TNF receptor 1 genetic risk mirrors outcome of anti-TNF therapy in multiple sclerosis

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Although there has been much success in identifying genetic variants associated with common diseases using genome-wide association studies (GWAS), it has been difficult to demonstrate which variants are causal and what role they have in disease. Moreover, the modest contribution that these variants make to disease risk has raised questions regarding their medical relevance. Here we have investigated a single nucleotide polymorphism (SNP) in the TNFRSF1A gene, that encodes tumour necrosis factor receptor 1 (TNFR1), which was discovered through GWAS to be associated with multiple sclerosis (MS)1,2,3, but not with other autoimmune conditions such as rheumatoid arthritis4, psoriasis5 and Crohn’s disease6. By analysing MS GWAS1–3 data in conjunction with the 1000 Genomes Project10 we provide genetic evidence that strongly implicates this SNP, rs1800693, as the causal variant in the TNFRSF1A region. We further substantiate this through functional studies showing that the MS risk allele directs expression of a novel, soluble form of TNFR1 that can block TNF. Importantly, TNF-blocking drugs can promote onset or exacerbation of MS7–11, but they have proven highly efficacious in the treatment of autoimmune diseases for which there is no association with rs1800693. This indicates that the clinical experience with these drugs parallels the disease association of rs1800693, and that the MS-associated TNFR1 variant mimics the effect of TNF-blocking drugs. Hence, our study demonstrates that practical experience can be informed by comparing GWAS across common autoimmune diseases and by investigating the functional consequences of the disease-associated genetic variation.

The largest MS GWAS1 reports rs1800693 as the most associated SNP in the TNFRSF1A region by over two orders of magnitude (odds ratio for risk allele = 1.12 (1.11–1.14), P = 4.1 × 10−14). To assess whether this SNP is mainly driving the association we examined the haplotype structure across the region in 379 individuals of European ancestry using whole-genome sequence from the 1000 Genomes Project and we performed statistical imputation into a UK cohort of 1,853 MS patients and 5,174 controls4. Among genotyped SNPs the strongest signal is seen at rs1800693, and the variants in strongest association with this SNP were also genotyped in the study. Statistical imputation revealed no other variant with stronger association to MS within the region, including the previously reported nonsynonymous SNP rs4149584 (Supplementary Fig. 1), and analysis of association after controlling for the effect of rs1800693 removed almost all of the signal (Fig. 1a). These observations all support variation at rs1800693 as being primarily responsible for the MS association in the TNFRSF1A region.

To further substantiate the causality of rs1800693, we next sought to investigate the functional consequences of the variation at this SNP. As rs1800693 is proximal to the TNFRSF1A exon 6/intron 6 boundary, we proposed that it may influence splicing of TNFR1 exon 6 (ref. 13). In an in vitro minigene splicing assay, only the risk ‘G’ allele resulted in skipping of exon 6 (Fig. 1b). In primary human immune cells we found that the presence of the risk allele correlated with increased expression of transcripts lacking exon 6, with the gene dosage effect observed being consistent with the genetic effect on disease risk (Fig. 1c and Supplementary Fig. 2). Moreover, these transcripts are translated in the primary immune cells, as binding of polysomies to these transcripts demonstrated active translation (Supplementary Fig. 3). As full-length TNFR1 (FL-TNFR1) expression did not vary in a genotype-dependent fashion (Supplementary Figs 4 and 5), this indicates that the effect of rs1800693 may be specifically mediated by its influence on the generation of a functional protein isoform lacking exon 6 (∆6-TNFR1), and we therefore investigated the properties of this molecule.

