 randomly selected individuals (150 PsA, 150 KORA) were used to estimate error and recombination rates with MACH1 [24]. 100 iterations of the Markov chain were used for each chromosome, using the HapMap CEU r22 dataset as a reference. After this, maximum likelihood estimation of the missing alleles was also performed with MACH1, using the full initial data set, the calculated error and recombination rates as well as the HapMap data as input. From the MACH1 output of 2.5 million HapMap SNPs, only those imputed genotypes with at least 95% posterior probability were used; all others were marked as unknown.

Finally, a filter for at least 95% known genotypes, a minor allele frequency of at least 5% and HWE p value of ≥ 0.001 was applied, yielding an imputed data set of 1,585,307 SNPs in 603 cases and 899 controls.

Association analysis

Association was initially tested in a chi-square test for independence (allelic, $n = 3004$) for all 1,585,307 SNPs using PLINK [25]. Genomic control [26] yielded an inflation factor of 1.219 (based on the median chi square value), suggesting some additional structure within the data. Principal component analysis of the unimputed data set with EIGENSOFT [27] showed visible clustering of both patients and controls in 3 groups along the second principal eigenvector and removed further 31 cases and 11 control individuals as outliers (Supplementary Fig. 3). Accordingly, a within-cluster Cochran-Mantel-Haenszel $2\times2\times K$ ($K = 3$) test with the imputed data was performed with PLINK in the final data set of 572 cases and 888 control individuals, yielding an inflation factor $\lambda_{GC} = 1.177$ (Supplementary Fig. 2a). All loci with a corrected p-value at least 1 order of magnitude below the theoretical quantile expected under H_0 were then visualized in GPGraphics (Uebe et al. in preparation) [28]. Three loci (*HLA-C*, *IL12B* and *TRAF3IP2*) that showed high significance data were then selected for replication testing. To further reduce inflation, EIGENSOFT was used on the full, imputed data, which contained only the most likely (95% posterior probability) imputed genotypes for the final data set of 572 cases and 888 control individuals.

Replication and Quality control

For replication, genotyping of rs13191343, rs12188300, rs13196377, rs13190932 (p.R74W) and rs13210247 was performed with TaqMan technology (Applied Biosystems, Foster City, USA) using pre-designed or self-designed assays in the German, Italian and Swedish study groups, and with Sequenom i-Plex for the samples from the United Kingdom and Ireland. rs13191343 was genotyped with a TaqMan assay, exclusively. In 548 PsA patients, genotypes of SNPs from the genome wide association study and the replication analysis with TaqMan technology were 99.6% concordant. All SNPs had a genotyping rate of ≥ 95% in every single study group, individual DNAs with a genotyping rate of less than 80% were excluded. Genotypes for rs13196377 in British and Irish individuals were imputed based on perfect LD with rs13190932 in HapMap data. Association in the validation data set ($K = 5$, grouped by nationality) and the combination of GWAS and replication sets ($K = 8$) was determined using $2\times2\times K$ Cochran-Mantel-Haenszel tests. For the comparative statistics of PsA versus PsV patients, descriptive statistics using the sample odds ratio as an effect estimator was used.

Sequencing

Sequencing was performed in eight German PsA patients homozygous for the *TRAF3IP2* risk haplotype and 16 individuals homozygous for non-risk haplotypes as previously described [29].

Analysis of rs33980500 (p.D10N) and *TRAF3IP2* haplotypes

rs33980500 was genotyped by TaqMan technology in German, Irish, Italian and UK individuals. Haplotypes within *TRAF3IP2* were calculated with PHASE [29]. For combined analyses within the PsA phenotype, individuals were stratified according to nationality, and a within-cluster Cochran-Mantel-Haenszel test (2×2×3) was performed.

