NFKB2 polymorphisms associate with the risk of developing rheumatoid arthritis and response to TNF inhibitors: Results from the REPAIR consortium

Jose Manuel Sánchez-Maldonado1,2, Manuel Martínez-Bueno3, Helena Canhão5, Rob ter Horst5, Sonia Muñoz-Peña1, Ana Moñiz-Diez1, Ana Rodríguez-Ramos4, Alejandro Escudero4, Signe B. Sorensen5,10, Merete L. Hetland11,12, Miguel A. Ferrer13, Bente Glintborg11,12, Ileana Filipescu14, Eva Pérez-Pampín15, Pablo Conesa-Zamora16, Antonio García13, Alfons den Broeder17, Salvatore De Vita18, Svend Erik Jacobsen19, Eduardo Collantes9, Luca Quartuccio10,18, Mihai G. Netea5,6, Yang Li5,7, João E. Fonseca20,21, Manuel Jurado1,2, Miguel Ángel López-Nevo1,2, Marieke J. H. Coenen17, Vibeke Andersen5,10, Rafael Cáñiz1,2,13 & Juan Sainz1,2*

This study sought to evaluate the association of 28 single nucleotide polymorphisms (SNPs) within NFKB and inflammasome pathway genes with the risk of rheumatoid arthritis (RA) and response to TNF inhibitors (TNFi). We conducted a case-control study in a European population of 1194 RA patients and 1328 healthy controls. The association of potentially interesting markers was validated with data from the DANBIO (695 RA patients and 978 healthy controls) and DREAM (882 RA patients) registries. The meta-analysis of our data with those from the DANBIO registry confirmed that anti-citrullinated protein
antibodies (ACPA)-positive subjects carrying the NFKB2^rs105056890^ allele had a significantly increased risk of developing RA (P^Meta_ACPA^ = 0.0006) whereas no significant effect was found in ACPA-negative individuals (P^Meta_ACPA^ = 0.35). An ACPA-stratified haplotype analysis including both cohorts (n = 4210) confirmed that ACPA-positive subjects carrying the NFKB2 allele had an increased risk of RA (OR = 1.39, P = 0.0042) whereas no effect was found in ACPA-negative subjects (OR = 1.04, P = 0.82). The meta-analysis of our data with those from the DANBIO and DREAM registries also revealed a suggestive association of the NFKB2^rs105056890^ SNP with larger changes in DAS28 (OR = 1.18, P = 0.007). Functional experiments showed that peripheral blood mononuclear cells from carriers of the NFKB2^rs105056890^ allele (in LD with the rs1056890, r^2 = 1.00) showed increased production of IL10 after stimulation with LPS (P = 0.0026). These results provide first evidence of a role of the NFKB2 locus in modulating the risk of RA in an ACPA-dependent manner and suggest its implication in determining the response to TNFi. Additional studies are now warranted to further validate these findings.

Rheumatoid arthritis (RA) is a chronic inflammatory disease more frequently diagnosed in females than males, that has a prevalence of about 0.5–1%3. RA perpetuates and amplifies itself through a wide number of molecular mechanisms involving several immune cell types and multiple inflammatory mediators that are released from the damaged tissue4. Although the complexity of inflammatory pathways implicated in RA development and progression remains in part unknown, there are convincing evidences supporting the view that NFKB pathway and its connection with the NLRP3-inflammasome plays a pivotal role in the modulation of the expression of multiple inflammatory genes implicated in RA development5 and drug response or disease progression6.

Activated NFKB has been detected in the synovium of RA patients at both early and late stages of joint inflammation7–8 and once NFKB is activated (for instance, through the interaction of antigen presenting cells and T cells), it triggers two major signaling pathways in the implicated cells: the canonical and the non-canonical NFKB pathway. Whereas the canonical pathway regulates the activation of NFkB1, RELA and c-RELA and leads to rapid but transient NFKB activation, the non-canonical NFKB pathway selectively activates p100-sequestered NFKB members (predominantly NFKB2 p52 and RELB) and produces a long-lasting signaling. Even though a cross-talk between the canonical and non-canonical NFKB pathways has been previously reported, the activation of the canonical NFKB pathway is generally associated with inflammation whereas the induction of the non-canonical NFKB pathway was linked to development processes9. In RA, it is well known that the acute activation of the canonical pathway on antigen presenting cells and T cells quickly leads to the production of a wide range of essential proinflammatory mediators including cytokines (TNFα, IL1α, IL1β, IL2, IL12p40 and IFNγ), chemokines (IL8, CXCL11), immunoreceptors (CD80, CD23, CD48, CD69, IL2R, TNFRs, and CCR5), cell adhesion molecules (ELAM-1, ICAM-1, VCAM-1 and P-selectin) and growth factors (GM-CSF, IGFBP2, and PDGF) that often facilitate synovial hyperplasia by promoting cell proliferation and apoptosis inhibition of RA fibroblast-like synovial cells10. On the contrary, the activation of the non-canonical pathway involves a slow build-up of long-lasting signals that have been implicated in developmental processes including B-cell development11, secondary lymphoid organ development12,13 and osteoclast differentiation14 but also development of myeloid-related CD4+CD8α− dendritic cells and macrophages15, key players in modulating immune responses in RA.

Besides the role of NFKB in the inflammatory process, recent evidences have shown that the NLRP3-inflammasome is a cytosolic multiprotein complex highly expressed in peripheral blood mononuclear cells of RA patients and in the synovial tissues of osteoarthritis patients. The NLRP3 inflammasome is capable of alerting immune system to the presence of tissue damage and to induce the processing of the IL1β, IL18 and IL33 pro-cytokines into biologically active proinflammatory mediators that drive cartilage destruction16. In addition, it has been reported that the presence of mutations in NLRP3-inflammasome-related proteins (CARD8 and NLRP3) predispose to RA17,18 and that genetic variation in this pathway might also modulate inflammatory activity in early stages of the disease and thereby affect disease progression17,18.

Considering the aspects detailed above, but also previous studies suggesting that the NFKB and NLRP3-inflammasome pathways are genetically determined19, we decided to conduct a case-control study to investigate whether 28 single nucleotide polymorphisms (SNPs) within the NFKB1, NFKB2, NFKBIB, IKBKB, GBP6, IRF4, NLRP3, REL, RELA, KLRC1, KLRC1, KLRC4, LOC105376246 (ncRNA), TLR4, TLR5, TLR9, TLR10 and TRAF1 |C5| genes influence the risk of developing RA and the response to TNF inhibitors (TNFi). In addition, we investigated the correlation of selected SNPs with steroid hormone levels and their role in modulating immune responses after stimulation of whole blood, peripheral mononuclear cells (PBMCs) and macrophages with lipopolysaccharide (LPS), phytohemagglutinin (PHA) and Pam3Cys.

