Growing evidence supports the hypothesis that narcolepsy with cataplexy is an autoimmune disease. We here report genome-wide association analyses for narcolepsy with replication and fine mapping across three ethnic groups (3,406 individuals of European ancestry, 2,414 Asians and 302 African Americans). We identify a SNP in the 3′ untranslated region of P2RY11, the purinergic receptor subtype P2Y11 gene, which is associated with narcolepsy (rs2305795, combined \( P = 6.1 \times 10^{-10} \), odds ratio = 1.28, 95% CI 1.19–1.39, \( n = 5,689 \)). The disease-associated allele is correlated with reduced expression of P2RY11 in CD8+ T lymphocytes (339% reduced, \( P = 0.003 \)) and natural killer (NK) cells (\( P = 0.031 \)), but not in other peripheral blood mononuclear cell types. The low expression variant is also associated with reduced P2RY11-mediated resistance to ATP-induced cell death in T lymphocytes (\( P = 0.0007 \)) and natural killer cells (\( P = 0.001 \)). These results identify P2RY11 as an important regulator of immune-cell survival, with possible implications in narcolepsy and other autoimmune diseases.

Narcolepsy-cataplexy affects 1 in every 2,000 individuals and is primarily caused by the loss of around 70,000 hypocretin (hcrt, also known as orexin)-producing neurons in the hypothalamus. The disease is associated with HLA-DQB1*06:02 (ref. 3) and a TRA@ (encoding the T cell receptor alpha) locus. Further, autoantibodies against Tribbles homolog 2 (Trib2), a protein expressed in hcrt cells, have recently been detected in the sera of some individuals with recent-onset narcolepsy. These findings strongly suggest narcolepsy is caused by an autoimmune attack on hcrt neurons. This disease may offer a unique model to study immune surveillance of neurons, a topic of growing importance.

Following on our recently published genome-wide association study (GWAS) of 807 individuals with narcolepsy-cataplexy (cases) and 1,074 HLA-DQB1*06:02–positive controls, we conducted replication of ten suggestive loci from this study in an additional 1,525 individuals of European ancestry (594 cases and 931 controls). We selected SNPs having \( P < 5 \times 10^{-6} \) for replication. A total of 18 SNPs, representing ten genomic regions, met this criterion (Supplementary Table 1). Of these SNPs, only one, rs4804122, was significantly associated in the current replication study after Bonferroni correction for 18 markers (\( P < 0.01 \); see Table 1 for nominal \( P \) value). This SNP is located downstream of P2RY11 on chromosome 19 in a region of high linkage disequilibrium (LD) spanning several genes (PPAN, P2RY11, EIF3G and DNM1T1; Fig. 1).

The last of these 18 markers is rs9275523, a SNP located between HLA-DQA1 and HLA-DQA2 (\( P = 5.1 \times 10^{-6} \), odds ratio (OR) = 0.59, minor allele frequency = 0.071 in 799 cases and minor allele frequency = 0.116 in 1,068 controls). The association with this marker is consistent with prior data indicating that the genetic influences of HLA on narcolepsy predisposition are not mediated solely through DQB1*06:02 heterozygosity. DQB1*06:02 homozygotes and DQB1*06:02/03:01 heterozygotes are, for example, at higher risk for narcolepsy as compared to DQB1*06:02 heterozygotes in general, whereas DQB1*06:02/06:01, DQB1*06:02/05:01 and DQB1*06:02/06:03 heterozygotes are at a decreased risk for narcolepsy. Heterodimerization of DQA1*01:02 and DQB1*06:02 with other DQA1 and DQB1 alleles of the DQ1 group may account for these protective effects by reducing the abundance of the disease susceptibility DQA1*01:02/DQB1*06:02 heterodimer. We therefore expected the finding of a secondary association in the HLA-DQ locus, and this finding further indicates a complex influence of HLA-DQ or other loci in high LD with HLA-DQ on narcolepsy. A recently published study also reported an association of a SNP located in the HLA-DQA2 region, rs2858884, with narcolepsy, independent
of DQB1*06:02 (ref. 8). In our initial GWAS sample of 1,881 individuals of European ancestry, rs2858884 had a nominal P value of 0.013, which was well below the association observed for rs9275523 (Supplementary Table 1).