TNFR1 exon 6 skipping results in a frameshift and a premature stop codon, which translates into a protein comprising only the amino-terminal 183 amino acids of FL-TNFR1, followed by a novel 45 amino acid sequence, as confirmed by tandem mass spectrometry (Supplementary Fig. 6). Consequently, ∆6-TNFR1 lacks the extracellular carboxy-terminal portion of the fourth cysteine-rich domain (CRD) of FL-TNFR1, the transmembrane domain, and the intracellular region that is essential for proper subcellular localization. Whereas FL-TNFR1 localizes to the Golgi apparatus, ∆6-TNFR1 demonstrated a more diffuse intracellular distribution (Fig. 2), consistent with the absence of the Golgi-retention motif. Nevertheless, we observed partial subcellular colocalization of FL-TNFR1 and ∆6-TNFR1 (Supplementary Fig. 7), suggesting the potential for an interaction between the two isoforms. As ∆6-TNFR1 retains the pre-ligand assembly domain (PLAD/CRD1), required for TNFR1 trimerization at the cell surface, it could exert a functional effect on FL-TNFR1 by associating with it to form heteromers that would therefore have modified properties. To investigate the existence of such an interaction, we used FL-TNFR1 and ∆6-TNFR1 proteins fused at their carboxy termini with fluorescent or luciferase proteins in fluorescence and bioluminescence detection (Supplementary Fig. 8). This demonstrated that ∆6-TNFR1 associates neither with the full-length protein nor with itself (Fig. 2c and Supplementary Fig. 8). Consistent with this and with the lack of transmembrane and cytoplasmic domains, ∆6-TNFR1 was not observed at the surface of transfected cells (Fig. 2d). Hence, as ∆6-TNFR1 cannot interact with FL-TNFR1, it
is unlikely to have a direct impact on the latter, intracellularly or at the cell surface.

To assess whether Δ6-TNFR1 has some intracellular function, regardless of its inability to associate with FL-TNFR1, we investigated TNFR1-mediated signalling in Δ6-TNFR1-transfected cells. As predicted by the absence of a death domain, which is necessary for both NF-kB-mediated signal transduction and apoptosis[14,15], no significant spontaneous NF-kB signalling or TNFR1-mediated apoptosis were observed upon Δ6-TNFR1 expression (Supplementary Figs 9 and 10). However, Δ6-TNFR1 could potentially retain some intracellular activity by accumulating in the endoplasmic reticulum and evoking a stress response[15]. Nevertheless, there was no evidence for increased endoplasmic reticulum localization of Δ6-TNFR1 (Fig. 2a, b), or induction of the unfolded protein response in Δ6-TNFR1-compared with FL-TNFR1-transfected cells (Supplementary Fig. 11).

Given that no intracellular Δ6-TNFR1 activity was observed, and that this isoform has no transmembrane region and does not associate with FL-TNFR1, we proposed that Δ6-TNFR1 could exist as a soluble, functional molecule. Soluble TNFR1 generation has been previously described through exosomal release of full-length receptor and through metallocathease-dependent cleavage of the FL-TNFR1 extracellular domain. Here, we demonstrate that skipping of exon 6 constitutes a novel mechanism of stable, soluble TNFR1 production: a higher level of soluble protein was found in supernatants of Δ6-TNFR1-transfected cells (Supplementary Fig. 11).

Figure 2 | Δ6-TNFR1 localization and analysis of isoform association. a, TNFR1 subcellular localization. ER, endoplasmic reticulum. b, FL-TNFR1/Δ6-TNFR1 localization analysis. Data = mean ± s.e.m.; FL-TNFR1, n = 20, Δ6-TNFR1, n = 27 cells; scale bar, 1 μm. c, FL-TNFR1/Δ6-TNFR1 association analysis. HEK 293T double-positive cells expressing enhanced cyan/yellow fluorescent protein (ECFP/EYFP)-tagged TNFR1 isoforms were analysed for FRET to assess isoform association. FL-TNFR1–ECFP + FAS180–EYFP co-transfection was used for background signal definition with non-interacting proteins. Percentage of FRET-positive events (indicating protein association) is shown. d, HEK 293T cells expressing haemagglutinin (HA)-tagged FL-TNFR1 or Δ6-TNFR1–EYFP were analysed for cell surface and total protein expression.
TNF antagonists were originally used in Crohn’s disease and ankylosing spondylitis. In accordance with this, common genetic variants in the TNFRSF1A region have not been identified for these conditions with the exception of ankylosing spondylitis, that has a distinct association signal compared to MS (Supplementary Fig. 15), indicating that Δ6-TNFRI is unlikely to influence the pathology of these conditions. Notably, the MS-associated rs1800693 SNP is also associated with primary biliary cirrhosis, but there is no controlled clinical study of TNF antagonists in this disease. Interestingly, side effects associated with the use of TNF antagonists in treating non-MS autoimmune diseases include clinical onset of MS and isolated demyelinating diseases, such as optic neuritis, which is often an early manifestation of MS. These side effects are relatively rare, indicating that they may only arise in individuals with a propensity for demyelinating disease that is unmasked upon treatment.

