

## Tyrosine kinase 2 variant influences T lymphocyte polarization and multiple sclerosis susceptibility

Nicolas Couturier,<sup>1</sup> Florence Bucciarelli,<sup>1</sup> Ramil N. Nurtdinov,<sup>1</sup> Marc Debouverie,<sup>2</sup> Christine Lebrun-Frenay,<sup>3</sup> Gilles Defer,<sup>4</sup> Thibault Moreau,<sup>5</sup> Christian Confavreux,<sup>6</sup> Sandra Vukusic,<sup>6</sup> Isabelle Cournu-Rebeix,<sup>7</sup> Robert H. Goertsches,<sup>8</sup> Uwe K. Zettl,<sup>8</sup> Manuel Comabella,<sup>9</sup> Xavier Montalban,<sup>9</sup> Peter Rieckmann,<sup>10</sup> Frank Weber,<sup>11</sup> Bertram Müller-Myhsok,<sup>11</sup> Gilles Edan,<sup>12</sup> Bertrand Fontaine,<sup>7</sup> Lennart T. Mars,<sup>1</sup> Abdelhadi Saoudi,<sup>1</sup> Jorge R. Oksenberg,<sup>13</sup> Michel Clanet,<sup>1</sup> Roland S. Liblau<sup>1</sup> and David Brassat<sup>1</sup>

1 INSERM U563 and Pôle des neurosciences, University of Toulouse 3, 31000 Toulouse, France

2 Department of Neurology, University of Nancy Medical Centre, 54000 Nancy, France

3 Department of Neurology, University of Nice Medical Centre, 06000 Nice, France

4 Department of Neurology, University of Caen Medical Centre, 14033 Caen, France

5 Department of Neurology, University of Dijon Medical Centre, 21000 Dijon, France

6 Pierre Wertheimer Neurological Hospital, Neurology A and EDMUS Coordinating Centre, INSERM, U842, University of Lyon I, 69000 Lyon, France

7 Department of Neurology, AP-HP, University Pierre et Marie Curie–Paris 6, UMR S975, INSERM, UMR 975, 75013 Paris, France

8 Institute of Immunology, University of Rostock, 2500 Rostock, Germany

9 Clinical Neuroimmunology Unit, Vall d'Hebron University Hospital, 08035 Barcelona, Spain

10 Neurologischen Klinik Bamberg, 96047 Bamberg, Germany

11 Max-Planck-Institute for Psychiatry, Sections of Neurology, Clinical Chemistry, and Statistics, 58352 Munich, Germany

12 CIC-P 02-03 INSERM, University Hospital, UEB, 35033 Rennes, France

13 Department of Neurology, University of California, San Francisco, 94143 San Francisco, CA, USA

Correspondence to: Prof. David Brassat,  
INSERM U563 CPTP,  
Equipe Auto-immunité et Immunorégulation,  
CHU Purpan, BP3028,  
F-31024 Toulouse, France  
E-mail: dbrassat@yahoo.fr

The tyrosine kinase 2 variant rs34536443 has been established as a genetic risk factor for multiple sclerosis in a variety of populations. However, the functional effect of this variant on disease pathogenesis remains unclear. This study replicated the genetic association of tyrosine kinase 2 with multiple sclerosis in a cohort of 1366 French patients and 1802 controls. Furthermore, we assessed the functional consequences of this polymorphism on human T lymphocytes by comparing the reactivity and cytokine profile of T lymphocytes isolated from individuals expressing the protective *TYK2<sup>GC</sup>* genotype with the disease-associated *TYK2<sup>GG</sup>* genotype. Our results demonstrate that the protective C allele infers decreased tyrosine kinase 2 activity, and this reduction of activity is associated with a shift in the cytokine profile favouring the secretion of Th2 cytokines. These findings suggest that the rs34536443 variant effect on multiple sclerosis susceptibility might be mediated by deviating T lymphocyte differentiation toward a Th2 phenotype. This impact of tyrosine kinase 2 on effector differentiation is likely to be of wider importance because other autoimmune diseases also have been associated with polymorphisms within tyrosine kinase 2. The modulation of tyrosine kinase 2 activity might therefore represent a new therapeutic approach for the treatment of autoimmune diseases.

**Keywords:** autoimmunity; multiple sclerosis; susceptibility T lymphocyte polarization; TYK2

**Abbreviations:** IFN $\beta$  = interferon beta; IFN $\lambda$  = interferon lambda; IL = interleukin; JAKs = Janus kinase superfamily; STAT = signal transducers and activators of transcription family; TNF = tumour necrosis factor; TYK2 = tyrosine kinase 2

## Introduction

Multiple sclerosis is a major cause of disability in young adults (Compston and Coles, 2008). Multiple sclerosis pathogenesis is not fully understood, but a large body of evidence supports an underlying autoimmune process triggered by environmental factors (Ascherio and Munger, 2007a, b) in genetically susceptible individuals (Oksenberg *et al.*, 2008). Genetic factors in multiple sclerosis have been suggested by epidemiological studies and a strong genetic effect was identified almost 40 years ago within the human leukocyte antigen locus (Jersild *et al.*, 1972). Non-human leukocyte antigen susceptibility genes have been described only recently, mostly through genome-wide association studies. Interleukin (*IL*)-2R and *IL*-7R were found first (Gregory *et al.*, 2007; International Multiple Sclerosis Genetics Consortium *et al.*, 2007; Lundmark *et al.*, 2007; Wellcome Trust Case Control Consortium *et al.*, 2007; Maier and Hafler, 2008), followed by DNA variants within or next to *CD6* (De Jager *et al.*, 2009a), *CD58* (Australia and New Zealand Multiple Sclerosis Genetics Consortium, 2009; De Jager *et al.*, 2009b), *CD40* (Ascherio and Munger, 2007b), *CD226* (Baranzini *et al.*, 2009), *CLEC16A* (International Multiple Sclerosis Genetics Consortium *et al.*, 2007; Australia and New Zealand Multiple Sclerosis Genetics Consortium, 2009), *CYP27B1* (Australia and New Zealand Multiple Sclerosis Genetics Consortium, 2009), *IRF8* (De Jager *et al.*, 2009a), *TNFRSF1A* (De Jager *et al.*, 2009b), *VAV1* (Jagodic *et al.*, 2009) and others. In contrast to human leukocyte antigen genes, all non-human leukocyte antigen variants identified so far are responsible for very modest increases in multiple sclerosis susceptibility (odds ratio 1.2–1.5).