Our next step was to attempt replication of the new chromosome 19 association in other ethnic groups. Notably, rs4804122 had no effect in a sample of 2,414 Asians and 302 African Americans (Table 1). This led us to explore differential LD patterns for this marker across ethnic groups. Based on data from the International HapMap Project, we selected five SNPs in high LD with rs4804122 in individuals of European ancestry but which were in lower LD with rs4804122 in Asians. We also genotyped one additional marker, rs3745601, a functional SNP located in the 3′ untranslated region (3′UTR) of P2RY11 which was previously reported to be associated with myocardial infarction and elevated levels of C-reactive protein10, even though this SNP is in low LD with rs4804122. We genotyped these six SNPs in 3,406 individuals of European ancestry (1,401 cases and 2,005 controls), 2,414 Asians (1,130 cases and 1,284 controls) and 302 African Americans (113 cases and 189 controls). A SNP located in the 3′ untranslated region (3′UTR) of P2RY11, rs2305795, showed the most significant association with narcolepsy across all ethnic groups (Table 2 and Fig. 1). The rs2305795 association was significant in individuals of European ancestry (P = 5.19 × 10−8), in Asians (P = 0.042), and also in the combined study sample (combined P = 6.1 × 10−10, OR = 1.28). These associations remained significant after Bonferroni correction for the six fine-typing markers in the replication sample. The replication of this association across ethnic groups also illustrates the value of transethnic mapping in narcolepsy, as previously found in HLA and TCR studies3,4,11. We found no significant interaction between rs2305795 and previously identified loci (data not shown).

To determine whether the presence of the disease-associated SNP was correlated with a significant change in the expression of any of the genes in the linkage region (Fig. 1), we quantified mRNA expression in peripheral blood mononuclear cells (PBMCs) of PPAN, P2RY11, PPAN-P2RY11, EIF3G and DNMT1 in 60 individuals with narcolepsy and 56 control subjects of European ancestry (Supplementary Table 2). Expression of P2RY11 mRNA correlated strongly with rs2305795 genotype (52.6% lower expression with the disease-associated allele, P = 0.002; Fig. 2a) and sex (lower expression in females, P = 0.039) but not disease status, HLA-DQB1*06:02 genotype, age or body mass index (BMI). The lack of effect of disease status is not surprising, considering the current narcolepsy-catalepsy model suggesting rapid hypocretin cell destruction with minimal residual immune response once the destruction is complete (a ‘hit and run’ hypothesis). A weaker correlation was also found with DNMT1 mRNA expression (lower expression with the disease-associated allele, P = 0.029). As expression of DNMT1 positively correlates with P2RY11 independently of rs2305795 (r² = 0.48, P < 0.0001), this effect is likely secondary. We found expression of the readthrough PPAN-P2RY11 transcript12 to be very low.

