always a possibility in pharmacogenetic studies, especially those conducted in different countries; and (3) ethnic differences, including different patterns of LD between the underlying causative allele (which is as yet unknown) and marker SNPs tested in our study. We did observe subtle differences in local patterns of LD between Asians and Europeans using genetic data from the 1000 Genomes Project (Figure S5). We note that the rs6427528 minor allele A has a frequency of ~5–10% in European and East Asian populations, and ~50% in the African YRI population (HapMap2 and 1000 Genomes); therefore, it may be of interest to test African American samples in replication.

What are the options for increasing sample size in pharmacogenetic studies, thereby providing an opportunity to replicate our *CD84* genetic and expression findings? While it might seem trivial to collect more samples through traditional registries, this is extremely challenging for phenotypes pertaining to treatment efficacy. To underscore this point, we highlight our study design, where we organized samples and clinical data from 16 different collections across 7 different countries in order to obtain the samples for the current study. Going forward, non-traditional strategies to collect biospecimens linked with clinical data (e.g., online registries, electronic medical records) may be required to achieve clinical collections of sufficient size to discover pharmacogenomic predictors of efficacy.

In conclusion, we conducted the largest GWAS to date for response to anti-TNF therapy in RA patients. Our genetic and expression data suggest that *CD84* genetic variants and/or expression levels could be developed as predictive biomarkers for etanercept treatment response in RA patients of European ancestry.

Methods

Samples and clinical data

All patients met 1987 ACR criteria for RA, or were diagnosed by a board-certified rheumatologist. In addition, patients were required to have at least moderate disease activity at baseline (DAS>3.2). All patients gave their informed consent and all institutional review boards approved of this study. A total of 13 collections from across 5 countries were included in GWAS [11,12,13,22]: Autoimmune Biomarkers Collaborative Network (ABCoN) from the U.S. (N = 79); the Genetics Network Rheumatology Amsterdam (GENRA, N = 53); the Dutch Behandelstrategieen voor Rheumatoide Arthritis (BeSt, N = 85); the U.K. Biological in Rheumatoid arthritis Genetics and Genomics Study Syndicate (BRAGGSS, N = 140); the U.S. Brigham Rheumatoid Arthritis Sequential Study (BRASS, N = 55); the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA, N = 298); the Immunex Early Rheumatoid Arthritis study (eRA N = 57); the Swedish Karolinska Institutet study (KI, N = 77); the Netherlands collection from Leiden University Medical Center (LUMC, N = 43); and the U.S. Treatment of Early Aggressive RA (TEAR, N = 109). We refer to these collections as the American College of Rheumatology Research and Education Foundation (REF) collection, as funding for GWAS genotyping was provided by the "*Within Our Reach*" project. We included additional samples from BRAGGSS (N = 595) [12]; the Dutch Rheumatoid Arthritis Monitoring registry (DREAM) in the Netherlands, and the ApotheekZorg (AZ) database (which facilitates the Dutch distribution of adalimumab; N = 880) [23,24], together referred to as DREAM; and the French Research in Active Rheumatoid Arthritis (ReAct, N = 272) [25].

Additional samples were collected for replication of SNPs in the *1q23* locus. These included the Rheumatic Diseases Portuguese Register (Reuma.pt, N = 378) from the Portuguese Society of Rheumatology (SPR), which captures more than 90% of patients treated with biological therapies and managed in rheumatology departments across Portugal [26]. Additional replication samples (N = 374) of East Asian ancestry were included from the IORRA and Kyoto University Hospital registries, part of the Japanese Genetics and Allied research in Rheumatic diseases Networking consortium (GARNET) [27].

Clinical data were collected in each cohort, including disease activity scores at baseline and at least one time point after treatment, gender, age, methotrexate use, as well as autoantibody status (RF or CCP). The composite disease activity scores for 28 joints (DAS28) included laboratory values for erythrocyte sedimentation rate (ESR) for most samples and C-reactive protein (CRP) for 191 samples in the REF collection (ABCoN, BRASS and eRA cohorts). DAS28 values were available at baseline and at 3–12 months after initiating anti-TNF therapy. Our primary phenotype was defined as ΔDAS = baseline DAS - end DAS, and responder status was also determined according to EULAR criteria for start and end DAS [15]. Clinical variables were assessed for association with phenotype in multivariate linear or logistic regression models for both the ΔDAS and EULAR responder-status phenotypes. Clinical variables that were significant in these analyses were retained as covariates in genetic association tests, except for methotrexate co-therapy. Including a covariate for methotrexate co-therapy reduced sample size substantially due to missing clinical data, so results were compared for our primary analysis and a secondary analysis with the covariates (and with reduced sample size) and the results were verified not to be impacted (not shown).