The tyrosine kinase 2 (*TYK2*) variant rs34536443 was reported to be associated with multiple sclerosis susceptibility by Ban *et al.* (2009), and this finding was replicated soon after the independent studies with subjects from different ancestral backgrounds (Johnson *et al.*, 2010; Mero *et al.*, 2010). This polymorphism occurs in exon 21 of the gene and modifies the *TYK2* amino acid sequence at position 1104. Whereas the major G allele encodes a proline, the minor C allele encodes an alanine (as *TYK2*<sup>P1104A</sup> variants hereafter). Previous observations suggested that the *TYK2*<sup>P1104A</sup> modification may influence the enzymatic activity of the protein (Kaminker *et al.*, 2007; Tomasson *et al.*, 2008). *TYK2*, a member of the Janus kinase superfamily (JAKs) (Wilks, 2008), interacts with the intracellular domain of type I interferon receptor IFNAR (Barbieri *et al.*, 1994; Theofilopoulos *et al.*, 2005). The phosphorylation of two tyrosines (positions 1054 and 1055) in the A-loop of *TYK2* results in the activation of the signalling pathway (Lucet *et al.*, 2006). The phosphorylation of the intracellular domain of IFNAR by *TYK2* and JAK1 kinases is followed by the phosphorylation of STAT1 and STAT2. The phosphorylated STAT1/STAT2 heterodimers associate with IRF-9 and translocate into the nucleus to activate the expression

of IFN-regulated genes. Interestingly, interferon beta (IFN $\beta$ ) is an approved multiple sclerosis drug that reduces symptoms and has some impact on disease progression (Jacobs *et al.*, 2000; Kappos *et al.*, 2009).

*TYK2* was also found to be associated with *IL*-6, *IL*-10, *IL*-12, *IL*-23 and interferon lambda (IFN $\lambda$ ) receptors, where it plays a significant role in the activation of these cytokine pathways (Watford and O'Shea, 2006; Uzé and Monneron, 2007; Ghoreschi *et al.*, 2009a). Nevertheless, how *TYK2* influences multiple sclerosis susceptibility is not known. In this study, after validating the role of rs34536443 in multiple sclerosis susceptibility in French patients with multiple sclerosis and the lack of association with response to IFN $\beta$  treatment in a European cohort, we evaluated how this variant influences the immunological response. Our findings confirm that the rs34536443 variant affects *TYK2* enzymatic activity and thereby associated cytokine pathways. Moreover, the modification of *TYK2* activity was associated with an exacerbation of the Th2 polarization in individuals carrying the multiple sclerosis-protecting allele. The impact of *TYK2* polymorphism on the adaptive immune system might explain its role in multiple sclerosis susceptibility.

## Materials and methods

### Clinical samples

*TYK2* polymorphism was genotyped in a French case–control cohort including 1248 individuals and in 640 French Caucasian trio families. The clinical characteristics of these two cohorts are summarized in Supplementary Table 1. Samples from patients with multiple sclerosis of the case–control cohort ( $n = 726$ ) were obtained from 20 academic multiple sclerosis clinics. Control samples ( $n = 522$ ) were obtained from healthy blood donors. French trio families, composed of one affected subject and the two parents (with four European Caucasian grandparents), were recruited through a national media campaign followed by the selection of individuals who satisfied the criteria for multiple sclerosis (Poser *et al.*, 1983; Goodkin *et al.*, 1991; Polman *et al.*, 2005) for the REFGENSEP (réseau français pour la génétique de la sclérose en plaques). The pharmacogenetics cohort was composed of IFN $\beta$ -treated patients with a relapsing–remitting form of multiple sclerosis. These patients were recruited from France, Germany and Spain for the UEPHA-MS (European Association for Pharmacogenetics of Multiple Sclerosis) network and the Max-Planck Institute for Psychiatry in Munich between 2000 and 2005. The status for neutralizing antibodies against IFN $\beta$  is not available for this cohort. According to clinical criteria collected during the first 2 years of IFN $\beta$  treatment, these patients were classified in two groups: responders ( $n = 240$ ) were patients without relapse and without disability progression, and non-responders ( $n = 299$ ) were patients with at least two relapses or a disability progression of at least one point. Patients with intermediate criteria were not included in the analysis. Clinical characteristics of the pharmacogenetics cohort are provided in Supplementary Table 2.

All the subjects gave their informed consent and the local ethics committee of Paris-Pitié-Salpêtrière-France approved the study.

## Genotyping methods

Genomic DNA was purified from fresh peripheral blood leukocytes by standard methods. We genotyped the rs34536443 variant using a TaqMan 5' allele-discrimination assay (Applied Biosystems). The polymerase chain reaction was performed as recommended by Applied Biosystems. An ABI Prism 7900 Sequence detection system and SDS 2.2.2 software (both from Applied Biosystems) were used for allele discrimination. In each cohort, the rs34536443 marker did not deviate significantly from Hardy-Weinberg equilibrium.

## Primary antibodies and quantitative real-time polymerase chain reaction

Rabbit antibodies for JAK1, STAT1, STAT2, and their phosphorylated forms (Tyr<sup>1022</sup> and Tyr<sup>1023</sup> for phospho-JAK1, Tyr<sup>701</sup> for phospho-STAT1 and Tyr<sup>690</sup> for phospho-STAT2) were purchased from Cell Signalling. Anti-phospho-TYK2 (Tyr<sup>1054</sup> and Tyr<sup>1055</sup>) antibody was also purchased from Cell Signalling. Mouse antibodies for TYK2 and  $\beta$ -actin came from BD Biosciences. IRDye 680 goat anti-mouse IgG and IRDye 800CW goat anti-rabbit IgG were from Li-Cor Biosciences. Monoclonal rat antibodies used for flow cytometry experiments came from BD Pharmingen (anti-IL-12, anti-IL-10) and Caltag Medsystems (IgG1 isotype). Primers used for quantitative real-time polymerase chain reaction came from Invitrogen and are listed in Supplementary Table 3.

## T cell isolation and expansion from peripheral blood mononuclear cells

From heparinized blood samples, peripheral blood mononuclear cells were isolated through density gradient centrifugation via lymphocyte separation medium (Eurobio). For polyclonal expansion of T cells, peripheral blood mononuclear cells were cultured with  $3 \times 10^6$  Dynabeads CD3/CD28 T cell expander (Invitrogen) in RPMI 1640 (Gibco) containing 10% foetal bovine serum (Gibco), 50  $\mu$ M 2-mercaptoethanol (Gibco), 10 mM HEPES, 1% L-glutamine and 1% penicillin-streptomycin. After 2 days, 50 U/ml of human IL-2 (AbCys) was added to the culture medium for 13–20 days. After 15 days, cells expressing the T cell receptor  $\alpha/\beta$  represent 97% of all cells of the culture. Among T cell receptor  $\alpha/\beta$ -expressing cells, the ratio CD4<sup>+</sup>/CD8<sup>+</sup> is 2:1. We observed no significant difference between TYK2<sup>GC</sup> and TYK2<sup>CC</sup> for the cell culture characteristics.