**Table 1** Association of rs4804122 with narcolepsy

<table>
<thead>
<tr>
<th>Cohorts (n)</th>
<th>MAF cases (n)</th>
<th>MAF controls (n)</th>
<th>OR (95% CI)</th>
<th>Nominal P</th>
<th>P (BD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>European ancestry</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original sample (1,881)</td>
<td>0.383 (614)</td>
<td>0.468 (911)</td>
<td>0.71 (0.61–0.82)</td>
<td>3.69 × 10⁻⁶</td>
<td>0.285</td>
</tr>
<tr>
<td>Replication sample (1,525)</td>
<td>0.391 (603)</td>
<td>0.453 (907)</td>
<td>0.77 (0.66–0.89)</td>
<td>5.42 × 10⁻⁴</td>
<td>0.252</td>
</tr>
<tr>
<td>All (3,406)</td>
<td>0.395 (1,391)</td>
<td>0.459 (1,968)</td>
<td>0.76 (0.69–0.84)</td>
<td>1.16 × 10⁻⁷</td>
<td>0.173</td>
</tr>
<tr>
<td>Asians</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese (1,269)</td>
<td>0.264 (582)</td>
<td>0.279 (681)</td>
<td>0.93 (0.78–1.10)</td>
<td>0.389</td>
<td>0.617</td>
</tr>
<tr>
<td>Japanese (869)</td>
<td>0.221 (424)</td>
<td>0.245 (432)</td>
<td>0.87 (0.70–1.09)</td>
<td>0.224</td>
<td>N/A</td>
</tr>
<tr>
<td>Koreans (276)</td>
<td>0.238 (105)</td>
<td>0.240 (169)</td>
<td>0.99 (0.66–1.49)</td>
<td>0.967</td>
<td>N/A</td>
</tr>
<tr>
<td>All (2,414)</td>
<td>0.245 (1,111)</td>
<td>0.262 (1,282)</td>
<td>0.91 (0.80–1.04)</td>
<td>0.172</td>
<td>0.894</td>
</tr>
<tr>
<td>African Americans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All (302)</td>
<td>0.314 (113)</td>
<td>0.307 (189)</td>
<td>1.04 (0.72–1.48)</td>
<td>0.851</td>
<td>N/A</td>
</tr>
<tr>
<td>All samples (6,122)</td>
<td>0.328 (2,615)</td>
<td>0.377 (3,439)</td>
<td>0.83 (0.76–0.89)</td>
<td>1.03 × 10⁻⁶</td>
<td>0.1568</td>
</tr>
</tbody>
</table>

MAF, minor allele frequency; BD, Breslow Day test (performed in combination with Mantel-Haenszel test of association).

*Including genotypes for 174 cases, 150 controls from original sample that were typed on the Affymetrix 500K Array Set.

Figure 1** Risk locus on 19q13.2, showing gene organization and linkage disequilibrium in the region of interest (10,071,000–10,130,000 bp). At top, the D′ and LOD-based LD plot using data from the combined Chinese and Japanese populations (CHB/JPT). Below, the D′ and LOD-based LD plot for individuals of European ancestry only (CEU/TSI). We calculated D′ values from the HapMap v3R2 CHB, JPT, CEU and TSI populations. In addition, r² values between the original marker, rs4804122 (green), and the best transethnic marker, rs2305795 (orange), derived from our own data are indicated. rs2305795 falls in the 3′UTR of P2RY11.
Table 2 Fine typing of SNP markers in the chromosome 19 region