Material and Methods

Discovery population. The discovery population consisted of 1194 RA patients and 1328 healthy controls ascertained through the REPAIR consortium (Table 1). RA patients fulfilled the 1987 revised American College of Rheumatology (ACR)20 and the ACR/EULAR 2010 classification criteria21. The study followed the Declaration of Helsinki. Study participants were of European origin and gave their written informed consent to participate in the study, which was approved by the ethical review committee of participant institutions. The Ethics committee of each participant institution approved the study protocol: Virgen de las Nieves University Hospital (2012/89); Santa Maria Hospital-CHLN (CE 877/121.2012); University Clinical Hospital of Santiago de Compostela (2013/156). A detailed description of the discovery population has been reported elsewhere22–24.
RA patient populations

Table 1. Demographic and clinical characteristics of RA patients. Data are means ± standard deviation or n (%). Abbreviations: RF, rheumatoid factor; ACPA: anti-cyclic citrullinated peptide antibodies; DAS28, disease activity score; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs. ND, not determined. *Clinical data for 708 RA patients that were available for genotyping. †RF was available for 1113, 688 and 345 patients in the discovery, DREAM and DANBIO populations, respectively. *ACPA was available for 908, 127 and 535 patients in the discovery, DREAM and DANBIO populations, respectively.

Response to anti-TNF medications. Six hundred and four RA patients treated with TNFi (adalimumab, etanercept, infliximab, golimumab or certolizumab) were included in the drug response analysis of the discovery population. The change in disease activity score (DAS28) at baseline and at 6 months of treatment with TNFi was calculated for each patient. Linear regression analysis adjusted for age and sex was used to determine the association between selected SNPs and changes in DAS28. Subjects with missing values for DAS28 in any of these time points were not included in the analysis.

SNP selection and genotyping. NFκB- and inflammasome-related polymorphisms were selected on the basis of their potential functionality and linkage disequilibrium (LD) but also because of existing studies reporting their significant association with the risk of developing autoimmune and immune-related diseases or response to TNFi25–29. This strategy resulted in the selection of 28 genetic variants within the 17 loci that were genotyped in the discovery population (Table 2). Genomic DNA was extracted from peripheral blood using the Qiagen Mini Kit (Qiagen, CA, USA) or from saliva using standard procedures. Genotyping was carried out using KASPar® assays (LGC Genomics, London, UK) in a 384-well plate format (Applied Biosystems, CA, USA) according to manufacturer’s instructions. Five percent of samples were included as duplicates to ensure high-quality genotyping.

Statistical analysis. The Hardy-Weinberg Equilibrium (HWE) test was performed in the control group by a standard observed–expected chi-square ($\chi^2$). Logistic and linear regression analyses adjusted for age, sex and country of origin were used to assess the main effects of the selected SNPs on RA risk and the response to TNFi respectively. Statistical power was estimated using Quanto software (http://hydra.usc.edu/gxe/). Correction for multiple testing was performed using the Meff method for SNPs genotyped across all populations30. The threshold used for the risk and drug response analyses was 0.0008 (0.05/22 independent markers×3 inheritance models).

Linkage disequilibrium (LD) and haplotype analysis. We performed haplotype frequency estimation and haplotype association analysis adjusted for age, sex and country of origin using SNPstats31 and haplo.stats package in STATA. Haplotype frequencies were determined using the Expectation-maximization (EM) algorithm. Haplotypes were reconstructed using SNPtool and Haploview and block structures were determined according to the method of Gabriel et al.32.
### Table 2. Selected SNPs within NFKB-related genes

| Gene    | Chr. | dbSNP rs# | Nucleotide substitution | Effect-allele | Location     | Reported associations with autoimmune diseases, drug response and/or potential functional role |
|---------|------|-----------|-------------------------|---------------|--------------|------------------------------------------------------------------------------------------------|
| GBP6    | 1    | rs928655  | A/G                     | A             | Intronic     | Associated with etanercept response in moderate-to-severe plaque psoriasis56                        |
| IKBKB   | 8    | rs11986055| A/C                     | A             | Intrinsic    |                                                                                                  |
| IRF4    | 6    | rs1050975 | A/G                     | A             | 3′-UTR/ncRNA| Correlated with white blood cell count44                                                            |
| IRF4    | 6    | rs12203592| C/T                     | T             | Intrinsic    |                                                                                                  |
| IRF4    | 6    | rs1877175 | C/T                     | T             | 3′-UTR/ncRNA|                                                                                                  |
| IRF4    | 6    | rs7768807 | T/C                     | T             | 3′-UTR/ncRNA|                                                                                                  |
| KLRC1   | 12   | rs7301582 | C/T                     | T             | Intrinsic    | Associated with response to anti-TNF therapy in RA patients49                                      |
| KLRC1 | KLRC4 | 12     | rs1049174               | C/G           | C            | 3′UTR/Intrinsic Associated with response to anti-TNF therapy in RA patients49                     |
| KLRC1 | KLRC4 | 12     | rs1154831               | A/C           | A            | Intrinsic/Near gene Lack of association with response to anti-TNF therapy50                      |
| KLRC1 | KLRC4 | 12     | rs2255336               | A/G           | A            | Thre72Ala                                                                                         |
| LOC105376246 | 9 | rs2722824 | A/C                     | A             | Near gene    |                                                                                                  |
| NFKB1   | 4    | rs4648110 | A/T                     | A             | Intrinsic    |                                                                                                  |
| NFKB1   | 10   | rs11574851| C/T                     | T             | Ane698Am     |                                                                                                  |
| NFKB2   | 10   | rs12769316| C/T                     | T             | Near gene    |                                                                                                  |
| NFKB2 | PSD  | 10     | rs1056890               | C/T           | T            | Near gene/3′-UTR                                                                                   |
| NFKRB1  | 19   | rs3136645 | C/T                     | C             | ncRNA        | Associated with response to anti-TNF drugs in RA patients52                                         |
| NLRP3   | 1    | rs4612666 | C/T                     | T             | Intrinsic    | Overall association with the risk of RA at GWAS level53;55;56 Association with RA in ACPA-positive individuals at GWAS level55; Association with early-onset psoriasis56 and autoimmune diseases57 in large candidate gene association studies56 |
| REL     | 2    | rs13031237| G/T                     | T             | Intrinsic    | Associated with susceptibility to Behcet’s disease58                                               |
| REL     | 2    | rs842647  | A/G                     | A             | Intrinsic    |                                                                                                  |
| REL     | 2    | rs13017599| A/G                     | A             | Near gene    | Associated with RA and psoriatic arthritis at GWAS level53;55 and in a candidate gene association study56 |
| RELA    | 11   | rs11820662| C/T                     | T             | Intrinsic    | Eosinophil counts59                                                                                 |
| RELA    | 11   | rs2306365 | A/G                     | A             | Intrinsic    |                                                                                                  |
| RELA    | 11   | rs7119750 | C/T                     | T             | Intrinsic    |                                                                                                  |
| TLR10   | 4    | rs11096957| A/C                     | A             | Ane241His    | Associated with hip osteoarthritis54;55 and effectiveness of biologics for psoriasis treatment at GWAS level56 |
| TLR4    | 9    | rs4986791 | C/T                     | T             | Thr399Ile    | TLR4 lymphocyte 96 antigen complex level51; Associated with RA risk and response to anti-TNF drugs56; Associated with risk of developing inflammatory bowel disease56 |
| TLR5    | 1    | rs5744174 | C/T                     | C             | Phe616Leu    | Associated with response to anti-TNF drugs in RA patients53; Associated with the risk of Crohns disease56; response to anti-TNF treatment57; Associated with response to ustekinumab treatment in psoriasis patients58 |
| TLR9 | TWF2 | 3     | rs187084                | G/A           | T            | Near gene                                                                                         |
| TRAF1   | C5   | rs3761847 | A/G                     | A             | Near gene    | Associated with RA at GWAS level54;55                                                             |