## Western blot

Expanded T lymphocytes were incubated for 2 h at 37°C in RPMI 1640 culture medium (Gibco). Following this resting period, 1000 U/ml of IFN $\beta$  (Rebif, Merck-Serono) was added to the medium to stimulate lymphocytes for 15 min. T lymphocytes were then rapidly washed in ice-cold phosphate buffered saline and harvested in lysis buffer containing phosphatase inhibitors. Equivalent amounts of cell lysates were separated by electrophoresis using 10% Bis-Tris Nu-PAGE<sup>®</sup> gels (Invitrogen) and then transferred onto nitrocellulose membranes (Hybond-C Extra, Amersham Biosciences). After blocking (Tris buffered saline containing 5% non-fat dry milk), membranes were incubated with primary antibodies at 4°C overnight. The following secondary fluorescent antibodies were used: IRDye 680 goat

anti-mouse IgG (Li-Cor Biosciences) or IRDye 800CW goat anti-rabbit IgG (Li-Cor Biosciences). Laser scanning and quantitative analyses of the blots were performed using the Odyssey<sup>®</sup> Infrared Imaging System (Li-Cor Biosciences). Quantification of protein phosphorylation was carried out by measuring the intensity of fluorescence of the band corresponding to the phosphorylated protein normalized to  $\beta$ -actin expression. In parallel, total levels for each protein were quantified on a separate blot in the same way. Results are expressed as the ratio of the phosphorylated form to the total protein, after  $\beta$ -actin normalization.

## Isolation of RNA and gene expression analysis

Expanded T lymphocytes were incubated for 2 h at 37°C in RPMI 1640 (Gibco) culture medium. Following this resting period, T lymphocytes were stimulated for 2 h with 1000 U/ml of IFN $\beta$  (Rebif, Merck-Serono), 10 ng/ml of IL-6 (AbCys), or 100 ng/ml of IL-10 (AbCys). Total RNA was extracted using the RNeasy<sup>®</sup> RNA isolation kit (Qiagen) according to the manufacturer's instructions. A DNase (Qiagen) digestion was included to remove genomic DNA. RNA was converted into a representative complementary DNA pool using the Superscript<sup>®</sup> III First-Strand Synthesis System and random-hexamer primers (Invitrogen) according to the manufacturer's instructions. Complementary DNAs were stored at –20°C until use. Quantitative real-time polymerase chain reaction was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and quantitative PCR Mastermix Plus for Sybr Green I (Eurogentec). Messenger RNA levels for each target gene were quantified using GAPDH as a housekeeping gene. The relative expression was calculated with the traditional formula of  $2^{-\Delta\Delta C_t}$ , and a reference value was arbitrarily chosen as the mean of all samples.

## Analysis of T lymphocyte proliferation and *in vitro* cytokine production

Proliferation of expanded T lymphocytes was assessed after 48 and 72 h of culture by incorporation of [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) in the presence of Dynabeads CD3/CD28 T cell expander. Supernatants were analysed for seven cytokines (IL-4, IL-5, IL-13, IFN $\gamma$ , TNF $\alpha$ , IL-17A and IL-10) using custom multiplex immunoassays and Luminex instrumentation (Millipore). These functional T lymphocyte assays were performed in non-polarizing cytokine conditions with only the addition of 50 U/ml of human IL-2 in the culture medium.

## Protein modelling and alignment

The kinase domain structure of TYK2 was generated via the Swiss-Model (Peitsch, 1995; Arnold *et al.*, 2006; Kiefer *et al.*, 2009; <http://swissmodel.expasy.org>) using the X-ray structures of JAK2 and JAK3 domains. The representation was visualized with the molecular graphics visualization tool RasMol 2.7.5 (<http://www.bernstein-plus-sons.com/software/rasmol>). Using PolyPhen software (Ramensky *et al.*, 2002), we defined 40 vertebrate homologues for the TYK2 protein, all belonging to the JAK family in different species. For human TYK2, JAK1, JAK2 and JAK3, and mouse, opossum and frog TYK2 proteins, we calculated the conservation profile. Using Muscle software (Edgar, 2004), we compared the homologous part of TYK2 from 1050 to 1119 position with its pseudo-kinase domain and with SRC and EGFR proteins.

## Monocyte isolation, intracellular staining and flow cytometry

Monocytes were isolated from peripheral blood mononuclear cells by allowing them to adhere to plastic in Macrophage-SFM medium (Gibco) containing 5% foetal bovine serum (Gibco), 50  $\mu$ M 2-mercaptoethanol (Gibco), 10 mM HEPES, 1% L-glutamine and 1% penicillin-streptomycin. After 2 h, non-adherent cells were removed by gentle washing. In the remaining adherent cells were >90% pure monocytic cells.

After 20 h of culture, the macrophage monolayer was stimulated with phorbol myristate acetate (1  $\mu$ M; Sigma) and ionomycin (1  $\mu$ M; Sigma) in presence of GolgiStop (BD Biosciences) for 4 h. Macrophages were gently scraped and, after washing by centrifugation, the surface-expressed CD14 detected using an anti-CD14 monoclonal antibody. Intracellular staining with anti-cytokine monoclonal antibodies against IL-10, IL12 and IgG1 isotype control using a Cytofix/Cytoperm™ Plus Kit (BD Biosciences) was performed. Labelled cells were analysed with a LSRII flow cytometer (Becton Dickinson) using the BD FACS-Diva software.

## Statistical analysis

Chi-square analysis was performed to evaluate the differences in allele frequencies in the case-control cohort and responder/non-responder cohort using Prism 5 software (GraphPad Software). Allele frequencies were used to calculate the odds ratio and the 95% confidence interval. The  $\chi^2$  value was calculated from the 2  $\times$  2 contingency table, and *P*-values were determined using one degree of freedom. The transmission disequilibrium test was performed using Haploview 4.2 (Barrett *et al.*, 2005; <http://www.broadinstitute.org/haploview>) under default settings. This software examines the transmission patterns of all complete trios within each pedigree. rs34536443 Genotypes were tested for deviation from Hardy-Weinberg equilibrium using Haploview 4.2. The global odds ratio and the genotype-phenotype correlations with age of onset and multiple sclerosis severity score were performed using Unphased 3.1.4 (<http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/>). To test the differences in continuous values between the two groups, we applied an exact non-parametric permutation test with StatXact 8 (Cytel Statistical Software). All statistical tests were two-sided. *P*  $\leq$  0.05 were considered statistically significant.

## Results

### TYK2 is associated with multiple sclerosis susceptibility in the French population

In an effort to replicate the *TYK2* association with multiple sclerosis susceptibility in the French population, the rs34536443 polymorphism was genotyped in 3168 individuals corresponding to multiple sclerosis case-control and trio-family cohorts ascertained using well-established inclusion criteria (Table 1). The rare *TYK2*<sup>CC</sup> genotype was never observed in the French cohort. We confirmed a significant enrichment of the C allele in controls compared with cases (*P* = 0.003) and under-transmission of the C allele to children with multiple sclerosis (*P* = 0.012). A combined analysis revealed that the C allele decreased multiple sclerosis risk with an

odds ratio of 0.63 (0.47–0.84). Additionally, we tested if rs34536443 could influence disease severity in this data set (Johnson *et al.*, 2010). We found no association with age of onset (*P* = 0.76) or multiple sclerosis severity score (*P* = 0.35). Finally, because of the major role of *TYK2* in the IFN $\beta$  signalling pathway (Minegishi *et al.*, 2006), we tested the influence of the rs34536443 polymorphism on the response to IFN $\beta$  treatment in 539 well-characterized multiple sclerosis IFN $\beta$ -treated patients and found no association with treatment response (*P* = 0.67).