<table>
<thead>
<tr>
<th>Cohort (n)</th>
<th>SNP</th>
<th>Position (bp)</th>
<th>Associated allele</th>
<th>Freq cases (n)</th>
<th>OR (95% CI)</th>
<th>P (MH)</th>
<th>P (BD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>European ancestry (3,406)</td>
<td>rs2305795</td>
<td>10,087,052</td>
<td>A</td>
<td>0.608 (1,311)</td>
<td>1.33 (1.20–1.47)</td>
<td>29.64</td>
<td>5.19 × 10⁻⁸</td>
</tr>
<tr>
<td></td>
<td>rs4804122¹</td>
<td>10,102,944</td>
<td>T</td>
<td>0.605 (1,391)</td>
<td>1.32 (1.19–1.45)</td>
<td>28.09</td>
<td>1.16 × 10⁻⁷</td>
</tr>
<tr>
<td></td>
<td>rs11880388</td>
<td>10,114,573</td>
<td>A</td>
<td>0.536 (1,312)</td>
<td>1.16 (1.05–1.30)</td>
<td>9.06</td>
<td>0.00260</td>
</tr>
<tr>
<td></td>
<td>rs2228611</td>
<td>10,128,077</td>
<td>A</td>
<td>0.538 (1,316)</td>
<td>1.18 (1.06–1.30)</td>
<td>9.34</td>
<td>0.00224</td>
</tr>
<tr>
<td>Africans (302)</td>
<td>rs2305795</td>
<td>10,087,052</td>
<td>A</td>
<td>0.728 (1,105)</td>
<td>0.689 (1,249)</td>
<td>1.20 (1.06–1.37)</td>
<td>8.209</td>
</tr>
<tr>
<td></td>
<td>rs4804122¹</td>
<td>10,102,944</td>
<td>T</td>
<td>0.755 (1,111)</td>
<td>0.109 (0.82–1.28)</td>
<td>0.89</td>
<td>0.3437</td>
</tr>
<tr>
<td></td>
<td>rs11880388</td>
<td>10,114,573</td>
<td>G</td>
<td>0.590 (563)</td>
<td>0.757 (638)</td>
<td>1.09 (0.92–1.28)</td>
<td>0.879</td>
</tr>
<tr>
<td></td>
<td>rs2228611</td>
<td>10,128,077</td>
<td>G</td>
<td>0.594 (561)</td>
<td>0.754 (644)</td>
<td>1.11 (0.94–1.32)</td>
<td>1.63</td>
</tr>
<tr>
<td>All samples (6,122)</td>
<td>rs2305795</td>
<td>10,087,052</td>
<td>A</td>
<td>0.684 (106)</td>
<td>0.621 (116)</td>
<td>1.32 (0.89–1.96)</td>
<td>1.952</td>
</tr>
<tr>
<td></td>
<td>rs4804122¹</td>
<td>10,102,944</td>
<td>C</td>
<td>0.314 (113)</td>
<td>0.307 (189)</td>
<td>1.04 (0.72–1.48)</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>rs11880388</td>
<td>10,114,573</td>
<td>G</td>
<td>0.491 (106)</td>
<td>0.430 (114)</td>
<td>1.28 (0.88–1.68)</td>
<td>1.63</td>
</tr>
<tr>
<td></td>
<td>rs2228611</td>
<td>10,128,077</td>
<td>G</td>
<td>0.472 (107)</td>
<td>0.430 (115)</td>
<td>1.18 (0.81–1.72)</td>
<td>0.772</td>
</tr>
</tbody>
</table>

¹ Mantel-Haenszel (MH) test was performed on all cohorts except for the African American cohort (NA in the table) in order to account for diverse subgroups. Breslow Day (BD) test of heterogeneity results are presented in the context of MH testing. For ease of comparison of ORs, results for the associated allele (rather than the minor allele) are presented for each cohort. Most significant SNPs in each cohort are indicated in bold. Original GWA SNP. Differences in Asian genotype counts reflect samples genotyped at Stanford versus samples genotyped at Stanford combined with those genotyped in China.

(93.5% lower than P2RY11 expression) and to vary with sample storage conditions, and thus, we did not further analyze it. Gene expression levels of PPAN and EIF3G did not correlate significantly with rs2305795 or disease status (Supplementary Table 3). These results indicate that the A allele of rs2305795, the disease-associated allele, decreases P2RY11 mRNA expression in PBMCs.

P2RY11 is a member of a large family of more than 20 purinergic receptors. Purinergic signaling plays a fundamental role in immune regulation, modulating proliferation, apoptosis and chemotaxis in lymphocytes, monocytes and polymorphonuclear granulocytes. Unlike most other purinergic receptors, P2Y11 is a low-affinity receptor and detects high concentrations of extracellular ATP. P2RY11 is unique among purinergic receptors, as it is coupled to both Gq and Gs, with its activation leading to increases in both cAMP and IP₃ (ref. 15). In healthy tissue, ATP is mostly localized intracellularly (millimolar range) and not extracellularly (nanomolar range). During inflammation, however, ATP levels rise in the extracellular space and produce a cascade of concentration-dependent effects on the immune system. At lower concentrations, ATP induces immune-cell chemotaxis through the stimulation of P2Y2 and P2Y6 receptors. High levels of ATP are typically cytotoxic, an effect mediated by the P2X7 receptor.