**Replication populations and meta-analyses for RA risk and drug response.** For replication purposes, we genotyped the most promising SNPs associated with RA risk in a cohort of 695 Danish RA patients and 978 healthy controls55. Clinical data from RA patients were collected through the DANBIO registry (The National
Danish Registry for Biological Treatment of Rheumatic Diseases)\textsuperscript{34} and DNA samples were obtained from peripheral blood collected at the Statens Serum Institut (Copenhagen, Denmark), which routinely perform screening for tuberculosis before treatment with biological treatments. Healthy blood donors were recruited in Viborg and Sønderborg (Denmark). In order to replicate the most interesting associations with response to TNFi, we also used genetic data from a genome-wide association study (GWAS) on drug response conducted in 882 Dutch RA patients from the DREAM (Dutch RhEumatoid Arthritis Monitoring) registry. Imputed SNPs reporting potentially interesting overall or ACPA-specific associations with RA risk or drug response were genotyped in a subset of 708 patients. To further validate our results, we also genotyped the most interesting markers associated with drug response in 555 RA patients from the DANBIO registry that were treated with TNFi. A total of 2107 patients were treated with anti-TNF. Demographic and clinical details of the 3 cohorts are included in Supplementary Table 1. The study was approved by the Institutional review board of the Radboud university medical centre and by the Regional Ethics Committee of Central Denmark Region (M-20100153 and S-20120113). All patients provided written informed consent and clinical information was prospectively gathered from the medical records.

To test for genetic association, we conducted a meta-analysis of the discovery data with those from the 2 European registries and the $I^2$ statistic was used to assess statistical heterogeneity between studies. The pooled OR was computed using the random-effect model.

### Functional analysis of the NFKB and inflammasome-related variants.

Cytokine stimulation experiments were conducted in the 500 Functional Genomics (500FG) cohort from the Human Functional Genomics Project (HFGP; \url{http://www.humanfunctionalgenomics.org/}), which was designed to determine the influence of genomic variation on the variability of immune responses. The HFGP study was approved by the Arnhem-Nijmegen Ethics Committee (no. 42561.091.12) and biometric specimens (venous blood) were collected after informed consent was obtained. We assessed whether any of the 28 NFKB and inflammasome-related SNPs correlated with cytokine levels (TNFα, IFN-γ, IL1β, IL1RA, IL6, IL8, IL10, IL17, and IL22) after the stimulation of whole blood, peripheral blood mononuclear cells (PBMCs) or monocyte-derived macrophages from 408 healthy subjects with LPS (1 or 100 ng/ml), PHA (10 μg/ml), and Pam3Cys (10 μg/ml). After log transformation, linear regression analyses adjusted for age and sex were used to determine the correlation of selected SNPs with cytokine expression quantitative trait loci (cQTLs). All analyses were performed using R software (\url{http://www.r-project.org/}). In order to account for multiple comparisons, we used a significant threshold of 0.00025, i.e. 0.05/(22 independent SNPs × 9 cytokines).

Details on PBMCs isolation, macrophage differentiation and stimulation assays have been reported elsewhere\textsuperscript{35–37}. Briefly, PBMCs were washed twice in saline and suspended in medium (RPMI 1640) supplemented with 9 cytokines). PBMC stimulations were performed with 5 × 10⁶ cells/well in round-bottom 96-wells plates (Greiner) for 24 hours in the presence of 10% human pool serum at 37 °C and 5% CO₂. Supernatants were collected and stored in −20 °C until used for ELISA. LPS (100 ng/ml), PHA (10 μg/ml) and Pam3Cys (10 μg/ml) were used as stimulators for 24 or 48 hours. Whole blood stimulation experiments were conducted using 100 μl of heparin blood that was added to a 48 well plate and subsequently stimulated with 400 μl of LPS and PHA (final volume 500 μl) for 48 hours at 37 °C and 5% CO₂. Supernatants were collected and stored in −20 °C until used for ELISA. Concentrations of human TNFα, IFN-γ, IL1β, IL1RA, IL6, IL8, IL10, IL17, and IL22 were determined using specific commercial ELISA kits (PeliKine Compact, Amsterdam, or R&D Systems), in accordance with the manufacturer’s instructions.

Once we examined the correlation of NFKB and inflammasome-related polymorphisms with cytokine levels in our functional experiments, we also used the HaploReg SNP annotation tool (\url{http://www.broadinstitute.org/mammals/haploreg/haploreg.php}) to further investigate the functional consequences of each specific variant. We also assessed whether any of the potentially interesting markers correlated with mRNA expression levels of their respective genes using data from GTex portal (\url{https://www.gtexportal.org/home/}).

### Correlation between steroid hormone levels and NFKB- and inflammasome-related SNPs.

We also measured serum levels of seven steroid hormones (androstenedione, cortisol, 11-deoxy-cortisol, 17-hydroxy progesterone, progesterone, testosterone and 25 hydroxy vitamin D3) in the 500FG cohort, which includes 531 healthy subjects. Steroid hormones were analyzed by Liquid Chromatography Tandem-Mass Spectrometry (LCMSMS) after protein precipitation and solid-phase extraction as described in Ter Horst et al.,\textsuperscript{37} (see also Supplementary Material). Hormone levels and genotyping data were available for a total of 406 subjects.

After log-transform, correlation between steroid hormone levels and NFKB- and inflammasome-related SNPs was evaluated by linear regression analysis adjusted for age and sex. In order to avoid a possible bias, we excluded those subjects that were using oral contraceptives or those subjects in which this information was not known from the analysis. A total of 379 healthy subjects (107 women and 272 men) were finally available for analysis. A Bonferroni significance threshold was set to 0.00033 considering the number of independent SNPs tested (n = 22) and the number of hormones determined (n = 7).

### Results

This study was conducted in a discovery population comprised of 1194 RA patients and 1328 healthy controls. RA patients were slightly older than controls (59.22 ± 12.97 vs. 52.67 ± 8.99) and showed an increased female/male ratio compared to healthy controls (4.10 [959/234] vs. 1.39 [773/555]). Sixty percent of the RA patients presented positive values of anti-citrullinated protein antibodies (ACPA) and the median disease duration was of 17.60 years and the disease activity score 28 (DAS28) calculated at patient recruitment was of 5.74 (Table 1).