### rs34536443 Polymorphism does not modify *TYK2* expression level

The *TYK2* gene variant could influence messenger RNA expression, *TYK2* protein stability, and/or its spatial structure. To investigate these possibilities, we quantified *TYK2* messenger RNA and protein levels on expanded T lymphocytes isolated from *TYK2*<sup>GG</sup> and *TYK2*<sup>GC</sup> carrier individuals. We found no differences in *TYK2* expression at the messenger RNA level (Fig. 1A; *P* = 0.81) or at the protein level (Fig. 1B; *P* = 0.24). Therefore we conclude that the rs34536443 polymorphism does not modify the steady-state level of *TYK2* in T lymphocytes.

### rs34536443 Polymorphism affects IFN $\beta$ signalling pathway

The rs34536443 *TYK2* polymorphism induces a *TYK2*<sup>P1104A</sup> substitution positioned in the  $\alpha$ FG helix of the kinase domain (Fig. 2A). This  $\alpha$ FG helix is present in all members of the JAK family but is not found in other tyrosine kinases (Fig. 2B). According to the PolyPhen software (Ramensky *et al.*, 2002), the *TYK2*<sup>P1104A</sup> substitution would have important functional consequences. Multiple protein alignments of JAK family proteins and several *TYK2* proteins from non-human species showed that position 1104 of *TYK2* is conserved, whereas considerable variations in other positions of the  $\alpha$ FG helix are possible (Fig. 2B). It is tempting to speculate that the  $\alpha$ FG helix could participate in intramolecular regulatory interactions with other domains of *TYK2*. In that case, a modification of the  $\alpha$ FG helix, such as the *TYK2*<sup>P1104A</sup> substitution, could directly affect the *TYK2* activation state under cytokine stimulation. To test this hypothesis, we stimulated T lymphocytes with IFN $\beta$  and quantified *TYK2* phosphorylation on tyrosine 1054 and tyrosine 1055 by western blot. Without IFN $\beta$  stimulation, no phosphorylated *TYK2* was detected in either genotype, demonstrating the absence of *TYK2* kinase activation under basal conditions. After 15 min of IFN $\beta$  stimulation, the lymphocytes from *TYK2*<sup>GC</sup> carrier individuals showed half as much phosphorylated *TYK2* as those from *TYK2*<sup>GG</sup> carrier individuals (Fig. 2C; *P* = 0.02). This observation indicates that the rs34536443 polymorphism influences the *TYK2* activation state under IFN $\beta$  stimulation.

When activated, *TYK2* is directly implicated in JAK1 and STAT1/STAT2 heterodimer phosphorylation. The comparison of STAT2 phosphorylation after 15 min of IFN $\beta$  stimulation revealed 2.2-fold less phosphorylation in lymphocytes from *TYK2*<sup>GC</sup> than from *TYK2*<sup>GG</sup> carriers (Fig. 2D; *P* = 0.04). The same tendency was

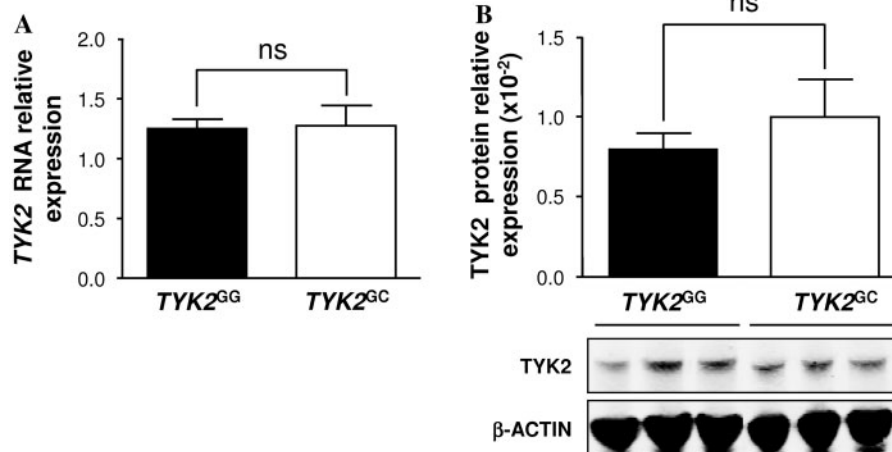
**Table 1** Association analysis of rs34536443 TYK2 polymorphism with multiple sclerosis

Cohort	Tyk2 <sup>GC</sup> frequency (%)	$\chi^2$	P-value	Odds ratio of C allele (95% confidence interval)
Susceptibility <sup>a</sup>				
Case-control	5.1 versus 9.6	8.6	0.003	0.52 (0.34–0.81)
Trios	2.4 versus 4.2 <sup>b</sup>	6.3	0.012	0.57 (0.37–0.89)
Combined analysis		10.58	0.001	0.63 (0.47–0.84)
Severity				
Age of onset		0.09	0.76	
Multiple sclerosis severity score		0.88	0.35	
Response to IFN $\beta$ treatment <sup>c</sup>				
Responder/non-responder	5.6 versus 4.8	0.18	0.67	0.84 (0.39–1.84)

a The susceptibility cohort included 726 multiple sclerosis cases, 522 controls and 640 trio families (1920 individuals).

b C allele transmission versus non-transmission to child with multiple sclerosis.

c The pharmacogenetics cohort included multiple sclerosis patients treated with IFN $\beta$ : 240 were classified as responders and 299 as non-responders.



**Figure 1** The rs34536443 polymorphism does not modify TYK2 expression. (A) TYK2 transcript quantification by quantitative real-time polymerase chain reaction. RNA was extracted from expanded lymphocytes of TYK2<sup>GG</sup> and TYK2<sup>GC</sup> individuals. Relative expression was calculated from duplicate samples normalized to GAPDH expression from TYK2<sup>GG</sup> ( $n = 20$ ) and TYK2<sup>GC</sup> ( $n = 19$ ) individuals. (B) TYK2 protein quantification by western blot. Proteins were extracted from expanded lymphocytes of TYK2<sup>GG</sup> ( $n = 10$ ) and TYK2<sup>GC</sup> ( $n = 10$ ) individuals. Total TYK2 protein was normalized to  $\beta$ -actin expression. Each bar represents mean  $\pm$  SEM; ns = not significant.

observed for JAK1 and STAT1, two other targets of TYK2 phosphorylation (Supplementary Fig. 1).

Furthermore, we analysed the transcriptional upregulation of a panel of IFN $\beta$ -inducible genes, including *MxA*, *OAS1*, *IRF1* and *SOCS3* (Fig. 3A–D). Consistent with our previous immunoblotting results, quantitative real-time polymerase chain reaction revealed that all IFN $\beta$ -inducible genes tested were significantly (or tended to be) less upregulated in response to IFN $\beta$  in the TYK2<sup>GC</sup> carrier group. This decreased induction ranged from 1.6- to 2.2-fold in TYK2<sup>GC</sup> versus TYK2<sup>GG</sup> carriers.