In rodents, functional studies have observed no direct effect of disease status on P2RY11 expression, subjects with various rs2305795 genotypes (mean ± s.e.m., n = 7–8 normal controls per genotype category). NK cells, CD56⁺ natural killer cells; B cells, CD19⁺ B cells; Monocy., CD14⁺ monocytes; DCs, myeloid/plasmacytoid dendritic cells. Shown are Bonferroni-corrected one-way analysis of variance (ANOVA) P values.

Figure 2 P2RY11 mRNA expression in PBMCs. (a) Expression in PBMCs from 116 subjects with various rs2305795 genotypes (mean ± s.e.m., 60 cases and 56 controls; AA, n = 49; AG, n = 51; GG, n = 16). As we observed no direct effect of disease status on P2RY11 expression, subjects were grouped by genotype. The P2RY11 rs2305795 AA genotype resulted in a 53% reduction in P2RY11 expression compared to the rs2305795 GG genotype and was associated with increased risk of narcolepsy. (b) P2RY11 expression by rs2305795 genotype in various immune cell subsets (mean ± s.e.m., n = 7–8 normal controls per genotype category). NK cells, CD56⁺ natural killer cells; B cells, CD19⁺ B cells; Monocy., CD14⁺ monocytes; DCs, myeloid/plasmacytoid dendritic cells. Shown are Bonferroni-corrected one-way analysis of variance (ANOVA) P values.
mitigates this effect, suggesting that immune-cell death in the presence of high ATP is controlled by a balance of activation of multiple purinergic receptors, including P2RY11. A similar survival effect of P2RY11 stimulation by ATP has been reported in neutrophils and endothelial cells following natural killer cell–mediated killing. Comparing cells with various rs2305795 genotypes, we found that the protecting effect of P2RY11 stimulation was less pronounced in subjects carrying the narcolepsy-associated, low expression rs2305795 A allele genotype, as demonstrated by the lower P2RY11–induced survival in PBMCs with this genotype (Fig. 3b). To determine whether these effects varied by immune cell subsets, we used fluorescence activated cell sorting (FACS) and found significant genotype effects in natural killer cells (P = 0.001), CD8+ T cells (P = 0.0007) and CD4+ T cells (P = 0.0009), but not in monocytes or B cells (Fig. 4). This result is in line with the expression data reported in Fig. 2b, although we also found genotype-dependent effects in CD4+ T cells, a population without P2RY11 expression differences, a finding possibly reflecting differential P2RY11 responses in various CD4+ T cell subsets.

How could reduced P2RY11 function, associated with the rs2305795 A allele, be involved in narcolepsy susceptibility? Our results demonstrate clear effects of the polymorphism on immune-cell viability. A possible pathway may thus be modulation by P2RY11 of immune response to a potential infectious narcolepsy trigger, such as Streptococcus pyogenes, or a modulatory effect of the autoimmune process leading to hypocretin cell destruction. Although our results suggest a novel function for P2RY11 in T cells and natural killer cells, relevant effects on other cells not measured here are possible, if not likely. For example, P2RY11 induces thrombospondin-1 secretion and inhibits lipopolysaccharide-stimulated interleukin-12 (IL-12) release in monocyte-derived dendritic cells, an effect that could have a cascade of indirect effects on the immune system. Further, activation of P2RY11 on dendritic cells induces maturation and stimulates IL-8 release. As IL-8 is an important mediator of neutrophil chemotaxis, modulation of the innate immune system could also be involved. Indeed, it has recently been shown that P2RY11 stimulation also modulates natural killer cell chemotaxis in response to CX(3)CL1 and CXCL12 (ref. 30). Finally, direct effects of P2RY11 on hypocretin cell apoptosis or microglial activation are also possible, as virtually nothing is known regarding localized expression and function of P2RY11 in the human brain.