Mammalian two-hybrid assay

A mammalian two-hybrid assay (Stratagene) was applied to analyze the interaction of wild type and mutant protein of *TRAF3IP2* with TRAF3 or TRAF6 [23]. A coding segment of human *TRAF3IP2* – corresponding to amino acid residues (aa) 1-355 – that harbours the TRAF-interacting domain [23] was PCR-amplified and cloned into the pCMV-AD prey vector. *TRAF3* (aa 51-543) and *TRAF6* (aa 70-522) [23] were cloned accordingly into the pCMV-BD bait vector. The bait, prey (10ng each) and the firefly luciferase reporter (250 ng) were co-transfected in HEK 293 cells. A renilla luciferase plasmid (pRL-TK vector, Promega) was co-transfected to control the transfection rate. 48 h after transfection, the luciferase expression was measured using the Dual-Luciferase Reporter Assay System (Promega). The risk alleles of rs33980500 (p.D10N) and/or of rs13190932 (p.R74W) were introduced into *TRAF3IP2* to generate the PsA-associated variants for functional testing using the QuikChange site-directed mutagenesis kit (Stratagene). Prior to transfection of the bait- and prey vectors, a quality control was performed by DNA sequencing. An independent positive control based on the tight interaction between murine p53 protein and viral SV40 large T antigen was performed confirming functionality of the system [Relative normalized luminescence: 406422 (CI: 287555 – 525283) units], while the non-interacting vector combination [bait: pBD-p53 and prey: pAD-TRAF2] served as negative control.

Analysis of Variance

Using a spreadsheet program, the normalized relative luminescence units were used to calculate the ratios of treatment to error variance of the TRAF6 data set. p-values were obtained from the cumulative F-distribution with 9 degrees of freedom in the numerator, 30 in the denominator, also within the same spreadsheet program.

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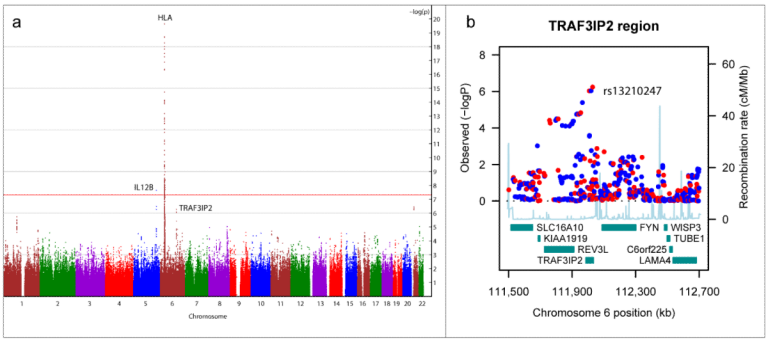

Figure 1.

Genome wide association results from the initial GWAS analysis. Negative decadic logarithms of genomic control corrected EIGENSTRAT p-values are plotted against genomic position (hg18). (**a**) Plot of 1,585,307 SNPs (after imputation) in 572 German psoriatic arthritis patients versus 888 German controls. The red line represents the genome wide significance threshold of 5×10^{-8}. (**b**) Regional association of the *TRAF3IP2* region. Genotyped SNPs appear in red, imputed SNPs in blue. Recombination rate (from HapMap data) is indicated by the light blue graph.

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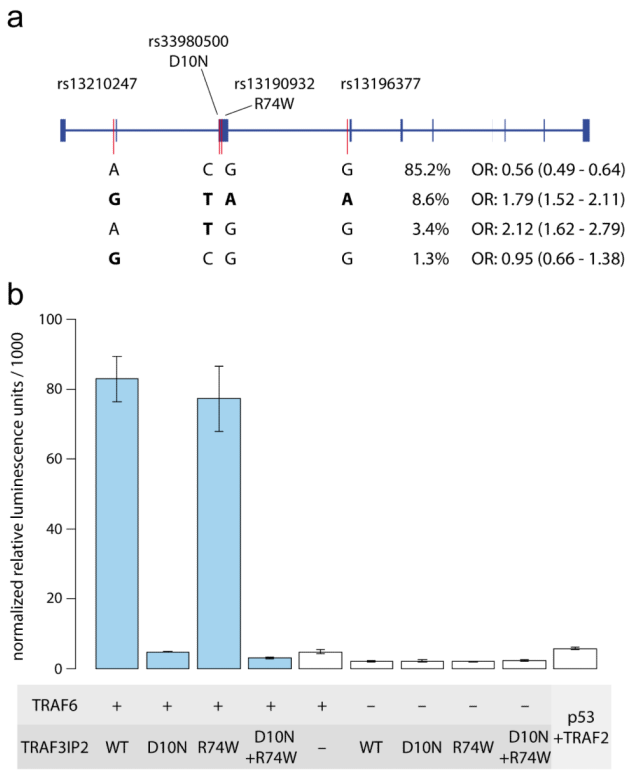

Figure 2.