### Association of selected SNPs with RA risk.

All SNPs were in Hardy-Weinberg equilibrium in the control group (P > 0.001). Logistic regression analysis adjusted for age, sex and country of origin showed that carriers
per-allele ORACPA overall haplotype analysis that revealed that carriers of the
1.62, \( P \leq 0.05 \) (ORDom-ACPA = 0.86, 95%CI 0.39–1.90, \( P = 0.90 \) and per-allele ORACPA = 1.39, 95%CI 1.06–1.83, \( P = 0.017 \) and per-allele ORACPA = 1.02, 95%CI 0.68–1.52, \( P = 0.93 \); Table 3). On the other hand, we found that seronegative subjects carrying the KLRC1 rs70301432T or KLRK1 rs1050975A/A genotype or the
1.30 (1.04–1.62)§ 0.019 1.51 (1.14–1.99)** 0.003 1.30 (0.91–1.86)** 0.15

| Gene | SNP ID | Chr. | Effect allele | OR (95% CI)** | \( P \) | OR (95% CI)** | \( P \) | OR (95% CI)** | \( P \) |
|------|--------|------|--------------|---------------|------|---------------|------|---------------|------|
| GBP6 | rs928655 | 1 A | 0.94 (0.81–1.08) | 0.37 | 0.88 (0.74–1.04) | 0.14 | 1.08 (0.84–1.38) | 0.54 |
| IKKβ | rs11986055 | 8 A | 0.93 (0.71–1.21) | 0.59 | 1.15 (0.83–1.62) | 0.40 | 0.99 (0.65–1.53) | 0.98 |
| IRF4 | rs1050975 | 6 A | 1.30 (1.04–1.62)** 0.019 1.51 (1.14–1.99)** 0.003 1.30 (0.91–1.86)** 0.15
| IRF4 | rs22203592 | 6 T | 0.97 (0.81–1.18) | 0.79 | 0.99 (0.78–1.24) | 0.92 | 0.83 (0.60–1.17) | 0.29 |
| IRF4 | rs1877175 | 6 T | 1.00 (0.86–1.16) | 0.98 | 0.97 (0.80–1.16) | 0.70 | 1.04 (0.82–1.32) | 0.74 |
| IRF4 | rs7678807 | 6 T | 0.95 (0.83–1.10) | 0.51 | 0.93 (0.78–1.19) | 0.36 | 1.03 (0.82–1.30) | 0.78 |
| KLRC1 | rs7301582 | 12 T | 1.15 (1.00–1.34)** 0.050 1.05 (0.84–1.30)** 0.67 1.56 (1.18–2.09)** 0.002
| KLRC1 | KLRC4 | 12 C | 1.18 (0.99–1.41)** 0.068 1.09 (0.88–1.35)** 0.42 1.38 (1.03–1.84)** 0.031
| KLRC1 | KLRC4 | 11 A | 1.00 (0.86–1.16) | 0.99 | 1.05 (0.88–1.26) | 0.59 | 0.92 (0.71–1.17) | 0.48 |
| KLRC1 | KLRC4 | 12 A | 1.10 (0.94–1.27) | 0.22 | 1.04 (0.87–1.25) | 0.68 | 1.33 (0.99–1.77)** 0.055
| LOC105376246 | rs2722828 | 9 A | 0.96 (0.83–1.10) | 0.53 | 0.93 (0.79–1.10) | 0.41 | 1.08 (0.86–1.36) | 0.50 |
| NFKB1 | rs4648110 | 4 A | 1.28 (0.85–1.93)** 0.23 | 1.65 (1.04–2.63)** 0.031 | 0.86 (0.39–1.90)** 0.90
| NFKB2 | rs11574851 | 10 T | 1.17 (0.93–1.48) | 0.19 | 1.39 (1.06–1.83) | 0.017 | 1.02 (0.68–1.52) | 0.93 |
| NFKB2 | rs12769316 | 10 T | 1.70 (1.04–2.78)** 0.034 1.70 (0.95–3.06)** 0.077 2.53 (1.24–5.14)** 0.011
| NFKB2 | PSD | rs1056890 | 10 T | 0.96 (0.84–1.09) | 0.54 | 0.95 (0.81–1.12) | 0.56 | 1.01 (0.82–1.25) | 0.90 |
| NFKBIB | rs3136645 | 19 C | 1.07 (0.91–1.24) | 0.42 | 1.15 (0.95–1.38) | 0.14 | 0.81 (0.62–1.04) | 0.10 |
| NLRP3 | rs4612666 | 1 T | 1.25 (1.05–1.49)** 0.013 1.29 (1.04–1.60)** 0.020 | 1.18 (0.89–1.56)** 0.26
| REL | rs13031237 | 2 T | 1.16 (0.91–1.48)** 0.24 | 1.15 (0.85–1.53)** 0.36 | 1.48 (1.02–2.15)** 0.040
| REL | rs842647 | 2 A | 1.08 (0.94–1.24) | 0.30 | 1.10 (0.93–1.31) | 0.27 | 1.05 (0.83–1.33) | 0.68 |
| REL | rs13017599 | 2 A | 1.06 (0.93–1.20) | 0.40 | 1.04 (0.89–1.21) | 0.64 | 1.17 (0.95–1.43) | 0.13
| RELA | rs11820062 | 11 T | 0.93 (0.82–1.06) | 0.29 | 0.91 (0.78–1.05) | 0.20 | 1.07 (0.88–1.31) | 0.49 |
| RELA | rs2036356 | 11 A | 1.07 (0.89–1.29) | 0.48 | 1.02 (0.81–1.28) | 0.86 | 1.16 (0.86–1.57) | 0.32 |
| RELA | rs7119750 | 11 T | 1.09 (0.91–1.32) | 0.34 | 1.04 (0.82–1.30) | 0.76 | 1.24 (0.93–1.65) | 0.15 |
| RELB | rs1096957 | 4 A | 1.12 (0.99–1.27) | 0.066 | 1.13 (0.98–1.32) | 0.10 | 1.08 (0.89–1.33) | 0.43
| RELF | rs4986791 | 9 A | 1.17 (0.89–1.54) | 0.25 | 1.15 (0.83–1.60) | 0.40 | 1.00 (0.63–1.58) | 0.99 |
| TRIL | rs5744174 | 1 C | 0.99 (0.87–1.13) | 0.86 | 1.03 (0.88–1.20) | 0.75 | 0.89 (0.72–1.10) | 0.27
| TRIL | rs928655 | 12 A | 0.97 (0.85–1.10) | 0.61 | 0.93 (0.80–1.09) | 0.39 | 1.02 (0.83–1.25) | 0.88
| TRAF1 | IC | | | | | | | |
| TRAF1 | CS | | | | | | | |