Genotyping and data processing

A total of eleven genotyping batches were processed separately. (1) BRASS samples were genotyped using Affymetrix 6.0 chip [28]; (2) WTCCC samples were genotyped on Affymetrix 500K chip [12]. All other cohorts were genotyped using Illumina platform arrays (see Table 1). Our American College of Rheumatology Research Education Fund (REF) collection was made up of smaller cohorts from throughout North America and Europe, including BRASS samples. Also included in REF: (3) ABCoN [13] and (4) EIRA [29] were separately genotyped on the Illumina 317K genotyping array; (5) eRA on the Illumina 550K chip; and (6) GENRA, BeSt, BRAGGSS (a subset of N = 53 samples), KI and LUMC were genotyped in one batch, and (7) BRAGGSS (N = 87) and TEAR were genotyped in a second batch, both using Illumina 660k chips, at the Broad Institute (8–10). DREAM and AZ samples were genotyped in three batches, one on 550K chip and two on 660K chips (manuscript in preparation), and (11) ReAct samples were genotyped on Illumina OmniExpress chips. Quality control (QC) filtering was done in each genotyping batch, including filtering individuals with >5% missing data, and filtering SNPs with >1% missing data, minor allele frequency (MAF) <1% and Chi-squared test of Hardy Weinberg equilibrium $P_{HWE}<10^{-5}$. We then used individual-pairwise identity-by-state estimates to remove occasional related and potentially contaminated samples. Data processing and QC were performed in PLINK [30]. Principal Components Analysis (PCA) was performed using EIGENSTRAT [31] (default settings) on the combined dataset using 20,411 SNPs genotyped across all datasets. Ethnicity outliers including all individuals of non-European decent were identified and removed, and the first three eigenvectors were used as covariates in GWAS.

Imputation was conducted on each of eleven datasets separately, using the IMPUTE v1 software [32] and haplotype-phased

HapMap Phase 2 (release 22) European CEU founders as a reference panel. Imputation of BRASS and EIRA was previously reported [28,33], and we followed the same imputation procedures for the remaining datasets. Imputation yielded posterior genotype probabilities as well as imputation quality scores at SNPs not genotyped with a minor allele frequency ≥1% in HapMap CEU. We removed imputed SNPs with imputation 'info' scores <0.5 or MAF <1% in any of the datasets.

Expression profile and eQTL data

Gene expression levels were quantified using mRNA derived from peripheral blood mononuclear cells (PBMCs) using Affymetrix Human Genome U133 Plus 2.0, for 255 multiple sclerosis patients in the Comprehensive Longitudinal Investigation of MS at the Brigham and Women's Hospital [34], either untreated (N = 83) or treated with interferon-beta (N = 105) or glatiramer acetate (N = 67). The raw intensity values were subject to quality control based on the recommended pipeline available in the simpleaffy and affyPLM R Bioconductor packages, and were then normalized using GCRMA (N = 228). The data are available on the Gene Expression Omnibus website (GSE16214). Expression levels for 17,390 probes mapping to 9,665 Ensembl transcripts were adjusted for confounding factors including age, gender, drug and batch using principle components and Bayesian factor analysis [35], and used in eQTL association analyses. Genotype data were collected on the Affymetrix 550K GeneChip 6.0 platform as a part of a previously published study [36]. Allelic dosages from imputed data (HapMap Phase II CEU samples; >2 million SNPs, MACH imputation quality >0.1 and MAF>=0.05) were used for association analysis. *Cis*-eQTLs were identified +/−1 Mb of transcription start sites (TSS) in the *1q23* locus region. Significance was evaluated by 10,000 permutations per gene, and false discovery rates were calculated based on cis-eQTL analyses in the total of 9,665 genes [37].