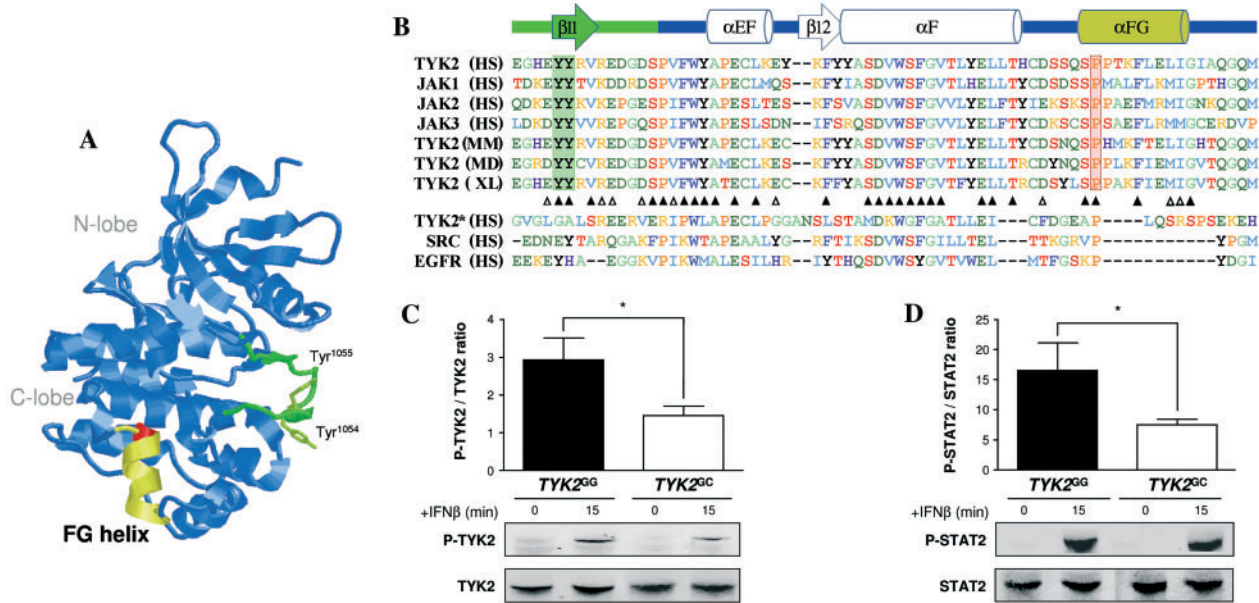
### rs34536443 Polymorphism affects IL-6 and IL-10 signalling pathways

We next examined whether the rs34536443 polymorphism could influence T lymphocyte response to IL-6 and IL-10 stimulation. These two cytokine pathways, like IFN $\beta$ , depend on TYK2

kinase for activation of the signalling cascade. To test the activation level of these pathways, *SOCS3* transcript upregulation was quantified by quantitative real-time polymerase chain reaction 2 h after IL-6 or IL-10 stimulation. We observed a reduced induction of *SOCS3* transcripts after IL-6 (Fig. 3E;  $P = 0.0003$ ) and IL-10 stimulation (Fig. 3F;  $P = 0.03$ ) in carriers of TYK2<sup>GC</sup> versus TYK2<sup>GG</sup>. This reduced induction of *SOCS3* was in the same range as that observed after IFN $\beta$  stimulation (2.1–3.6). Overall, these data confirm that the TYK2<sup>GC</sup> variant is associated with decreased activation of TYK2 kinase activity and subsequently of the IFN $\beta$ , IL-6 and IL-10 pathways.

### rs34536443 Polymorphism modifies T lymphocyte polarization

Previous experiments in mouse and human implicated TYK2 protein in lymphocyte differentiation (Minegishi *et al.*, 2006;



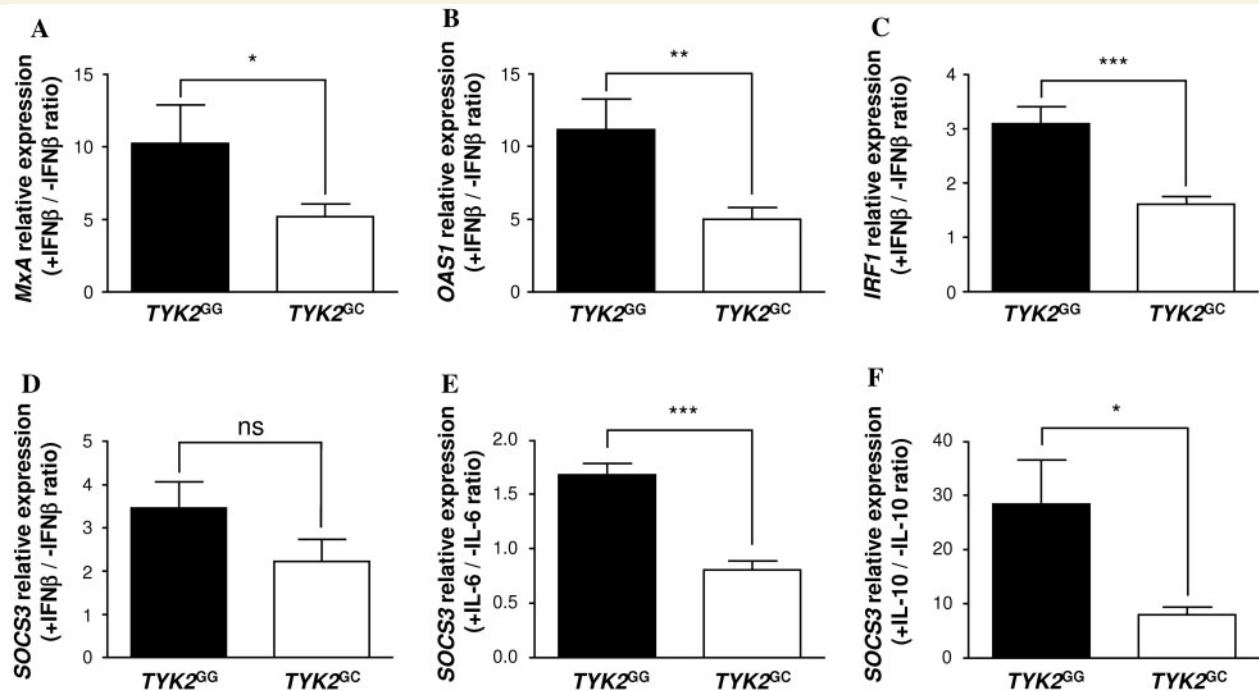
**Figure 2** The rs34536443 polymorphism localizes in the PTK domain of TYK2 and affects the activation state of the protein. (A) Three-dimensional PTK domain model of the TYK2 protein. The activation loop is green, where Tyr<sup>1054</sup> and Tyr<sup>1055</sup> (pale green) are shown in stick form. The  $\alpha$ FG helix is yellow and the TYK2<sup>P1104A</sup> amino acid modification is red. (B) Sequence alignment for a part of the kinase domain of human JAK family proteins and the kinase domain of SRC and EGFR. TYK2\* represents the pseudo-kinase domain of TYK2. Alignment starts from position 1050 for TYK2 and 761 for TYK2\* domains. HS, MM, MD and XL are for *Homo sapiens*, *Mus musculus*, *Monodelphis domestica* and *Xenopus laevis*, respectively. On the top of the alignment,  $\beta$ -strands are indicated as arrows,  $\alpha$ -helices as cylinders, and loops as lines. Colours used are the same as in (A). Solid triangles below the JAK family protein alignment represent completely conserved positions and open triangles represent positions where all amino acids belong to the same structural class (Smith and Smith, 1992). Quantification of the P-TYK2/TYK2 ratio (C) and the P-STAT2/STAT2 ratio (D) by western blot. Proteins were extracted from expanded lymphocytes stimulated with IFN $\beta$  (1000 U/ml). Total proteins and their phosphorylated forms were quantified on samples from TYK2<sup>GG</sup> ( $n = 10$ ) and TYK2<sup>GC</sup> ( $n = 9$ ), individual and normalized to  $\beta$ -actin expression. Each bar represents mean  $\pm$  SEM. \* $P < 0.05$ .