Table 3. Overall and ACPA-specific associations of NFKB-related polymorphisms and risk of developing RA (discovery population). Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval. §Estimates calculated according to an additive model of inheritance and adjusted for age, sex and country of origin. Estimates calculated according to a dominant model of inheritance and adjusted for age, sex and country of origin. Estimates calculated according to a recessive model of inheritance and adjusted for age, sex and country of origin. P ≤ 0.05 in bold. Data on anti-ccp was missing in 285 patients.
Table 4. Meta-analysis for the association of NFKB- and inflammosome-related polymorphisms and RA risk in ACPA+ patients. Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval. A random effect model was assumed for the meta-analysis of both cohorts. Estimates calculated according to an additive model of inheritance and adjusted for age and sex. *Estimates calculated according to a dominant model of inheritance and adjusted for age and sex. † Estimates calculated according to a recessive model of inheritance and adjusted for age and sex. P < 0.05 in boldface.

| Gene | SNP ID | Chr. | Effect allele | Discovery population ACPSA RA vs. controls (n = 1971) | Replication DANBIO Registry ACPSA RA vs. controls (n = 1741) | Meta-analysis ACPSA RA vs. controls (n = 3712) |
|------|--------|------|--------------|------------------------------------------------------|------------------------------------------------------|------------------------------------------------------|
|      |        |      |              | OR (95% CI) | P         | OR (95% CI) | P         | OR (95% CI) | P         | OR (95% CI) | P         | F²  |
| GBP6 | rs928655 | 1 A  | 0.88 (0.74–1.04) | 0.14 | 1.24 (0.97–1.58) | 0.079 | 1.03 (0.74–1.44) | 0.85 | 0.024 |
| IKKβ2 | rs11986055 | 8 A  | 1.15 (0.83–1.62) | 0.40 | — | — | — | — | — |
| IRF4 | rs1050975 | 6 A  | 1.51 (1.14–1.99) | 0.003 | 0.93 (0.65–1.32) | 0.68 | 1.12 (0.74–1.93) | 0.45 | 0.035 |
| IRF4 | rs12203592 | 6 T  | 0.99 (0.78–1.24) | 0.92 | — | — | — | — | — |
| IRF4 | rs1877175 | 6 T  | 0.86 (0.75–1.01) | 0.065 | 1.06 (0.84–1.33) | 0.61 | 0.93 (0.77–1.15) | 0.52 | 0.13 |
| IRF4 | rs2766887 | 6 T  | 0.93 (0.78–1.09) | 0.36 | — | — | — | — | — |
| KLRC1 | rs3701582 | 12 T | 1.15 (0.97–1.37) | 0.096 | 0.85 (0.67–1.08) | 0.19 | 1.00 (0.74–1.34) | 1.00 | 0.044 |
| KLRC1 | rs1049174 | 19 C | 1.06 (0.90–1.25) | 0.45 | 0.95 (0.76–1.19) | 0.66 | — | — | — |
| KLRC1 | rs1154831 | 12 A | 1.05 (0.88–1.26) | 0.59 | — | — | — | — | — |
| KLRC1 | rs2255336 | 12 A | 1.04 (0.87–1.25) | 0.68 | — | — | — | — | — |
| LOC105376246 | rs2722824 | 9 A | 0.93 (0.79–1.10) | 0.41 | — | — | — | — | — |
| RELA | rs4648110 | 4 A  | 1.16 (0.97–1.39) | 0.11 | — | — | — | — | — |
| NFKB2 | rs11574851 | 10 T | 1.39 (1.06–1.83) | 0.017 | 1.72 (1.14–2.59) | 0.009 | 1.48 (1.18–1.86) | 0.0006 | 0.40 |
| NFKB2 | rs12769316 | 10 T | 1.70 (0.95–3.06) | 0.077 | 1.91 (0.93–3.92) | 0.080 | 1.78 (1.13–2.80) | 0.013 | 0.81 |
| NFKB2 | rs1056890 | 10 T | 0.95 (0.81–1.12) | 0.56 | — | — | — | — | — |
| NFKBII | rs3136645 | 19 C | 1.15 (0.95–1.38) | 0.14 | — | — | — | — | — |
| NLRP3 | rs4612666 | 1 T  | 1.29 (1.04–1.60) | 0.020 | 1.06 (0.81–1.39) | 0.68 | 1.19 (0.99–1.44) | 0.072 | 0.27 |
| REL | rs1301237 | 2 T  | 1.15 (0.85–1.53) | 0.36 | 1.15 (0.78–1.70) | 0.47 | 1.15 (0.91–1.45) | 0.24 | 1.00 |
| REL | rs842647 | 2 A  | 1.10 (0.93–1.31) | 0.27 | — | — | — | — | — |
| REL | rs13017599 | 2 A | 1.04 (0.89–1.21) | 0.64 | 1.02 (0.83–1.25) | 0.86 | 1.03 (0.91–1.17) | 0.61 | 0.88 |
| RELA | rs11820062 | 11 T | 0.91 (0.78–1.05) | 0.20 | — | — | — | — | — |
| RELA | rs2006365 | 11 A | 1.02 (0.81–1.28) | 0.86 | — | — | — | — | — |
| RELA | rs7119750 | 11 T | 1.04 (0.82–1.30) | 0.76 | — | — | — | — | — |
| TLR10 | rs11096957 | 4 A  | 1.13 (0.98–1.32) | 0.10 | 0.75 (0.60–0.93) | 0.010 | 0.93 (0.62–1.39) | 0.72 | 0.002 |
| TLR4 | rs4986791 | 9 T  | 1.15 (0.83–1.60) | 0.40 | — | — | — | — | — |
| TLR5 | rs5744174 | 1 C  | 1.03 (0.88–1.20) | 0.75 | — | — | — | — | — |
| TLR9 | rs187084 | 3 T  | 0.93 (0.80–1.09) | 0.39 | — | — | — | — | — |
| TRAF1 | rs3761847 | 9 A  | 1.00 (0.86–1.17) | 0.99 | — | — | — | — | — |

This association did not survive multiple testing correction, it pointed to a role of the NFKB2 rs11574851 SNP to confer risk to RA development.