In summary, we report on an association of rs2305795 in the 3’UTR of P2RY11 and narcolepsy. This receptor is highly expressed in CD8+ T cells and natural killer cells and modulates immune cell viability. Additional studies of this receptor in narcolepsy and other autoimmune diseases are warranted.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Figure 3 PBMC cell death induced by ATP was inhibited by the stimulation of P2RY11 and varied by rs2305795 genotype. (a) The effect of ATP on cell viability and the dose response of co-incubation with the P2RY11–specific agonist NF546 and the antagonist NF340 (mean ± s.e.m., n = 7–8 rs2305795 AG control subjects). Overall one-way ANOVA P values are shown, with Tukey’s post test. *, significantly different from control with no ATP, P < 0.01; †, significantly different from treatment with 100 µM ATP but no NF546, P < 0.01; ‡, significantly different from treatment with 100 µM ATP and 100 µM NF546, P < 0.01. (b) Effect of the rs2305795 genotype on the percent of cells rescued from ATP-induced cell death by P2RY11 stimulation. Ten micromolar NF546 has a less potent effect on cell survival after ATP-induced cell death with the rs2305795 AA genotype compared to the rs2305795 GG genotype. Heterozygote subjects fall in between. Mean ± s.e.m., n = 9 subjects in each group.

To further explore how P2RY11 might regulate the immune system, we next quantified receptor expression in CD4+ T cells, CD8+ T cells, CD56+ natural killer cells, CD19+ B cells, CD14+ monocytes and dendritic cells (myeloid and plasmacytoid subsets, that is, a combination of CD11c+, CD141+ and CD304+ cells). P2RY11 expression has previously been shown to be higher in dendritic cells as compared to monocytes and CD4+ T cells, but in that previous study, P2RY11 expression in CD8+ and CD19+ cells was not measured. We found that P2RY11 expression is widespread in immune cells but is notably higher in CD8+ cells compared to dendritic cells (in which expression is 63.0% lower), CD19+ B cells (65.5% lower), CD4+ T cells (75.0% lower) and CD14+ monocytes (84.8% lower). Further, the effect of the disease-associated allele, the A allele of rs2305795, on gene expression was apparent in both CD8+ T cells and natural killer cells (72% and 70% reduction across genotypes; Fig. 2b) but not in other PBMC subtypes. The smaller genotype effect on expression in PBMCs (Fig. 2a) is consistent with a primary effect in CD8+ T cells and natural killer cells, which represent roughly 25% of total PBMCs.

As changes in gene expression do not necessarily translate into functional effects, we next studied whether ATP and P2RY11 have genotype-dependent effects on immune cells. As previously reported, we found that increasing concentrations of ATP induce PBMC cell death (Fig. 3a), an effect likely mediated by P2X7 receptor stimulation. Using the recently developed P2RY11 agonist NF546 and the antagonist NF340, we further discovered that P2RY11 stimulation
Accession codes. mRNA sequences for genes in this study are deposited in the NCBI Nucleotide database under the following accession codes: P2RY11: NM_0025566.4; PPAN: NM_020230.4; EIF3G: NM_0037553; and DNMT1: NM_001130823.1.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Gene expression using RT-PCR. Complementary DNA (cDNA) was synthesized from 200 ng of total RNA (cell subsets) or 400 ng of total RNA (PBMCs) using the High Capacity cDNA Reverse Transcription Kit (#4374966, Applied Biosystems). Gene expression was determined by RT-PCR (ABI 7000, Applied Biosystems) using TaqMan gene expression assays (Applied Biosystems). The probe numbers were P2RY11 (Hs01038858_m1), PPAN (Hs00223350_m1), PPN2-P2RY11 (Hs01568729_m1), EIF3G (Hs00959170_m1), DNMT1 (Hs00945899_m1), CD4 (Hs00181217_m1), CD8 (Hs00233520_m1), CD14 (Hs01649122_g1), CD19 (Hs00174333_m1), CD56 (Hs00941830_m1), CD1c (Hs00233509_m1), B2MA-2/CD303 (Hs00639958_m1), NRPL1/CD304 (Hs00826128_m1), B2M (#4337366F), UBC (Hs00824732_m1), GAPDH (#4337364F) and ACTB (#4337362F), with the latter four serving as endogenous control genes. RT-PCR of CD4, CD8, CD14, CD19, CD56, CD1c, CD303 and CD304 mRNAs were used to verify the purity of each sorted cell fraction and of the samples. Cell fractions had a 30–69,000-fold difference in expression between wanted and unwanted markers, except in CD19 cells, where the differences were 12–24-fold, and in dendritic cells, where the differences were 5.5–27-fold. Relative quantities of target mRNAs were calculated using the comparative threshold method (CT-method), with the geometric mean of UBC, GAPDH and ACTB expression as endogenous controls. The s.d. of the fold changes were calculated as s.d. = $2^{\Delta\Delta Ct}$ × ln2 × SD(ΔCt), with SD(ΔCt) being the s.d. of ΔCt of all samples in the group.