Haplotype and functional analysis of associated variants in *TRAF3IP2*. **(a)** Exon/intron structure of *TRAF3IP2* with location of associated SNPs. The four most common haplotypes in a combined set of 2,077 PsA cases and 2,648 control individuals of European origin and their frequency within cases are given. Risk alleles are in bold type. For each haplotype, odds ratios and 95% confidence intervals (in brackets) compare occurrence of this vs. all three other haplotypes. (**b**) Interaction of wild type TRAF3IP2 (Act1) and PsA-associated variants D10N and R74W with TRAF6 in a mammalian two hybrid dual-luciferase reporter assay. Depicted are means of relative normalized luminescence units reflecting the specific induction of firefly-luciferase by the interaction of the cotransfected ligand pairs TRAF3IP2 and TRAF6 [blue bars]. The investigated TRAF3IP2-variants include constructs harboring either single missense variant (D10N or R74W) or both (D10N+R74W). To exclude autoactivation activity, each prey and bait construct was separately cotransfected with reporter plasmids into HEK293 cells in the absence of any potential interaction partner [open bars]. P53 andTRAF2 as a pair of non-interacting proteins served as an additional independent negative control. Error bars represent 95%-confidence intervals.

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Table 1

Association analyses of genome-wide significant SNPs. Number of alleles (risk/non-risk allele) and allele frequencies (risk allele) in GWAS, replication and combined study and results of Cochran-Mantel-Haenszel test as well as EIGENSTRAT corrected GWAS data are shown

SNP	Locus	Associated Allele	Study	No. of Alleles Cases	No. of Alleles Controls	Allele Frequency Cases	Allele Frequency Controls	p-Value (CMH)	p-Value (EIGENSTRAT)	Odds Ratio	95% Confidence Interval
rs13191343	*HLA*	T	GWAS	351/749	270/1442	0.319	0.158	2.63×10^{-23}	2.25×10^{-20}	2.48	2.06-2.97
			Replication	863/2545	866/6398	0.253	0.119	7.61×10^{-51}		2.33	2.09-2.61
			Combined	1214/3294	1136/7840	0.269	0.127	2.32×10^{-72}		2.37	2.16-2.61
rs12188300	*IL12B*	T	GWAS	157/917	108/1598	0.146	0.063	5.60×10^{-13}	2.40×10^{-8}	2.54	1.96-3.29
			Replication	374/3068	534/6248	0.109	0.079	5.14×10^{-8}		1.50	1.29-1.73
			Combined	531/3985	642/7846	0.118	0.076	7.33×10^{-17}		1.70	1.50-1.93
rs13196377	*TRAF3IP2*	A	GWAS	138/990	105/1649	0.122	0.060	5.48×10^{-9}	9.36×10^{-7}	2.17	1.66-2.82
			Replication	283/3167	421/6789	0.082	0.058	2.83×10^{-6}		1.50	1.27-1.78
			Combined	421/4157	526/8438	0.092	0.059	1.39×10^{-12}		1.67	1.45-1.93
rs13190932 (p.R74W)	*TRAF3IP2*	A	GWAS	129/983	97/1641	0.116	0.056	1.03×10^{-8}	9.19×10^{-7}	2.19	1.66-2.88
			Replication	294/3180	381/6841	0.085	0.053	4.53×10^{-10}		1.71	1.44-2.02
			Combined	423/4163	478/8482	0.092	0.053	8.56×10^{-17}		1.83	1.59-2.12
rs13210247	*TRAF3IP2*	G	GWAS	142/1000	112/1664	0.124	0.063	1.49×10^{-8}	5.76×10^{-7}	2.08	1.61-2.69
			Replication	316/3152	440/6802	0.091	0.061	3.79×10^{-8}		1.56	1.33-1.84
			Combined	458/4152	552/8466	0.099	0.061	1.73×10^{-14}		1.69	1.48-1.94