Most importantly, an ACPA-stratified meta-analysis of our data with those from the DANBIO registry also revealed that each copy of the NFKB2 rs11574851T allele conferred an additive risk of developing RA in ACPA-positive subjects (OR Meta ACPA+ = 1.48, 95%CI 1.18–1.86, P = 0.0006) that was not detected in ACPA-negative individuals (Table 4 and Fig. 1). Of note, the association of the NFKB2 rs11574851 SNP with an increased risk of RA remained significant after correction for multiple testing and the direction of the effect was consistent with no significant heterogeneity between cohorts (P I² = 0.40; Fig. 1). The ACPA-stratified meta-analysis of both populations also showed an increased risk of RA in ACPA-positive and ACPA-negative subjects carrying the NFKB2 rs11574851T allele with RA remained significant after correction for multiple testing, these findings supported the notion of a relevant role of the NFKB2 locus in modulating the RA risk. In order to further confirm this hypothesis, we decided to evaluate whether there was an ACPA-specific haplotype that could influence the risk of developing RA. Interestingly, the ACPA-stratified haplotype analysis including both the discovery and DANBIO cohorts also confirmed that ACPA-positive subjects carrying the NFKB2 T haplotype (including the NFKB2 rs11574851T risk allele) had a significantly increased risk of RA (OR Haplotype-ACPA+ = 1.39, 95%CI 1.11–1.74, P = 0.0042) whereas no effect was detected in ACPA-negative individuals (OR Haplotype-ACPA− = 1.04, 95%CI 0.75–1.44, P = 0.82; Table 5). These results again pointed to an ACPA-specific effect of the NFKB2 locus to modulate the risk of RA. No additional overall or ACPA-specific associations were confirmed in the meta-analysis of both cohorts. On the basis of the effect found for the NFKB2 rs11574851 or NFKB2 rs12769316 SNPs on the risk of developing RA, we decided to analyse whether these SNPs might exert their biological function directly through the modulation of NFKB2-mediated immune responses or indirectly through the regulation of steroid hormone levels. To do
that we evaluated if there were any correlation between the \(\text{NFKB2}_{rs11574851}\) and \(\text{NFKB2}_{rs12769316}\) SNPs and levels of 9 cytokines (TNF\(\alpha\), IFN\(\gamma\), IL1\(\beta\), IL1RA, IL6, IL8, IL10, IL17, and IL22) after stimulation of whole blood, PBMCs or macrophages with LPS, PHA or Pam3Cys in a cohort of 408 healthy subjects. Although our functional experiments were well powered, we did not find any significant correlation between the \(\text{NFKB2}_{rs11574851}\) and \(\text{NFKB2}_{rs12769316}\) SNPs and cytokine or steroid hormone levels (data not shown). Although these results might suggest no impact of the \(\text{NFKB2}\) variants in modulating immune responses, it is important to mention that we could not evaluate whether the effect of the \(\text{NFKB2}_{rs11574851}\) and \(\text{NFKB2}_{rs12769316}\) SNPs on the modulation of immune responses could be dependent on ACPA status as the genetic analyses indicate.

**Association of selected SNPs with the response to anti-TNF drugs.** When we evaluated the effect of any of the selected SNPs on the response to TNFi (defined as a change in DAS28 after 6 months of treatment), we found a significant effect of the \(\text{NFKB2}_{rs1056890}\) SNP to modulate the response to TNFi at nominal level \((P < 0.05)\). Thus, each copy of the \(\text{NFKB2}_{rs1056890T}\) allele additively increased the drop in DAS28 by 22% after the treatment with TNFi (per-allele \(OR = 1.22\), 95% CI 1.03–1.44, \(P = 0.025\); Table 6). Importantly, when we attempted to replicate this association through a well-powered meta-analysis of our data from the discovery population with those from the DREAM and DANBIO registries \((n = 2107)\), we could confirm that carriers of the \(\text{NFKB2}_{rs1056890T}\) allele showed a significantly higher improvement in DAS28 after treatment with TNFi \((OR_{meta} = 1.18, 95\% \text{CI} 1.05–1.33, P = 0.0077, \hat{P} = 51.7\%, P_{het} = 0.13; \text{Fig. 2A})\). Although this association did not remain significant after correction for multiple testing and therefore need to be further validated, this finding suggested that the \(\text{NFKB2}_{rs1056890}\) SNP might modulate the response to anti-TNF drugs through the regulation of the \(\text{NFKB2}\)-related immune responses.

In order to test this hypothesis, we assessed whether the \(\text{NFKB2}_{rs1056890}\) SNP was associated with cytokine and steroid hormone levels in the HFGP cohort. Although this SNP was not included in the genome-wide association data available from the HFGP cohort, we could evaluate the association of this marker with cytokine and steroid hormone levels through the analysis of neighbouring SNPs in strong LD with it. Our stimulation experiments were well powered, and we did not find any significant correlation between the \(\text{NFKB2}_{rs1056890}\) and \(\text{NFKB2}_{rs12769316}\) SNPs and steroid hormone levels (data not shown). However, our functional experiments showed that the \(\text{NFKB2}_{rs1056890T}\) allele positively correlated with high levels of IL10, and we showed that the \(\text{NFKB2}\) locus might modulate the response to anti-TNF drugs through the modulation of the \(\text{NFKB2}\)-related immune responses.

**Discussion**

Our data provided, for the first time, evidence that \(\text{NFKB2}\) locus might modulate the risk of RA. The meta-analysis of the data obtained in the discovery population with those from the DANBIO cohort showed a potentially interesting overall association of the \(\text{NFKB2}_{rs11574851}\) SNP with the risk of RA that was further confirmed in an overall haplotype analysis. Most importantly, we found that the effect attributed to the \(\text{NFKB2}\) locus on RA risk depended on the ACPA status. An ACPA-stratified meta-analysis of the discovery and DANBIO populations including 3712 subjects revealed that ACPA-positive subjects carrying the \(\text{NFKB2}_{rs11574851T}\) allele had a significantly increased risk of developing RA whereas no effect was detected in ACPA-negative individuals. Of note, the association of the \(\text{NFKB2}_{rs11574851T}\) allele with an increased risk of RA in ACPA-positive subjects remained significant even after correction for multiple testing and was further confirmed in an ACPA-stratified haplotype analysis that showed that the presence of the \(\text{NFKB2}_{rs11574851T}\) allele was driving the effect of the \(\text{NFKB2}_{rs11574851T}\) haplotype on the risk of RA in ACPA positive subjects but not in ACPA-negative individuals.

The \(\text{NFKB2}\) gene is located on chromosome 10q24 and it encodes for a subunit of the \(\text{NFkB}\) complex \((p/100/p52)\) that is expressed in multiple immune cells and modulates the inflammation. Other important processes involved in the RA pathology such as Th1 immune responses, activation, abnormal apoptosis and osteoclast differentiation and proliferation are also implicated. It is broadly known that RA arises as a consequence of...
the interaction between genetic and environmental factors and that the NFKB pathway plays a central role in determining the onset of the disease and its progression. In addition, it has been reported that the genetic and environmental factors that predispose to RA development are substantially different between ACPA-positive and ACPA-negative subjects. Recent studies have demonstrated, for instance, that the effect attributed to the two major genetic risk factors for RA (shared epitope of the HLA-DRB1 and a SNP on the PTPN22 gene) is clearly dependent on the ACPA status having a more evident effect in ACPA-positive subjects than in those lacking of these antibodies. Furthermore, recent GWAS studies have reported the existence of a completely different genetic component or even a gene-smoking interaction pattern between ACPA-positive and ACPA-negative patients, again suggesting a relevant role of ACPA in determining the onset of the disease. However, up to now, little is known about the effect of ACPA on the control of the NFKB pathway. Interestingly, recent investigations have demonstrated that the treatment of PBMCs-derived macrophages with ACPA induced the activation of the NFKB pathway and subsequently the induction of the NLRP3-inflammasome and the production of pro-inflammatory cytokines. Mechanistically, it was demonstrated that ACPA induces the activation of the NFKB pathway through the induction of the interaction between CD147 and integrin 31 or ATG1B, which in turn activates the downstream Akt/NFKB signalling pathway, resulting in the upregulation of NLRP3 and pro-IL-1β expression and further NLRP3 inflammasome activation. Considering these interesting findings, we decided to assess in the HFGP cohort if there was any correlation between the NFKB2 SNPs and cytokine or steroid hormone levels. Despite the use of a large cohort of healthy subjects from the HFGP cohort, we could not find any significant correlation between the NFKB2 rs11574851 and NFKB2 rs12769316 SNPs and cytokine or steroid hormone levels. Although these results suggested that these variants might not exert their effect on RA risk through the modulation of NFKB2 or steroid hormone-mediated immune responses, we could not rule out the possibility of a true effect of these variants on the immune response as their effect might depend on the presence of ACPA (as suggested by our genetic data) or even specific haplotypes. In line with this hypothesis, in silico analysis using Haploreg data showed that the NFKB2 rs11574851 and NFKB2 rs12769316 SNPs mapped among histone marks in multiple primary T helper naïve and memory cells and primary B cells from peripheral blood and they were predicted to act as enhancers in T helper memory cells and to change motifs for Po6fu1, AP-4, CEBPB, Mef2 and RP58. Even though these data supported the idea of a role of NFKB2 variants in modulating immune responses, we think that additional experiments are still needed to determine whether ACPA or specific haplotypes are factors involved in modulating the effect of the NFKB2 locus on the risk of RA.