We have identified a disease-associated genetic variant that directs increased expression of a molecule that is analogous to drugs whose adverse effects can promote or exacerbate disease. This finding has broader implications. A criticism against the potential clinical value of GWAS findings has been the modest effect on disease risk conferred by most associated genetic variants. However, this notion does not consider that drugs targeting the same pathways as the genetic variants are likely to have a larger functional impact and thus these variants may be clinically relevant. We show that naturally occurring variation and therapeutic agents can have the same target, but their effect differs in broad scope. A criticism against the potential clinical value of GWAS findings has been the modest effect on disease risk conferred by most associated genetic variants. However, this notion does not consider that drugs targeting the same pathways as the genetic variants are likely to have a larger functional impact and thus these variants may be clinically relevant. We show that naturally occurring variation and therapeutic agents can have the same target, but their effect differs in broad scope.

**METHODS SUMMARY**

Full descriptions of statistical analyses, minigene assays, transcript quantification, polyclonal profiling, mass spectrometry, ELISAs, and flow cytometric, confocal and biophysical analyses are available in the online version of the paper at www.nature.com/nature.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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Human immune cell TNFR1 surface expression. The anti-human monoclonal antibodies used for immunostaining were Alexa-Fluor647 anti-CD120a/TNFR1 (clone H398; AbD Serotec), Alexa-Fluor700 anti-CD11c, eFluor-540 anti-CD123 (clones 3.9 and 66b respectively; eBioscience), FITC (fluorescein isothiocyanate) and phycoerythrin anti-HLA-DR (clone L243, BioLegend), peridinin-chlorophyll-protein complex (PerCP)-Cy5.5 anti-CD14 and Pacific Blue anti-CD16 (clones HCD14 and 3G8 respectively; BioLegend) and FITC Lin-1 cocktail (BD Biosciences). Alexa-Fluor667 mouse IgG2a (AbD Serotec) was used as an isotype control for CD120a/TNFR1 staining. Data obtained were analysed using Flowjo (Tree Star). TNFR1 surface expression was calculated by dividing the median fluorescence intensity (MFI) of the anti-TNFR1 staining by the MFI of the isotype control for each sample. Each day that donor samples were evaluated, FluoroSpheres were also analysed (Blank Beads and Calibration Beads, Dakocytomation). Data normalization was performed using the calibration beads to control for day-to-day variation in flow cytometer function.

Confocal microscopy. HeLa cells (ATCC) were transfected with constructs expressing TNFR1 fluorescent fusion proteins, a DsRed2-tagged beta subunit of the signal particle receptor (SRB) as a marker of the endoplasmic reticulum, and an ECFP-tagged trans-Golgi-resident protein N-acetylgalactosaminyltransferase-2 (GalNAc-T2) stalk region as a marker of the Golgi. TO-PRO-3 iodide (Molecular Probes, Invitrogen) was used for nuclear staining, with staining being performed for 48 h after transfection. Images were taken with an LSM510 confocal microscope (Zeiss) and processed using Zeiss LSM510 v.3.2 and Image J software. Colocalization was quantified using MetaMorph software (Molecular Devices).

FRET. FRET analysis by flow cytometry was performed as described previously. Briefly, HEK 293T cells were transfected with the indicated ECFP and EYFP fusion protein constructs and collected 24 h later for FRET analysis. FRET signals were expressed as the viable, ECFP/EYFP double-positive cells. FRET, FRET assays were performed and analysed as described previously. GFP- and RLuc fusion protein constructs for FRET studies were generated by cloning TNFR1 sequences into pGFP-N3 and pRLuc-N3 vectors (BioSignal Packard).