Spach et al., 2009). Considering the kinase activity modulation conferred by the rs34536443 polymorphism, we assessed whether it could influence T lymphocyte polarization *in vitro*. First, we used quantitative real-time polymerase chain reaction to analyse the expression of *T-bet*, *GATA3*, *ROR $\gamma$ t* and *Foxp3* in expanded T lymphocytes. These four lineage-specific transcription factors are expressed by Th1, Th2, Th17 and Treg lymphocytes, respectively. The quantification of *T-bet* (Fig. 4A;  $P = 0.41$ ), *ROR $\gamma$ t* (Fig. 4B;  $P = 0.75$ ) and *Foxp3* (Fig. 4C;  $P = 0.96$ ) transcripts revealed no difference between TYK2<sup>GC</sup> and TYK2<sup>GG</sup> carriers. In contrast, TYK2<sup>GC</sup> individuals exhibited a significant, 1.5-fold increase in *GATA3* transcripts (Fig. 4D;  $P = 0.006$ ). This suggests that the rs34536443 polymorphism affects the modulation of the Th2 lymphocyte polarization. We tested this hypothesis further by assessing the secretion of relevant cytokines (IFN $\gamma$ , TNF- $\alpha$ , IL-17A, IL-10, IL-4, IL-5 and IL-13) by T lymphocytes before and after polyclonal stimulation. The secretion of IFN $\gamma$ , TNF- $\alpha$ , IL-17A and IL-10 were not modified according to TYK2 variants (Supplementary Fig. 2). These results agreed with our quantitative real-time polymerase chain reaction results (Fig. 4), showing no influence of the rs34536443 polymorphism on master regulatory genes for Th1, Th17 and Treg lymphocyte polarization. In stark contrast, we observed twice as much IL-5 secretion (Fig. 5B;  $P = 0.04$ ) and 1.5 times as much IL-13 secretion (Fig. 5C;

$P = 0.01$ ) in TYK2<sup>GC</sup> carriers as compared with TYK2<sup>GG</sup> carriers after CD3/CD28 stimulation. For IL-4, increased secretion was only seen in the baseline condition without stimulation (Fig. 5A). In parallel, a thymidine proliferation assay showed that T lymphocytes isolated from individuals of the two genotypes proliferated equally well (data not shown). Therefore, the differences in cytokine release cannot be attributed to differential T lymphocyte proliferation linked to the rs34536443 polymorphism. Overall, these results highlight the importance of the rs34536443 polymorphism on Th2 lymphocyte polarization, with no detectable effect on Th1, Th17 and Treg lymphocyte polarization.

## rs34536443 Polymorphism does not modify the M1/M2 balance of peripheral blood monocytes

To test the possibility that the Th2 immune deviation observed in TYK2<sup>GC</sup> individuals was related to cytokine production by antigen-presenting cells, we analysed peripheral blood monocyte polarization in the context of TYK2 rs34536443 genotype. The monocyte-macrophage system exists in at least two distinct phenotypes of differentiation: pro-inflammatory macrophages (M1) and anti-inflammatory macrophages (M2), which are involved in Th1



**Figure 3** The rs34536443 polymorphism affects the expression of genes induced by cytokines implicating TYK2. (A–D) Quantification of the IFN $\beta$ -induced genes. (A) *MxA*, (B) *OAS1*, (C) *IRF1* and (D) *SOCS3* transcripts were quantified by quantitative real-time polymerase chain reaction performed on expanded T lymphocytes of *TYK2*<sup>GG</sup> ( $n = 11$ ) and *TYK2*<sup>GC</sup> ( $n = 12$ ) individuals. The relative gene expression is shown as the ratio +IFN $\beta$  / –IFN $\beta$  that represents the gene expression after 2 h of IFN $\beta$  stimulation (1000 U/ml) over the expression of the same gene in parallel cultures without stimulation. (E) Quantification of *SOCS3* upregulation after IL-6 stimulation. The +IL-6 / –IL-6 ratio represents *SOCS3* expression after 2 h of IL-6 stimulation (10 ng/ml) over its expression without stimulation (*TYK2*<sup>GG</sup> individuals,  $n = 9$ ; *TYK2*<sup>GC</sup> individuals,  $n = 7$ ). (F) Quantification of *SOCS3* upregulation after IL-10 stimulation. The +IL-10 / –IL-10 ratio represents *SOCS3* expression after 2 h of IL-10 stimulation (100 ng/ml) over its expression without stimulation (*TYK2*<sup>GG</sup> individuals,  $n = 9$ ; *TYK2*<sup>GC</sup> individuals,  $n = 7$ ). Relative expression was calculated from duplicate samples normalized to *GAPDH* expression. Each bar represents mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns = not significant.