Besides the role of the NFKB2 locus in determining the risk of RA, this study also showed a noticeable impact of the NFKB2 gene in the modulation of the response to TNFi. In particular, the meta-analysis of the discovery population with data from the DREAM and DANBIO registries, including 2107 RA patients, showed that carriers of the NFKB2 rs1056890T allele had an improvement in DAS28 after treatment with TNFi. We found that the direction of the effect of the NFKB2 rs1056890 SNP on drug response was consistent across populations and that the effect was statistically significant in 2 of the 3 populations analysed. Although at this point it tempting to speculate that this SNP constitutes a biomarker for good response to TNFi in RA patients that might help to design more individualized treatment strategies, the association did not remain significant after correction for multiple testing and, therefore, need to be confirmed in independent populations. Mechanistically, we found that the presence of neighbouring genetic markers in strong LD with the NFKB2 rs1056890 SNP were associated with increased levels of IL10, suggesting that the NFKB2 locus might be implicated in modulating IL10-mediated immune responses. Although the association of the NFKB2 rs1056890 SNP with IL10 levels neither survive correction for multiple testing, our results were in agreement with previous studies demonstrating that NFKB2 unlikely NFKB1 is implicated in the control of antigen presenting cell function and not in the activation of T and B cells. Likewise, recent studies have also identified genetic polymorphisms within the NFKB pathway as genetic biomarkers for response to TNFi in RA but also other autoimmune diseases, which further supported our hypothesis suggesting a key role of the NFKB2 gene in modulating the response to TNFi. In addition, in silico tools such as Regulome showed that the rs1056890 SNP has a score of 4, which means that this polymorphism could affect transcription factor affinity and DNase peak. Using haploreg it was also suggested that the NFKB2 rs1056890 SNP might play a role in modulating immune responses as it mapped among histone marks in primary T helper naïve and T helper memory cells, T regulatory and primary NK cells and it was predicted to alter binding motifs for NRSF, Sin3Ak-20 and PLAG1. These transcription factors have been implicated in bone-related diseases and their activation results in up-regulation of multiple target genes including immune-related genes such as macrophage colony stimulating factor (MCSF) and insulin growth factor (IGF)-2.

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**Table 5.** Overall and ACPA-stratified haplotype association analysis for RA. Estimates calculated according to a dominant model. Minimum haplotype frequency was set at 0.01. P < 0.05 in bold.