ATP-induced cell death. PBMCs (1 × 10^6 cells/ml) from 12 controls selected on the basis of their rs2305795 genotype (age, sex and gender matched between genotypic groups) were incubated for 1 h or 2 h in the presence of ATP in different concentrations (0.1, 1, 10 and 100 µM) and combined with NF54 (0.1, 1, 10 or 100 µM) and/or NF340 (0.1, 1 or 10 µM) (both compounds were synthesized as described previously). Both compounds were also tested alone (NF54: 0.1, 1, 10 and 500 µM; NF340: 0.1, 1, 10 and 100 µM), and no effect was seen on cell viability except a tendency towards a decrease with 1 µM NF54. All cell work was performed using Ultra-Low Attachment plates (24W: #3473, 96W: #7007, Corning Inc.), and care was taken to flush loosely attached cells off the plates for analysis. The cells were counted in a hemocytometer using Trypan blue exclusion of dead and dying cells. All measurements were performed in duplicates. In a second setup, all surviving cells were subsequently analyzed by FACS to determine their immune phenotypes.

FACS analysis of cell phenotypes. The purity of the different cell fractions was checked on a FACSscan using the following antibody combinations (antibodies are from BD Biosciences): (i) αCD14-FITC (#553597) and αCD4-PerCP-Cy5.5 (#560650); (ii) αCD8-FITC (#553566), αCD6-PE (#555156) and αCD3-PerCP; and (iii) αCD9-PE (#555413) and αCD3-PerCP. For analyzing the phenotypes of the PBMCs surviving the ATP treatment, we used a 7-marker panel consisting of: αCD14-FITC (#553597), αCD4-PerCP-Cy5.5 (#560650), αCD3-Pacific Blue (#558117), αCD19-APC (#555415), αCD56-PE (#555156) and αCD8-PE-Cy7 (#5574) and also including an Aqua Amine Live/Dead Cell Stain (L34957, Invitrogen). This analysis was performed on a BD LSRII (BD Biosciences) in duplicates. The data was combined with the cell counts as described above.

Statistical analysis. Genotype data was maintained in our database (Progeny Lab 7). Allelic tests of association were performed using the PLINK software package (v1.06 26, April 2009). Genome-wide association analysis of the original Affymetrix sample has been described previously. When studying multiple ethnic groups or subgroups (for example, Taiwanese, Chinese, Japanese and Korean populations), the Mantel-Haenszel test was used together with the Breslow Day test of homogeneity of the OR. For the statistical analysis of expression data, we used a Student’s t-test, one-way and two-way analysis of variance tests in GraphPad Prism Version 5.00 where appropriate, and general linear regression models in Systat 12 Version 12.00.08, with control for relevant covariates (age, sex and BMI), if significant.