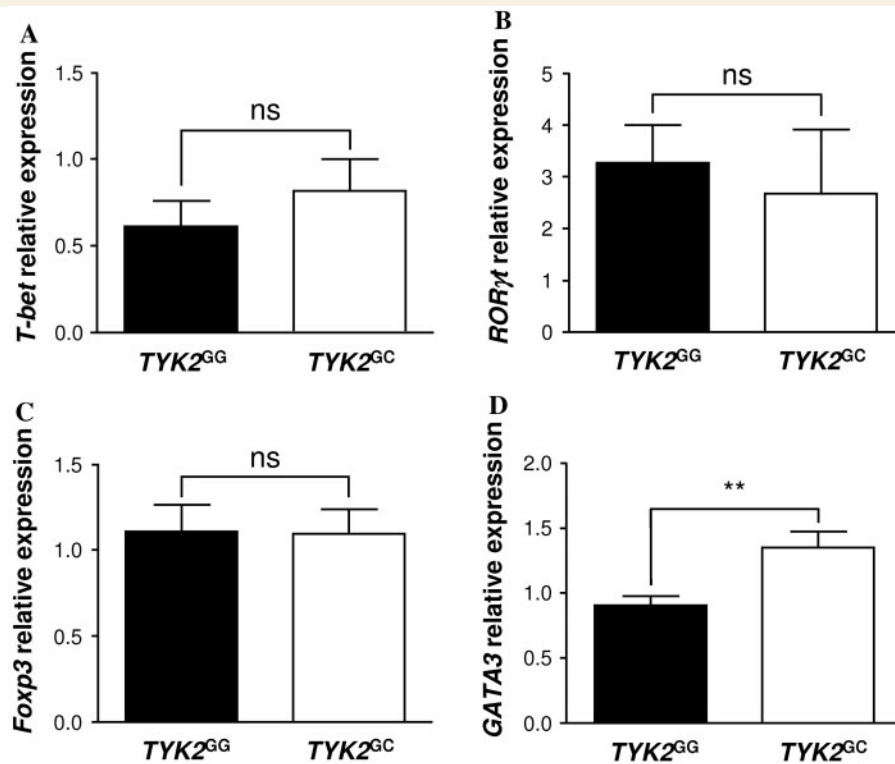
and Th2 responses, respectively (Mantovani, 2006). By flow cytometry, we quantified the percentage of IL-12<sup>+</sup>/IL-10<sup>–</sup>/CD14<sup>high</sup> cells (M1 macrophage phenotype) and the percentage of IL-12<sup>–</sup>/IL-10<sup>+</sup>/CD14<sup>high</sup> cells (M2 macrophage phenotype) in 10 *TYK2*<sup>GG</sup> and seven *TYK2*<sup>GC</sup> individuals (Fig. 6). We detected a low percentage of IL-12<sup>+</sup>/IL-10<sup>–</sup>/CD14<sup>high</sup> and IL-12<sup>–</sup>/IL-10<sup>+</sup>/CD14<sup>high</sup> cells in peripheral blood mononuclear cells (<5%), in accordance with previous studies (Luppi *et al.*, 2002). We observed no statistical difference in the M1/M2 balance between the *TYK2*<sup>GG</sup> and the *TYK2*<sup>GC</sup> carriers. This suggests that the rs34536443 polymorphism effect on Th2 lymphocyte polarization is not secondary to monocyte polarization.

## Discussion

We confirmed first the protective effect against multiple sclerosis risk (odds ratio = 0.63) conferred by the C minor allele of the rs34536443 variant in a French cohort composed of 1366 cases and 1802 controls. In agreement with Johnson *et al.* (2010), we also confirmed the absence of an association between the rs34536443 polymorphism, multiple sclerosis onset age and

disease severity (multiple sclerosis severity score). Moreover, despite the major implication of TYK2 in the IFN $\beta$  signalling pathway, the rs34536443 variant did not show any association with the response to IFN- $\beta$  treatment.

Second, we found that the rs34536443 variant influences TYK2 enzymatic activity (Figs 2 and 3). The rs34536443 polymorphism occurs in the  $\alpha$ FG helix of TYK2, where it exchanges a highly conserved proline at position 1104 with an alanine. Because of the highly conserved status of this region, it is expected that any modification would be biologically relevant. To assess the biological implication of TYK2 variants, we genotyped 300 individuals from our biological resource centre and identified 13 *TYK2*<sup>GC</sup> carriers (C allele frequency 4%). Using peripheral blood mononuclear cells from these patients, we found a reduction of TYK2 phosphorylation in T lymphocytes from *TYK2*<sup>GC</sup> individuals with downstream consequences on TYK2-related pathways, namely reduced phosphorylation of TYK2 substrates (JAK1, STAT1 and STAT2; Fig. 2) and blunted upregulation of a panel of cytokine-induced genes (*MXA*, *OAS1*, *IRF1* and *SOCS3*; Fig. 3). Together, these observations suggest that in *TYK2*<sup>GC</sup> carriers the tested TYK2-related cytokine pathways experience a global decrease in activity (Fig. 3).



**Figure 4** The rs34536443 polymorphism affects *GATA3* transcription factor expression. Quantification by quantitative real-time polymerase chain reaction of (A) *T-bet*, (B) *RORγt*, (C) *Foxp3* and (D) *GATA3* expression in expanded T lymphocytes. Relative gene expression was calculated from duplicate samples normalized to *GAPDH* expression from *TYK2*<sup>GG</sup> ( $n = 11$ ) and *TYK2*<sup>GC</sup> ( $n = 12$ ) individuals. Each bar represents mean  $\pm$  SEM. \*\* $P < 0.01$ ; ns = not significant.

Third, we investigated how *TYK2* may influence multiple sclerosis susceptibility. Owing to the importance of *TYK2* in cytokine receptor signalling and the central role of functional T cell subsets in multiple sclerosis, we studied the genetic effect of the rs34536443 variant on T cell polarization. *TYK2*<sup>GC</sup> minor allele carriers showed increased Th2 response as compared with *TYK2*<sup>GG</sup> carriers. We observed increased *GATA3* expression (Fig. 4) and IL-4, IL-5 and IL-13 secretion (Fig. 5). Th1, Th17 and Treg signature cytokines were not affected. Hence, the Th2 immune deviation favoured by the C allele of the rs34536443 variant might explain the protective effect conferred in multiple sclerosis susceptibility.

Like the majority of autoimmune diseases, multiple sclerosis is a pro-Th1 and pro-Th17 disease. In experimental autoimmune encephalomyelitis, the animal model for multiple sclerosis, the injection of pathogenic Th1 or Th17 cells is able to induce disease (Axtell *et al.*, 2010), in contrast to Th2 cells, which are unable to do so (Ramírez and Mason, 2000). Moreover, co-culture of Th2 cells with pathogenic cells regulates the induction of experimental autoimmune encephalomyelitis by a mechanism implicating the secretion of Th2 cytokines, including IL-4 and IL-13 (Racke *et al.*, 1994; Young *et al.*, 2000). Our data suggest that the rs34536443 variant effect on multiple sclerosis susceptibility might be directly mediated by deviation of the T lymphocyte differentiation toward a Th2 phenotype. We are fully aware, however, that with the ubiquitous expression of *TYK2*, the rs34536443

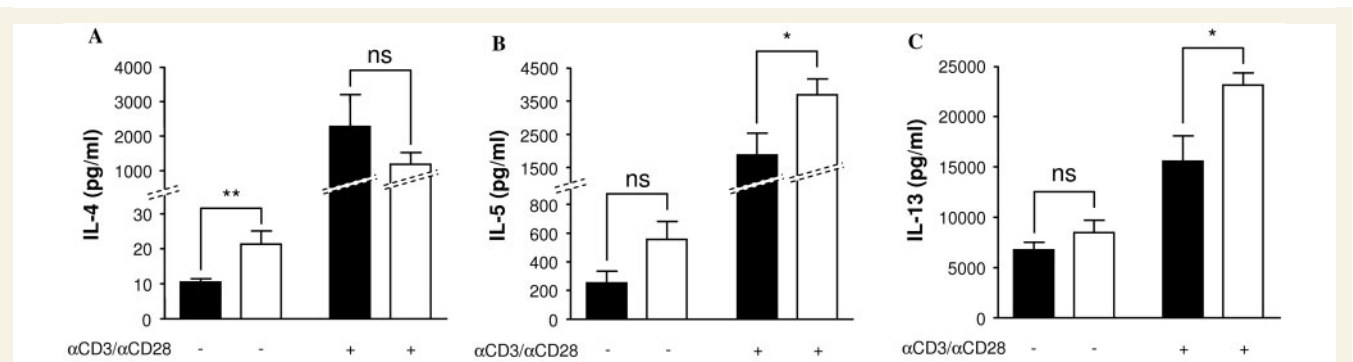
variant could modulate functions of non-T immune cells or glial cells. Previous reports noted the requirement of *TYK2* kinase activity in dendritic cells to promote the induction of Th1 cell differentiation (Tokumasa *et al.*, 2007) and that the *TYK2* gene was associated with central nervous system repair and remyelination (Bieber *et al.*, 2010).