Additional expression profile data were available for subsets of samples that were part of two cohorts in our GWAS. Expression data from patients enrolled in the BRASS registry have been previously published [38]. Expression data were collected on Affymetrix Gene Chip U133 Plus 2 microarrays. BRASS patients had either cross-sectional expression data (n = 132, assayed at the time the patient was enrolled in BRASS) or pre- and post-treatment expression data (n = 17 samples, 8 treated with etanercept). Of these, n = 87 patients had expression and GWAS data. For patients with pre- and post-treatment data, we used the "baseline" pre-treatment expression data for cross-sectional analysis. In ABCoN, 65 RA patients (n = 23 treated with etanercept) had both pre- and post-treatment expression data, as well as ΔDAS clinical data [39], and n = 45 patients had expression and GWAS data. As with BRASS, we use the "baseline" pre-treatment expression data for cross-sectional analysis. For ABCoN expression profile data were collected on Illumina Human WG6v3 microarrays and were quantile normalized according to Illumina recommended protocols. Within both BRASS and ABCoN, expression data were normalized to the mean and standard deviation within each collection. For prospective analyses of expression data and ΔDAS, we combined BRASS and ABCoN to include 31 etanercept-treated patients and 78 anti-TNF-treated patients.

Statistical analyses

In our primary GWAS analysis, we tested each SNP for association with ΔDAS using linear regression adjusted for baseline DAS and the first 3 PCA eigenvectors in each collection. In our secondary GWAS analysis, we modeled SNPs predicting EULAR good response *versus* EULAR non-response using logistic regression, again adjusting for start-DAS value and the first three eigenvectors. Association analysis was done using SNPTEST [32] assuming an additive genetic model. Genomic control λ_{GC} values [40] for genotyped SNPs only and all SNPs were calculated, and no inflation or deflation was observed in the distributions of association test results. We then conducted inverse variance-weighted meta-analysis to combine results across the four datasets, and conducted Cochran's Q tests for heterogeneity using the β coefficients [41]. We further divided samples into 3 subsets according to drug (etanercept, infliximab or adalimumab). GWAS analysis for each group followed the same analysis procedure. Meta-analysis and heterogeneity tests were conducted using SAS. Expression analyses utilized linear regression or Spearman rank correlation, also using SAS. We tested for effects of cohort, age, gender and concurrent methotrexate, and results are shown using significant covariates as indicated.

Supporting Information

Figure S1 Quantile–quantile (QQ) plots for ΔDAS and response analysis, with genomic control λ_{GC} values.
(TIF)

Figure S2 GWAS results for the good response versus non-response phenotype. Shown are strengths of association (−Log10 P-value) for each SNP versus position along chromosomes 1 to 22. A) All samples (n = 1,708). B) Etanercept-treated patients (n = 472). C) Infliximab-treated patients (n = 599). D) Adalimumab-treated patients (n = 636).
(TIF)

Figure S3 Forest plot of replication results for the CD84 SNP rs6427528, in patients treated with anti-TNF drugs other than etanercept (infliximab & adalimumab).
(TIF)

Figure S4 Forest plot of CD84 result in patients treated with etanercept, subset by all collections.
(TIF)

Figure S5 Patterns of linkage disequilibrium (LD) at the CD84 locus in HapMap. Shown patterns of LD for CEU (top panel) and CHBJPT (bottom panel).
(TIF)

Table S1 Sample information for each of thirteen clinical batches.
(DOC)

Table S2 Clinical multivariate model for the ΔDAS phenotype.
(DOC)

Table S3 GWAS results for all SNPs achieving $P<10^{-6}$ from any analysis.
(XLS)

Table S4 Sample and clinical data summary for replication samples.
(DOC)

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Author Contributions

Conceived and designed the experiments: JC EAS RMP. Performed the experiments: JC EAS RMP. Analyzed the data: JC EAS RMP. Contributed reagents/materials/analysis tools: SS CM DD GT TR MUM HC KI CT YO SW JA HY SM AT KO FM TM NG MK AWM JDI AGW KLH MH MED P-PT JBAC IEvdH-B GJW PLCMvR MvdL H-JG NAS CFA TWJH REMT RPK SLB LAC LWM JEF NdV BES PLDJ SR MEW PKG XM AB LP MJHC EWK. Wrote the paper: JC EAS RMP. All authors reviewed and approved the manuscript.

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