| NFKB2 | rs11574851 | rs12769316 | 999999 | Freq | RA patients (n = 4210) OR (95% CI) | P | Freq | ACPA-positive patients (n = 3117) OR (95% CI) | P | Freq | ACPA-negative patients (n = 2688) OR (95% CI) | P |
|-------|------------|------------|-------|------|-----------------------------------|---|------|-----------------------------------------------|---|------|-----------------------------------------------|---|
| 1     | C          | C          | 0.8181| 1.00 | —                                  | — | 0.8224| 1.00                                          | — | 0.8295| 1.00                                          | — |
| 2     | C          | T          | 0.1139| 1.14 (0.99–1.31) | 0.066 | 0.1706| 1.10 (0.92–1.32) | 0.30 | 0.1088| 1.10 (0.92–1.32) | 0.91 |
| 3     | T          | T          | 0.0571| 1.18 (0.98–1.42) | 0.13 | 0.0530| 1.39 (1.11–1.74) | 0.0042| 0.0538| 1.04 (0.75–1.44) | 0.82 |
| 4     | T          | C          | 0.0109| 2.21 (1.37–3.56) | 0.0011| —      | —                                              | — | —      | —                                              | — |
| Gene   | SNP ID    | Chr | Effect allele | Discovery population (n = 604) | Replication DREAM registry (n = 882) | Replication DANBIO Registry (n = 621) | Meta-analysis (n = 2107) |
|--------|-----------|-----|---------------|---------------------------------|--------------------------------------|--------------------------------------|--------------------------|
| GBP6   | rs928655  | 1   | A             | 1.05 (0.87–1.27)               | 0.61                                 | 0.90 (0.80–1.00)                     | 0.058 ND                 |
| IKBKB  | rs11986055| 8   | A             | 0.74 (0.48–1.11)               | 0.14                                 | 0.85 (0.66–1.07)                     | 0.17 0.94 (0.64–1.39) 76 |
| IRF4   | rs1050975 | 6   | A             | 0.95 (0.72–1.24)               | 0.69                                 | 0.99 (0.83–1.17)                     | 0.87 1.24 (0.94–1.65) 13 |
| IRF4   | rs12203592| 6   | T             | 1.01 (0.77–1.33)               | 0.93 ND                              | ND                                    | 1.03 (0.90–1.18) 67    |
| IRF4   | rs1877175 | 6   | T             | 1.09 (0.90–1.33)               | 0.37                                 | 0.92 (0.82–1.13)*                    | 0.15 0.90 (0.75–1.09) 30 |
| IRF4   | rs7768807 | 6   | T             | 0.86 (0.72–1.03)               | 0.10                                 | 1.04 (0.93–1.16)*                    | 0.52 0.96 (0.86–1.07) 47 |
| KLRC1  | rs7301582 | 12  | T             | 1.05 (0.86–1.27)               | 0.62                                 | 1.00 (0.88–1.12)                     | 0.04 0.99 (0.80–1.22) 92 |
| KLRK1 | KLRC4     | 12  | C             | 1.08 (0.91–1.29)               | 0.37                                 | 0.96 (0.86–1.08)                     | 0.53 1.07 (0.90–1.27) 47 |
| KLRK1 | KLRC4     | 12  | A             | 0.89 (0.73–1.10)               | 0.28                                 | 1.05 (0.93–1.19)*                    | 0.40 ND ND               |
| KLRK1 | KLRC4     | 12  | A             | 1.09 (0.90–1.33)               | 0.38                                 | 1.01 (0.89–1.16)                     | 0.81 1.04 (0.93–1.15) 54 |
| LOC105376246 | rs2722824 | 9   | A             | 1.03 (0.86–1.23)               | 0.77                                 | 0.94 (0.85–1.05)                     | 0.32 ND ND               |
| NFKB1  | rs4648110 | 4   | A             | 1.07 (0.88–1.29)               | 0.51                                 | 1.00 (0.89–1.13)*                    | 0.95 ND 1.02 (0.92–1.13) 71 |
| NFKB2  | rs11574851| 10  | T             | 0.97 (0.73–1.29)               | 0.83                                 | 0.92 (0.72–1.18)*                    | 0.53 0.78 (0.57–1.06) 11 |
| NFKB2  | rs12769316| 10  | T             | 0.92 (0.75–1.13)               | 0.43 ND                              | ND                                    | 0.86 (0.70–1.06) 16    |
| NFKB2 | PSN       | rs1056890| 10 | T             | 1.22 (1.03–1.44)               | 0.025                                | 1.08 (0.98–1.19)                     | 0.11 1.31 (1.10–1.57) 00030 1.18 (1.05–1.33) 00077 0.12 |
| NFKB2B | rs3136645 | 19  | C             | 0.90 (0.73–1.11)               | 0.34 ND                              | ND                                    | ND ND ND ND ND ND ND ND |
| NLRP3  | rs4612666 | 1   | T             | 1.05 (0.87–1.25)               | 0.62                                 | 1.20 (1.05–1.37)*                    | 0.006 0.96 (0.80–1.14) 62 |
| REL    | rs13031237| 2   | T             | 1.07 (0.91–1.26)               | 0.40                                 | 1.03 (0.94–1.14)                     | 0.49 1.08 (0.92–1.28) 36 |
| REL    | rs842647  | 2   | A             | 1.03 (0.86–1.24)               | 0.72                                 | 0.96 (0.87–1.06)                     | 0.45 ND ND               |
| REL    | rs13017599| 2   | A             | 1.07 (0.91–1.27)               | 0.41                                 | 1.03 (0.94–1.14)                     | 0.50 1.03 (0.86–1.21) 78 |
| RELA   | rs11820062| 11  | T             | 1.07 (0.90–1.26)               | 0.45                                 | 0.92 (0.84–1.01)*                    | 0.081 ND ND             |
| RELA   | rs2306365 | 11  | A             | 0.91 (0.71–1.16)               | 0.45                                 | 1.19 (1.03–1.37)                     | 0.021 ND ND             |
| RELA   | rs719750  | 11  | T             | 0.93 (0.73–1.18)               | 0.54 ND                              | ND                                    | ND ND ND ND ND ND ND ND |
| TLR10  | rs11996957| 4   | A             | 1.00 (0.85–1.19)               | 0.98                                 | 0.99 (0.89–1.09)                     | 0.80 ND ND               |
| TLR4   | rs4986791 | 9   | T             | 1.15 (0.78–1.70)               | 0.47                                 | 1.18 (0.98–1.41)*                    | 0.077 ND ND             |
| TLR5   | rs5744174 | 1   | C             | 0.99 (0.83–1.17)               | 0.89 ND                              | ND                                    | ND ND ND ND ND ND ND ND |
| TLR9 | TWF2 | rs187084 | 3   | T             | 1.02 (0.86–1.21)               | 0.81                                 | 0.98 (0.88–1.08)*                    | 0.67 ND ND               |
| TRAF1 | C5       | rs3761847| 9  | A             | 1.08 (0.91–1.29)               | 0.37                                 | 1.05 (0.95–1.16)                     | 0.33 ND ND               |

Table 6. Meta-analysis for the association of NFKB-related polymorphisms and relative change of DAS28 score (ADAS28). Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval. A random effect model was assumed for the meta-analysis of both cohorts. *Estimates calculated according to an additive model of inheritance and adjusted for age, sex and country of origin (or age and sex in the replication stages). Estimates based on imputed genotypes. P < 0.05 in boldface. No significant heterogeneity (heterogeneity chi-squared) was observed in any meta-analysis reported above.

Figure 2. Meta-analysis of the association of the NFKB2 rs1056890 SNP with response to TNFi [A] and with correlation with higher levels of IL10 after stimulation of PBMCs (n = 377) with LPS [B]. [A] Association estimates according to a random effect model. Pmeta = 0.00077. [B] Correlation with IL10 was analysed using genotype data of the NFKB2 rs1005044 SNP, a marker in strong LD with the rs1056890 (r² = 1.00).
Conclusions
In conclusion, this study reports, for the first time, a consistent association of the NFKB2 intronic polymorphism and NFKB2 haplotype with an increased risk of developing RA in ACPA-positive subjects. In addition, this study suggests a possible role of the NFKB2 locus in the modulation of the response to TNFi. Mechanistically, the functional experiments in the 500FG cohort suggested that the effect attributed to the NFKB2 gene in the modulation of the response to TNFi might be mediated by IL10-mediated immune responses. However, additional studies are still warranted to shed light into the biological processes that link NFKB2 SNPs and RA risk and drug response.

Data availability
All data used in this project have been meticulously cataloged and archived in the BBMRI-NL data infrastructure (https://hfgp.bbmri.nl/) using the MOLGENIS open source platform for scientific data46. This allows flexible data querying and download, including sufficiently rich metadata and interfaces for machine processing (R statistics, REST API) and using FAIR principles to optimize Findability, Accessibility, Interoperability and Reusability46. Genetic data from the discovery and DANBIO populations can be accessed at ftp.genyo.es and data from the DREAM registry are available at https://www.synapse.org/#!Synapse:syn3280809/wiki/194735 and https://www.synapse.org/#!Synapse:syn3280809/wiki/194736.

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Author contributions

R. Caliz and J. Sainz designed the study and drafted the manuscript. J. Sanchez-Maldonado, A. Moñiz-Díez, S. Muñoz-Peña, and A. Rodriguez-Ramos were responsible for genotyping. H. Canhão, A. Escudero, S.B. Sorensen, M.L. Hetland, M.A. Ferrer, B. Glintborg, I. Filipescu, E. Pérez-Pampin, P. Conesa-Zamora, A. Garcia, A. den Broeder, S. de Vita, S.E.H. Jacobsen, E. Collantes, L. Quartuccio, J.E. Fonseca, M. Jurado, M.A. López-Nevot, M.J.H. Coenen, V. Andersen, R. Caliz and J. Sainz coordinated the sample collection and H. Canhão, I. Filipescu, A. García, and M.A Ferrer were involved in the records review and data acquisition. M. Martínez-Bueno and J. Sainz performed the analysis of functional data. All authors contributed to and approved the final version of the manuscript.

Competing interests

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Additional information

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Correspondence and requests for materials should be addressed to J.S.

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