Yet, the evaluation of the *TYK2* rs34536443 polymorphism effect on monocyte polarization did not reveal a modification of the M1/M2 balance toward an anti-inflammatory M2 phenotype in *TYK2*<sup>GC</sup> carriers. This suggests that the Th2 deviation observed in *TYK2*<sup>GC</sup> carriers is a direct effect on T cells and not on monocyte polarization.

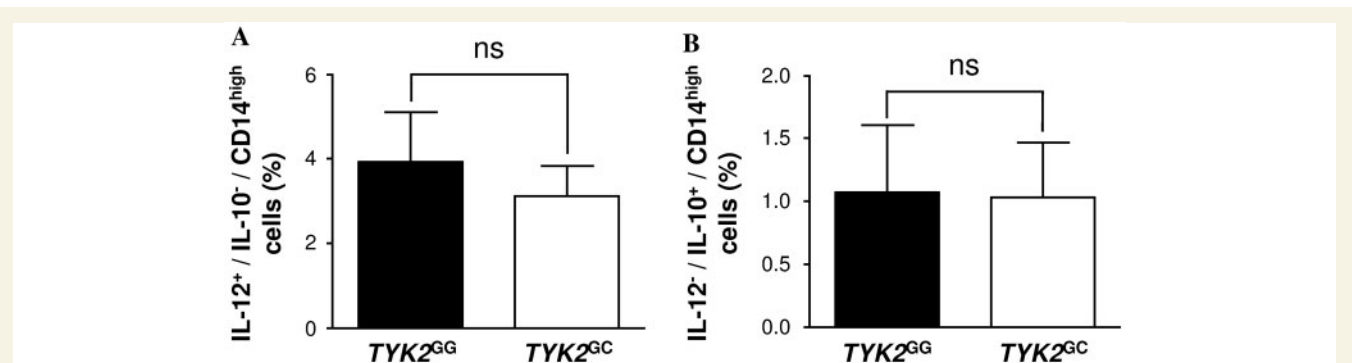
Moreover, studies on experimental autoimmune encephalomyelitis have shown the true importance of *TYK2* activity in disease susceptibility, especially by its expression in T lymphocytes (Spach *et al.*, 2009). The transfer of *TYK2*<sup>-/-</sup> CD4<sup>+</sup> pathogenic cells failed to induce a disease in wild-type animals, in contrast to CD4<sup>+</sup> pathogenic cells expressing *TYK2* (Oyamada *et al.*, 2009). Moreover, results indicated that the loss of pathogenicity conferred by *TYK2* deficiency was not due to an impaired proliferation or incapacity to migrate into the central nervous system (Spach *et al.*, 2009).

In our study, the protective *TYK2*<sup>GC</sup> genotype was associated with a moderate biological effect as compared with the *TYK2*<sup>GG</sup> genotype (1.5- to 2.0-fold global decrease). Such a moderate effect would be expected, considering the weak disease risk





**Figure 5** The rs34536443 polymorphism influences Th2 cytokine secretion. Expanded T lymphocytes were cultured for (A and B) 72 h or (C) 48 h in the presence of human IL-2 (50 U/ml) without or with an anti-CD3/anti-CD28 stimulation. The concentration of (A) IL-4, (B) IL-5 and (C) IL-13 was measured in the supernatants by multiplex immunoassay. Black bars indicate the  $TYK2^{GG}$  individuals ( $n = 10$ ) and white bars indicate the  $TYK2^{GC}$  individuals ( $n = 11$ ). Cytokine quantification was calculated from duplicate samples. Each bar represents mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ ; ns = not significant.



**Figure 6** The rs34536443 polymorphism does not modify the M1/M2 balance of peripheral blood monocytes. Adherent macrophages were stimulated with phorbol myristate acetate and ionomycin in presence of GolgiStop. After 4 h of stimulation, the percentage of (A) IL-12<sup>+</sup>/IL-10<sup>-</sup>/CD14<sup>high</sup> and (B) IL-12<sup>-</sup>/IL-10<sup>+</sup>/CD14<sup>high</sup> cells was quantified by flow cytometry. Black bars indicate the  $TYK2^{GG}$  individuals ( $n = 10$ ) and white bars indicate the  $TYK2^{GC}$  individuals ( $n = 7$ ). Each bar represents mean  $\pm$  SEM; ns = not significant.

conferred by the C allele (odds ratio = 0.63). As opposed to the polymorphism's weak biological effect observed in this study, the complete loss of TYK2 kinase activity linked to a Mendelian mutation was associated with profound perturbation of the immune system (Minegishi *et al.*, 2006).

In addition to multiple sclerosis, the *TYK2* gene has been associated with systemic lupus erythematosus susceptibility (Sigurdsson *et al.*, 2005; Graham *et al.*, 2007; Suarez-Gestal *et al.*, 2009) and, more recently, with type 1 diabetes (Wallace *et al.*, 2010) and Crohn's disease (Franke *et al.*, 2010). Interestingly, two systemic lupus erythematosus and type 1 diabetes variants are in strong linkage disequilibrium with rs34536443: rs2304256 ( $D' = 0.75$ ) and rs12720356 ( $D' = 1.0$ ). Hence, the rs34536443 variant might be the real variant associated with systemic lupus erythematosus and type 1 diabetes susceptibility. If this hypothesis is confirmed, the blunted TYK2 kinase activity might be a common mechanism for protection against several autoimmune diseases. Therapeutic inhibition of tyrosine kinase is an active field, with eight drugs approved for cancer treatment by the US Food and Drug Administration (Ghoreschi *et al.*, 2009b). In

autoimmune diseases, imatinib, an inhibitor of breakpoint cluster region-Abl fusion protein, receptor tyrosine kinases, platelet derived growth factor receptor and kinase inhibitor tyrosine, was shown to be effective in a mouse model of type 1 diabetes (Louvét *et al.*, 2008). Furthermore, JAK3 inhibitors were effective in trials for active rheumatoid arthritis treatment in humans (Kremer *et al.*, 2009). Overall, the present results and previous findings suggest that TYK2-related pathways are good candidate targets for disease-modifying drugs in the treatment of autoimmune diseases, including multiple sclerosis.

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## Conflict of interest

D.B. has participated in scientific advisory boards and received honoraria for lectures or educational activities from Bayer Schering Pharma, Sanofi-Aventis, Teva Pharma, Merck Serono, Novartis and Biogen Idec. F.W. has received honoraria for speaking from Bayer-Schering AG, Biogen-Idec and Orion Pharma; consultancies from Bayer-Schering and Orion Pharma; and grant support from Bayer-Schering and Merck-Serono. The authors have declared that no conflicts of interest exist.

## Supplementary material

Supplementary material is available at *Brain* online.

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