Flow cytometric analysis of TNFR1-transfected cells. HEK 293T cells were transfected with the indicated N-terminally HA-tagged TNFR1–EYFP fusion protein constructs. TNFR1 expression was measured in EYFP-positive cells using anti-HA.11 (clone 16B12; Covance) and APC-polyclonal goat anti-mouse IgG primary antibody. Anti-HA.11 (clone H398; AbD Serotec), Alexa-Fluor700 anti-CD11c, e-Fluor450 anti-CD123 (clone H398; AbD Serotec), TO-PRO-3 iodide (Molecular Probes, Invitrogen) was used for nuclear staining, with staining being performed for 48 h after transfection. Images were taken with an LSM510 confocal microscope (Zeiss) and processed using Zeiss LSM510 v.3.2 and Image J software. Colocalization was quantified using MetaMorph software (Molecular Devices).

Lentiviruses. The lentiviral expression plasmid pHRsinUbEm was a gift from P. J. Lehner. TNFR1 expression plasmids were co-transfected with the vesicular stomatitis virus-G envelope plasmid pMD2.G and packaging plasmid psPAX2 (containing HIV-1 Gag, Rev and pack) into HEK 293T cells to generate lentiviral particles. Viral titres were determined by serial dilution and transduction of HEK 293T cells.

Spontaneous apoptosis analysis. For spontaneous apoptosis measurement by mitochondrial integrity analysis, HeLa cells were transfected with the indicated TNFR1-ECFP construct. Mitochondrial membrane potentials (ΔΨm) were analysed by staining cells with the mitochondrion-selective probe tetramethylrhodamine methyl ester (TMRM). The TMRM signal was measured by flow cytometry in ECFP-positive cells. For spontaneous apoptosis measurement by cell cycle analysis, HEK 293T cells were transfected with the indicated TNFR1 lentivirus at a multiplicity of infection of 50. Cell DNA content was measured by staining cell populations using propidium iodide (PI) staining. Following exclusion of debris and cell doublets, cells with a sub-diploid (G0/G1) DNA content were considered apoptotic.

Unfolded protein response analysis. The XBP1 luciferase reporter assay was performed as for the NF-kB/RE luciferase reporter assay but with the use of an XBP1 luciferase reporter construct and HT1080 cells (ATCC). Endoplasmic reticulum stress-induced splicing of the 26-bp intron of human XBP1 was analysed.

Cell culture supernatant TNFR1 measurement. HEK 293T cells were transfected with the indicated N-terminally HA-tagged TNFR1–EYFP fusion constructs. Culture supernatants or anti-TNFR1 antibodies (R&D Systems; Santa Cruz Biotechnology) were used to coat Nunc microtitre plates. HA-tagged TNFR1 proteins were detected using biotinylated anti-HA.11 (clone 16B12; Covance) or biotinylated anti-TNFR1 antibodies (R&D Systems; Santa Cruz Biotechnology) and Europium-conjugated streptavidin (Perkin Elmer). For western blots, HA-tagged TNFR1 proteins detected using anti-HA.11 (clone 16B12; Covance)
and IRDye 800CW goat-anti-mouse IgG secondary antibody, and visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences). **Δ6-TNFR1 measurement in human serum.** Endogenous Δ6-TNFR1 was measured in control and patient sera by ELISA. An anti-Δ6-TNFR1-specific monoclonal Fab antibody was generated using HuCAL antibody technology (AbD Serotec) that uses a human combinatorial antibody library and the CysDisplay method of phage display. The anti-Δ6-TNFR1-specific antibody was coated onto Nunc microtitre plates at a concentration of 2 μg ml⁻¹ overnight. The anti-human TNFR1 mouse monoclonal antibody (clone 16805; R&D Systems) was used at 2 μg ml⁻¹ for detection, in conjunction with Europium-conjugated anti-mouse IgG. The sensitivity limit of the assay was determined to be ~1.25 ng ml⁻¹.

**Recombinant protein expression, purification and TNF immunoprecipitation.** HEK 293T cells were transiently transfected with TNF–Flag or Fc fusion constructs. Fc fusion proteins were purified from culture supernatant using protein A Sepharose (Sigma). Following Fc-fusion protein pre-incubation with TNF–Flag, immunoprecipitation was performed using protein A Sepharose. An Fc fusion of the first cysteine-rich domain of DR5 (DR5.CRD1–Fc) was used as a negative control. Fc fusion proteins were purified from culture supernatant using protein A Sepharose. An Fc fusion of the first cysteine-rich domain of DR5 (DR5.CRD1–Fc) was used as a negative control.

**Tandem mass spectrometry.** Purified Δ6-TNFR1–Fc protein and transfected cell supernatants were solubilized and digested with chymotrypsin (Roche). The digested protein mixture was separated with a 140 min gradient from 5 to 60% acetonitrile (uHPLC, Proxeon) and loaded onto a LTQ-Orbitrap or a Q-Exactive mass spectrometer (Thermo Fisher). The instrument was operated in a data-dependent top10 acquisition modus. Raw data were searched using the MaxQuant software suite (version 1.2.2.0) against the complete IPI human database (v3.68, 87061 entries) with an additional entry for the human Δ6-TNFR1 isoform. The fragmentation spectra were plotted and annotated by the Viewer interface of MaxQuant.

**Surface plasmon resonance.** Surface plasmon resonance spectroscopy was performed using the BIAcore T100 system. TNF–Flag was directly immobilized onto anti-Flag-coated chips from culture supernatants. Experiments were carried out using serial dilutions of the indicated purified Fc-fusion proteins. Binding curves were analysed using BIAcore T100 Evaluation software to fit the data and determine dissociation constants (K_d), assuming 1:1 binding.

**TNF neutralization assay.** Varying concentrations of purified Fc fusion proteins ranging from 1–10,000 ng ml⁻¹ were incubated with 3 × 10⁴ HEK-Blue TNFα/IL-1β cells (InvivoGen) in the presence of 25 ng ml⁻¹ of TNF. The assay was performed according to the manufacturer’s instructions.

**Statistical analysis of functional data.** All statistical tests were performed using GraphPad Prism, GraphPad StatMate 2.0, and the R statistical software package. Regression analysis was used to test for correlations between rs1800693 genotype and TNFR1 transcript levels and TNFR1 cell surface expression in human immune cell subsets, assuming a linear genotype-to-phenotype relationship (1 degree of freedom F-test); age and sex were not included as covariates as for all data sets no age or sex effects were observed (P > 0.05). For the TNFR1_A6 transcript levels, the slope calculated by the linear regression analysis for the CD14⁺ monocytes, polymorphonuclear cells and CD3⁺ T cells was 0.0014, 0.0026 and 0.0003, respectively. Using a Bonferroni correction for multiple testing (taking P = 0.05 and considering three independent hypotheses for the three different immune cell subsets for RNA-level analyses), the significance threshold estimated was P = 0.017. At this significance threshold we obtained >90% power to detect the differences observed with our sample size. The percentage of TNFR1_A6 transcript level variation accounted for by genotype at rs1800693 was estimated by least-squares regression analysis at 67, 52 and 55% for the CD14⁺ monocytes, polymorphonuclear cells and CD3⁺ T cells, respectively. Linear regression analysis was used to quantify the correlation in TNFR1_A6 transcript levels between the different immune cell subsets. For all regression analyses there was no significant departure from linearity (P > 0.05) as determined using a runs test. For the immune cell TNFR1 surface expression, using a Bonferroni correction (considering six independent hypotheses for six independent immune cell subsets), the significance threshold was P = 0.008; at this threshold we had >80% power to detect a 50% difference between the homozygous groups, given our sample size. For the statistical analysis of the confocal colocalization quantification, two-tailed, paired Student’s t-tests were performed. For all other analyses, two-tailed, unpaired Student’s t-tests were performed. For all Student’s t-tests a 5% significance threshold was used.

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